Momordica Charantia Polysaccharides Modulate the Differentiation of Neural Stem Cells via SIRT1/B-Catenin Axis in Cerebral Ischemia/Reperfusion

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Research

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Abstract

Background

Stroke is the leading cause of long-term motor disability and cognitive impairment. Recently, neurogenesis has become an attractive strategy for the chronic recovery of stroke. It is important to understand the molecular mechanism that promotes neural stem cell (NSC) neurogenesis for future NSC-based therapies. Our previous study showed that Momordica charantia polysaccharides (MCPs) exerted neuroprotective effects on stroke via their antioxidant and anti-inflammation activities. However, it remains unknown whether MCPs promote NSC neurogenesis after cerebral ischemic/reperfusion injury (IRI).

Methods

We investigated MCPs' function in differentiation of NSCs in vitro experiments. Primary NSCs and neural stem cell line C17.2 were cultured and subjected to glutamate stimulation to establish the cell model of ischemia / reperfusion injury (IRI). We evaluated the effect of MCPs on NSC differentiation in IRI cell model by Western blot and immunofluorescence staining. The SIRT1 activity of NSCs post glutamate stimulation were also evaluated by CELL SIRT1 COLORIMETRY ASSAY KIT. In addition, molecular mechanism was clarified by employing the activator and inhibitor of SIRT1.

Results

MCPs had no effects on the differentiation of neural stem cells under physiological conditions, while shifted NSC differentiation potential from the gliogenic to neurogenic lineage under pathological conditions. Activation of SIRT1 with MCPs was responsible for the neuronal differentiation of C17.2-NSCs. The neuronal differentiation effect of MCPs was attributed to upregulation SIRT1-mediated deacetylation of β-catenin. MCPs-induced deacetylation via SIRT1 promoted nuclear accumulation of β-catenin in NSCs.

Conclusion

Our findings indicate that the deacetylation of β-catenin by SIRT1 represents a critical mechanism of action of MCPs in promoting NSC neuronal differentiation. It provides an improved understanding of molecular mechanism underlying neuroprotective effects of MCPs in IRI, indicating its potential role on treating ischemic stroke especially chronic recovery.

Introduction

Stroke is a major disease that affects millions of people annually across the world [1, 2, 3]. As far as we have known that, after the onset of cerebral ischemia, a set of time-dependent cellular events are activated including not only glutamate receptor-mediated excitotoxicity, apoptosis, blood-brain barrier (BBB) dysfunction and inflammation but also endogenous neural repair[4, 5].

Accumulating evidence has suggested that the neurogenesis occurs in rodents, as well as in primate and patients after ischemic stroke [6, 7, 8]. However, the spontaneous repair capacity after cerebral ischemia is limited because proximately eighty percent of the new neurons produced by ischemia die within two weeks, and only a very few populations can survive over a long period of time [9, 10]. Therefore, that pharmacological interventions related to promoting endogenous neurogenesis and prolonging the survival of differentiated neurons after stroke are believed to be a promising strategy for brain repair.

Momordica charantia (MC), belonging to the gourd family, is an important multipurpose edible, medicinal plant widely distributed throughout Asia. In recent study, we have provided experimental evidence for that polysaccharide is main effective components extracted from MC. Additionally, MCPs have a protective role on nerve injury after stroke by scavenging free radicals [11]. The other teams previous research also demonstrated that MCPs have high antioxidant capacity for scavenging reactive oxygen species (ROS) [12, 13]. The side effects of currently prescribed synthetic antioxidants have prompted the use of alternative traditional herbal medicines and dietary supplements antioxidants associated with fewer adverse effects in the treatment of ischemia stroke [14]. It is well established that variety of natural molecules present in the diet, such as plant polysaccharide, could protect the brain and delay aging. Emerging studies show a role for antioxidant-rich foods such as fruits, vegetables, and nuts in improving cognitive damage by preventing or delaying the onset of cognitive decline during aging and neurodegeneration [15, 16]. As naturally compounds found in daily foods, MCPs are well known for their anti-oxidation, anti-inflammation, anti-tumor, hypoglycemic and anti-diabetic effects [17], however, little is known about its effects from neurogenesis regulation perspective.

Silent information regulator factor 2-related enzyme 1 (sirtuin1, SIRT1) is a deacetylase which is widely expressed in the whole adult brain[18] and has been shown to interact with set of protein targets involved in Wnt signaling[19], glucose homeostasis[20, 21], and calcium signaling[22, 23], making SIRT1 an attractive candidate target for controlling senescence, feeding behavior, energy expenditure and oxidative stress[24]. Studies of the aging brain have shown that the expression of SIRT1 decline with age, which is accompanied by a higher incidence of aging related neurodegenerative diseases in mammals, such as ischemic stroke [25, 26, 27]. In addition, SIRT1 has been found to delay aging and considered to be one of the determining factors of the biology of stem cells, including neural stem cells (NSCs) and neural progenitor cells (NPCs). SIRT1 has been implicated in NSCs proliferation and differentiation through deacetylate variety of important histone and non-histone proteins and many transcription factors [28, 29]. These phenomena raise the possibility that SIRT1 may play a crucial role on neurogenesis after ischemic stroke.

During recent years, canonical Wnt/β-catenin signaling has been found to play a key role in controlling the maintenance of neural stem cells and the development of neural tissues and progenitor cells [30]. The accumulation of β-catenin in the nucleus is the key step in canonical Wnt/β-catenin signaling pathway [31]. Furthermore, the post-translational modification of β-catenin, including phosphorylation and deacetylation, is an important mechanism of regulating of β-catenin stability and subcellular location, as well as its transcriptional activity [32, 33]. However, it has not previously been explored whether MCPs regulate nuclear translocation of β-Catenin via SIRT1 to promote differentiation of NSCs after stroke. Therefore, in the present study, we aim to explore whether MCPs plays an important role in NSC differentiation, and if SIRT1/β-catenin axis plays important roles in post-stroke neurogenesis.

1. Material And Methods

MCP preparation

MCP (purity≥99%) was extracted from M. charantia by water extraction and alcohol precipitation, followed by the elimination of proteins and starch. The extraction method was provided by Professor Min Wang from China Pharmaceutical University. The detail procedure was described as our previous publication [11].

Antibody and reagents

The following primary antibodies were used in this study: mouse anti-SIRT1 antibody [19A7AB4] (1 μg/ml, ab110304, Abcam), rabbit anti-β-catenin antibody [E247] (1:5,000; ab32572, Abcam), mouse anti-beta III Tubulin antibody [TUJ-1] (1:1000, ab14545, Abcam), mouse MAb anti-GFAP antibody (1:1,000; ab106509, Abcam),mouse anti-CNPase antibody [11-5B] (5 μg/ml, ab6319, Abcam), mouse anti-acetyl Lysine antibody [1C6] (1:1,000; ab22550, Abcam), rabbit monoclonal anti-Lamin B1 antibody (1:3000, #13435, Cell Signaling Technology, USA), β-actin (1:5000, #4970, Cell Signaling Technology, USA). The secondary antibodies used in our experiment were goat anti-mouse IgG (1:10000) and goat anti-rabbit IgG (1:10000), which were purchased from Sigma-Aldrich (St. Louis, MO, USA). SIRT1 siRNA (#5239398) was obtained from Life techenologies (USA). Resveratrol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Nicotinamide was acquired from Beyotime biotechnologies (USA).

2.2. Cell culture and treatment

C17.2 cells, an immortalized NSC line, were kindly provided by Professor Jiangang Shen (School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong, SAR, China). C17.2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS, Gibco) and 2 mM L-glutamine at 37°C in humidified incubators with a 5% CO2 atmosphere and passaged at 50% confluence every two days. In the experiment, C17.2 cells were incubated with glutamate (100 μM) for 30 mins and then were reperfused for different period of times to mimic ischemic insults in vitro. Subsequently, each experimental group of C17.2 cells were removing glutamate prior to treatment with MCPs. C17.2-NSCs were exposed to

Resveratrol (25 μM), Nicotinamide (Sirtuin inhibitors, 25 μM) or MCPs (5, 50 μg/ml) for 24 h. Resveratrol and Nicotinamide were added 24 h before glutamate treatment.

Primary cortical neural stem cells (E16-NSC) culture

All procedures involving the use of animals were approved by the local ethical review committee. Primary cortical neural stem cells (E16-NSC) were isolated from cerebral cortexes of Sprague-Dawley rat embryo on E16. Briefly, whole cerebral neocortices were removed from the rat fetuses, then mechanically dissociated. The single-cell suspension was transfected to 6cm culture dishes and cultured as floating neurospheres in a humidified atmosphere with 5% CO_2 at 37°C. Neurospheres were maintained in neural-basal medium (DMEM/F-12, 1:1, Hyclone), which was supplemented with B27-supplement, 20 μg/l basic bFGF and 20 ng/ml EGF (both from Invitrogen), 2 mmol/l glutamine (Invitrogen), 10,000 U/l penicillin and 10 mg/l streptomycin (both from Hyclone). After 5-7 days, the neurospheres were trypsinised (0.05% trypsin with 0.02% EDTA□Sigma–Aldrich) as single-cell and seeded in poly-D-lysine-treated culture plates at a density of 5×10^4 cells. then cells were cultured for 2 to 3 days at 37 °C in a 7.5% CO_2 atmosphere and used for in vitro experiment.

siRNA transfection

Small interfering RNAs (siRNAs, 5239398) targeting sitr1 were obtained from Life techenologies. 6-well plates were plated with C17.2-NSCs for transfection, and when cells reached 60% to 70% confluence, the lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) were used to transfected cells with either SIRT1-specific small interfering RNA (siRNA) (50 nM) or negative control siRNA (50 nM) mixed with Lipofectamine RNAiMAX (all from Life Technologies) according to the manufacturer's instructions. All transfected cells were harvested at 48 h after transfection for subsequent experiment.

2.3. Cellular immunofluorescence staining

For differentiation detection, the expression of Tuj1, GFAP, and CNP were analyzed by cellular immunofluorescence staining. The cells were seeded on glass coverslips in DMEM supplemented with 10% FBS. Coverslips were rinsed with PBS and then fixed with 4% paraformaldehyde solution for 10 minutes at room temperature (RT). After fixation, the cells were incubated in blocking buffer (1× PBS with 10% normal goat serum and 0.3% Triton X-100) for 1 hour at RT. Then cells were rinsed three times with 0.01 M PBS (pH 7.4), incubated with the primary antibody (anti-Tuj1, anti-GFAP, or anti-CNP), overnight at 4°C, washed with PBS for three times, and then incubated with the secondary antibody (Alexa Fluor 488–conjugated goat anti-mouse antibody ,1:200, Invitrogen) for 2 hour at RT, and DAPI (10 mg/mL) was added 15 minutes to display the nuclei. Images of 10 randomly selected fields of view were captured under a fluorescence microscope.

Protein Sample preparation

The cells were washed three times with iced PBS and lysed using 300~400 μL lysis buffer (containing 50 mM Tris-HCl, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 1 mM Na_3VO_4, 1 mM p-nitrophenyl phosphate, 0.5 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin) on ice for 30 minutes before collection. The mixture was then centrifuged to isolate the supernatant, which contained the cytoplasmic protein. The pellet was washed by adding 400 μL of lysis buffer and then centrifuged to discarde the supernatant. The pellet was re-suspended with 100 μL of RIPA lysate (containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM p-nitrophenyl phosphate, 0.5 Mm PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin). After three times ultrasonic treatments for 5 seconds at intervals of 5 seconds and centrifugation at 12,000g for 20 minutes at 4°C, the supernatant was the nuclear protein.

Immunoprecipitation

Coimmunoprecipitation (co-IP) was performed according to the following procedures. Briefly, protein samples were precleared for 1 h at 4°C using 20 μl of protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) to remove nonspecific proteins. After cetifugation, supernatants were incubated with 1 μg of primary antibodies overnight at 4°C. Targeted immune complexes were captured with 2 h incubation of Protein A. Samples were eluted three times with immunoprecipitation buffer. Targeted proteins were eluted by boiling at 100°C for 5 min in SDS-PAGE loading buffer and then isolated by centrifugation. Then immunoprecipitates were subjected to Western blot analysis.

Immunoblotting

Western blot analysis was performed according to the standard protocol. Briefly, Proteins were separated by by 4-12 % SDS-PAGE and transferred to PVDF) membranes (Merck KGaA, Darmstadt, Germany) for 90 min at 15 V. The membranes were then blocked with 5 % non-fat dry milk in Tris-Buffered Saline and Tween 20 (TBST) and then incubated with appropriate primary antibodies at 4℃ overnight, followed by washing with TBST, then incubating with horseradish peroxidase-conjugated secondary antibodies in TBST for 1 hour at RT. Then the bands were visualized by ECL (Pierce) and detected by BioRad digital imaging system (Bio-Rad Laboratories, Inc). For quantification, the density of the bands on the membrane were quantified using Image J 1.48 software.

Assessment of SIRT1 activity

Assessment of SIRT1 activity was performed according to the manufacturer's instruction in CELL SIRT1 COLORIMETRY ASSAY KIT (GenMed Scientifics Inc., U.S.A).

Data analysis and statistics

All values are presented as the means ± S.D. Statistical analysis of the results was carried out by Student’s t-test or ANOVA, followed by Duncan’s new multiple range method or the Newman-Keuls test. P-

values < 0.05 were considered be statistically significant. All experiments were repeated at least thrice in an independent manner.

Results

MCPs have no effects on the differentiation of neural stem cells under physiological conditions.

To determine whether MCPs treatment affects NSC differentiation under physiological conditions, we observed the expression of three NSC differentiation marker genes, including Tuj1, GFAP, CNP at 24 h after reperfusion in C17.2-NSCs. As displayed in Fig. 1, no significant difference was discovered between the Con group and the different concentrations of MCPs treatment groups (1.0, 3.0, 5.0, 10.0, 20.0 ug/ml) in Tubulin, GFAP or CNP expression. The data suggest that MCPs treatment could not affect NSC differentiation under physiological conditions.

MCPs shift NSC differentiation potential from the gliogenic to neurogenic lineage under pathological conditions.

To exclude the effect of C17.2-NSCs themselves differentiation on the experiment and to determine the time point of MCPs administration, C17.2 cells were treated with 30 minutes of glutamate (100 μM) and then were subsequently reperfused for different period of times to mimic ischemic insults in vitro. Firstly, we examine differentiation of C17.2-NSCs without the presence of MCPs by monitoring the protein level of three NSC differentiation markers. The results from Western blot assays indicated that the expression of TUJ1, GFAP and CNP gradually declined on day 0 and reached its lowest point on day3 and then started to increase on day5 after glutamic acid stimulation compared with the control cells (Fig. 2a, b). Upon glutamic acid treatment for 14 days, expression of TUJ1 dramatically decreased again and the other two cell markers GFAP and CNP still maintained higher level (Fig. 2a, b). It has been shown that MCPs has a protective role on nerve injury after stroke by scavenging free radicals [11]. According to above result, in next experiments, C17.2 cells were administrated with MCPs on day3 after stimulation with glutamic acid to further detect the effect of MCPs on C17.2 differentiation.

Next, as shown in Fig. 2c, d, upon MCPs treatment, the expression of TUJ1 was significantly increased in different concentrations MCP-treatment groups compared with that in the Control group and Glu/3d group. While the expression of GFAP dramatically decreased compared with that in the Control group and Glu/3d group. In contrast, no change of the protein level of CNP were found compared with the Glu/3d group (Fig. 2c, d). Taken together, these results suggested that MCPs could obviously promote C17.2-NSC differentiation into neuron under the ischemic pathological state. Furthermore, it was clearly that, with MCPs treatment, NSC differentiation toward the astroglial lineage was dramatically turned to the neuronal lineage under IRI conditions.

To further demonstrate the effect of MCPs, we detected the lineage-specific differentiation markers β-III-tubulin (for neurons) by TUJ1 immunofluorescent staining (Fig. 2e, f). To be more definitive about the effects that MCPs induced differentiation under the ischemic pathological conditions, we then tested

effect of MCPs on another primary neural stem cells derived from rat brains, E16-NSCs (16d rat embryonic cortical neural stem cells) as a model of embryonic neural stem cells. This result showed that MCPs addition produced a significant increased the number of TUJ1-positive cells compared with the Glu/3d groups (Fig. 2f). Based on these findings, we consider that MCPs may enhance the neuronal differentiation of E16-NSCs, which is in agreement with the results observed from immortalized C17.2-NSCs.

Activation of SIRT1 with MCPs is responsible for the neuronal differentiation of C17.2-NSCs

Studies have demonstrated that upregulation of SIRT1 repressed the proliferation of NPCs and directed their differentiation toward the astroglial lineage at the expense of the neuronal lineage under oxidative stress conditions in vitro and in vivo [34]. Therefore, for insight into further evaluating neuronal differentiation mechanisms of MCPs treatment in C17.2-NSCs post glutamate stimulation, we measured SIRT1 protein expression by Western blot. As shown in Fig. 3a-b, the expression of SIRT1 in Glu group was obviously higher compared to that of Con group. Similarly, in MCPs group it was significantly increased compared with Con group. But MCPs administration did not remarkably affect SIRT1 protein expression when compared to that in Glu group. Next, we detected the activity of SIRT1 by SIRT1 activity assay. The result (Fig. 3c) showed that the activity of SIRT1 in both concentration of MCPs groups were upregulated compared with Glu group, indicating that MCPs treatment enhanced activity of SIRT1.

In order to further study the role of SIRT1 in MCPs induced neuronal differentiation, the blockage of SIRT1 activity by Nicotinamide (SIRT1 inhibitors) were used to investigated NSC differentiation. Western blot result showed that MCPs enhanced TUJ1 expression, in contrast, the Nicotinamide (NA) abolished the promotion effect of MCPs on neuronal differentiation (Fig. 3d, e). Additionally, we used SIRT1-siRNA (represented as MCP + siRNA group) to suppress the expression of SIRT1 in the differentiation process, and then detected the immunofluorescence staining of TUJ1 in C17.2-NSCs after glutamate treatment (Fig. 3f). As shown in Fig. 3f, g, MCPs strongly upregulated the number of TUJ1-positive cells, while this effect was significantly reversed by SIRT1-siRNA treatment. These data clearly indicate that MCPs-mediated neuronal differentiation of NSC under IRI pathological conditions is related to the upregulating SIRT1 activity.

MCPs-induced deacetylation via SIRT1 promotes nuclear accumulation of β-catenin in NSCs

The above results of our studies showed the importance of SIRT1 in MCPs-mediated neuronal differentiation of C17.2-NSCs in glutamate-induced damage. These prompted us to further investigate the underlying mechanisms of SIRT1-mediated neuronal differentiation of NSCs after MCPs treatment. It is important to note that β-catenin is a deacetylation substrate of SIRT1, and acetylation/deacetylation modification of β-catenin participates in regulating its protein stability and transcriptional activity [35]. Recently, Leonard Guarente's study showed that SIRT1 could deacetylates β-catenin and promotes its nuclear localization and activity in mesenchymal progenitor cells [36]. Therefore, the level of acetylation β-catenin was measured by western blot analysis in C17.2-NSCs under ischemia-mimic condition. The results revealed that, as the time of reperfusion was prolonged, the level of acetylation β-catenin was

significantly increased (Fig. 4a, b). Then we tested the subcellular distribution of β-catenin in nuclear and cytoplasmic respectively. The results showed that treatment with MCPs (5 or 50 μM) in C17.2-NSCs strongly resulted in a decrease in cytoplasm presence of β-catenin and an increase nuclear accumulation of β-catenin (Fig. 4c, d, e). Collectively, the above results implied that MCPs could affect distribution of β-catenin, and it may be closely related to the level of β-catenin acetylation via SIRT1. To confirm whether SIRT1 could regulate nuclear accumulation of β-catenin, Resveratrol, an agonist of SIRT1, was used as the positive control for the evaluation of the effect of MCPs. As demonstrated in Fig. 4f, g, Resveratrol significantly reduced the acetylation level of β-catenin and promoted its nuclear migration under the glutamate insults in C17.2-NSCs, while the nicotinamide, SIRT1 deacetylase inhibitor, increased the acetylation level of β-catenin and inhibited intracellular migration of β-catenin. To further validate whether the MCPs could play the same effect, we detected the acetylation levels of β-catenin in the presence of MCPs or SIRT1 siRNA, and observed that MCPs remarkably downregulated the acetylation level of β-catenin and promoted its nuclear accumulation which were reversed by the SIRT1 siRNA (Fig. 4h, i). These results confirmed that effect of MCPs was mediated by the SIRT1/β-catenin axis.

Discussion

In this study, our results revealed that MCPs could promote NSC neurogenesis after ischemic stroke. Our in vitro results identified previously unrecognized mechanisms that MCPs led to the activation of SIRT1 under glutamate induced injury, which positively enhanced the nuclear accumulation of β-catenin and contributed to NSC differentiation into neuron in mimic IRI cell model. These findings indicate that MCPs may have the potential as a protective agent on late-stage recovery of ischemic stroke in clinical practice.

Recent years, polysaccharides have been widely used in medicine because of their low toxicity and obvious pharmacological effects [16]. As naturally compounds found in daily foods, water extract of MCPs is well known for its anti-oxidation, anti-inflammation, anti-tumor, hypoglycemic and anti-diabetic functions [13]. Several reports have shown that MCPs treatment ameliorates oxidative stress injury in cardiovascular and cerebrovascular disease [37]. We have previously investigated that MCPs had a protective role on nerve injury after stroke by scavenging free radicals [11]. However, the mechanism underlying MCPs-mediated neuroprotection in stroke and its pro-differentiation effect on injured brain tissues is still far from being understood. This is the first investigation detailing the neuronal differentiation action of MCPs in IRI.

To gain insight into the molecular mechanisms of the differentiation effect of MCPs on endogenous NSCs, firstly, an immortalized murine-derived neural stem cell line, C17.2-NSCs, was treated with different concentration of MCPs and then were detected the expression of NSC differentiation marker genes. We observed MCPs had no effects on the differentiation of neural stem cells under physiological conditions. This result suggests that MCPs (at different concentration ranges, 1.0, 3.0, 5.0, 10.0, 20.0 ug/ml) is safe for normal physiological NSCs and indicates that such concentration ranges may be physiologically acceptable for cultured cells in vitro. Next, in order to exclude the effect of C17.2 cells themselves differentiation on the experiment and to determine the time point of MCPs administration, C17.2-NSCs

were treated with glutamate and then were reperfused of different periods to mimic IRI. According to this experiment, we found that the differentiation features of C17.2-NSCs in IRI are consistent with previous reports. Therefore, in the following experiment we selected 3d after glutamate-incubation as the time point for MCPs administration. When MCPs was administered to C17.2-NSCs after glutamate-incubation, we observed that MCPs could obviously promote C17.2-NSC differentiation into neuron, in contrast, the astroglial differentiation of C17.2-NSCs were significantly inhibited. Astrocytes are the most abundant cells in the CNS and reactive astrogliosis accompany many pathological situations that affect the CNS, such as ischemic damage [38]. Compared with nonreactive astrocytes, reactive astrocytes show either deleterious effects on the progression of tissue damage or beneficial roles during recovery and repair. During recovery stage after IR, overactivated astrocytes can induce multiple damage effects and inhibit survival of newborn neurons [39]. The results of this study appear to suggest that the protective roles of MCPs is to enhance the neuronal differentiation of NSCs on one hand, and another possibility may be that MCPs could change astroglial differentiation which is more important in the recovery stage of CNS injuries. This effect of MCPs on inhibiting the over-activation of glial cells could potentially improve the microenvironment of NSCs survival or could promote neurite outgrowth and synaptic formation during brain repair stage. We have also investigated another primary neural stem cell derived from rat brain, E16-NSCs, and found that MCPs obviously enhances the neuronal differentiation, which strongly supported the results observed from immortalized C17.2-NSCs.

In latest years, therapeutic ability of NSCs has been matter of intense research, fueled by the hope of exploiting their regenerative potential for the treatment of different kinds of neurological impairments [40, 41]. Adult neurogenesis is the process whereby NSCs mature, migrate, and functionally integrate into the pre-existing neuronal network of an adult brain. Thus, the regenerative potential of endogenous neural stem cells in the adult brain has been proved as a likely source for regenerative tool to compensate for neuronal damage after ischemia stroke [42]. However, the molecular mechanisms underlying the regulation of neurogenesis in ischemic stroke remain to be elucidated. Our previous reports have demonstrated that MCPs mitigated ischemic stroke-induced damages by effectively eliminating oxygen free radicals and inhibiting JNK3 activation [11]. Our team has conducted research on the regulation of MCPs on neurogenesis after cerebral ischemia, and we will elaborate the proliferation effect of MCP on NSCs in another article. Our object of the present study is to focus on effect of MCPs on neuronal differentiation of NSCs under ischemia reperfusion injury and to understand its underlying mechanism.

To date, seven SIRTs in mammals (SIRT1- SIRT7) have been identified in different subcellular organelles and mediate different cellular functions depending on their typical substrate [24, 43, 44]. Interestingly, due to important effects on metabolism and physiological activities in CNS, SIRT1 has attracted great attention as medicinal targets particularly on neurodegenerative diseases [45]. Mountains of works have confirmed that SIRT1 regulates a wide variety of biological functions and cellular processes through the deacetylation of several histone/non-histone protein residues [46]. By deacetylating a variety of proteins, SIRT1 can mediate a broad range of vital biological functions including gene expression, DNA repair and apoptosis, neurogenesis, and aging. Thus, SIRT1 promotes cellular longevity through a number of mechanisms [47]. More importantly, cross talk between the gene regulation pathways of multiple

transcription factors and SIRT1 determines the expansion and differentiation of stem cells [48]. In general, SIRT1 has drawn more attention because it is deemed to be one of the determining factors of the biology of stem cells. Although increasing evidence have demonstrate that SIRT1 plays a prominent role in the survival of differentiated neurons under various cellular stresses,some other studies have shown different findings. A review article of Cai et al. analyzed the opposing effects of SIRT1 on NSC lineage choice. They suggested a possible explanation that SIRT1 can be neuroprotective or neurotoxic depending on conditions, cellular stress, and cellular type. The conclusion emphasized the importance that SIRT1 function in NSC differentiation depends heavily on the redox state of the cells [24].

With respect to that MCPs display free-radical scavenging properties, of course, it is entirely possible that MCPs change the redox state of the cells ultimately enhancing the activity of SIRT1. Therefore, in order to test whether the differentiation effect of MCPs was due to SIRT1 in more detail, we showed that MCPs administration did not remarkably affect SIRT1 protein expression. But our further studies demonstrated that the activity of SIRT1 in MCPs-treatment group was upregulated compared with glutamate-treatment group, indicating that MCPs treatment enhanced activity of SIRT1 during the differentiation process. We also observed that when administration with MCPs, NSCs differentiation was promoted into neuron. In contrast, subjecting to NA (a known SIRT1 inhibitor) or knocking down SIRT1 with SIRT1 siRNA, NSCs neuronal differentiation was significant downregulated. These results suggested that SIRT1 activation may contribute to the neuronal differentiation effects of MCPs under the ischemic-linked pathological conditions. One possible explanation for this finding is that the strong antioxidation of MCPs might change the intracellular redox state of NSCs after glutamate-induced injury, and SIRT1 might be more prone to play its deacetylase activity. This may explain our observation of MCPs promoting neuronal differentiation of NSCs in pathological models, accompanied by an increase in SIRT1 activity.

In our study as discuss above, SIRT1 is critical to MCPs-induced NSCs differentiation, however, what function might SIRT1 exert there? Intriguingly, numerous deacetylation substrates of SIRT1 have been identified, for example, one of them is β-catenin, the important transcription factor in Wnt signaling pathway [49, 50]. It is well-documented that the protein stability and nuclear localization of β-catenin are required for Wnt pathway target genes,and usually some of those genes are closely related to neurogenesis. Several studies have validated that deacetylation of β-catenin by SIRT1 is correlated with β-catenin nuclear accumulation for its transcriptional activity [32, 35]. For example, a recent study done by Simic group reported that SIRT1-mediated deacetylation was required for nuclear accumulation of β-catenin in Mesenchymal stem cells (MSCs) for their self-renewal and differentiation [36]. Therefore, we hypothesis that SIRT1/β-catenin axis participates in NSCs differentiation via regulating Wnt signaling pathway in IRI. Therefore, in the final part of study, we observed a significant increased acetylation of β-catenin in glutamate-induced injury of the C17.2-NSCs. More importantly, cell fractionation studies showed that MCPs treatment in C17.2-NSCs strongly resulted in a decrease in cytoplasm presence of β-catenin and an increase nuclear accumulation of β-catenin. Further experiments were carried out to verify whether the regulatory effect of MCP on the cellular localization of β-catenin was related to SIRT1. We found that, along with hyperacetylated β-catenin was decreased in nucleus upon SIRT1 inhibition after stimulation with glutamate. In contrast, incubating with MCPs or RSV (SIRT1 agonist) rescued the nuclear

localization defect and reversed the hyperacetylation of β-catenin in C17.2-NSCs. This part of data strongly suggests that MCPs could induce nuclear accumulation of β-catenin via SIRT1 in C17.2-NSCs. And this effect of MCPs is probably related to deacetylase activity of SIRT1. Although the detailed mechanisms underlying this interesting phenomenon remain to be defined, one plausible explanation is that MCPs is a distinct SIRT1 activators which suggest an important potential therapy for ischemic/reperfusion injury.

Conclusion

In conclusion, our study demonstrates that MCPs have the potential to upregulate the activity of SIRT1 by changing intracellular redox state and subsequently motivate the deacetylation of β-catenin, thereby leading to the nuclear accumulation of β-catenin, consequently promoting the neuronal differentiation of NSCs in mimic IRI [Fig. 5]. Thus, MCPs may have the potential as a novel nerval protective agent in clinical practice. Our study reveals a previously unknown effect of MCPs to promotion neuronal differentiation via activating SIRT1/β-catenin pathway in IRI. This finding suggests that MCPs, besides its commonly known functions of anti-oxidation, anti-inflammation and anti-tumor, regulate neurogenesis process as well. Our study suggests that water extract of MCPs as a potential therapeutic strategy in the treatment of ischemic stroke.

Abbreviations

NSCs: Neural stem cells; IRI: ischemia/reperfusion injury; MCPs: Momordica charantia polysaccharides; siRNA: Small interfering RNA; BBB: blood-brain barrier; ROS: reactive oxygen species; SIRT1: Silent information regulator 1; NPCs: neural progenitor cells

Declarations

Ethics approval and consent to participate

The study was conducted under the approval of the institutional guidelines, and the experimental procedures were approved by the Animal Ethics Committee of Xuzhou Medical University. Each participant was informed written consent prior to the study.

Availability of data and materials

The datasets that support our conclusions of the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Zhaoli Hu, Fengying L, Xiaoling Zhou, Feng Zhang contributed equally to this work. Suhua Qi and Zhaoli Hu designed this study. Zhaoli Hu, Fengying L, Xiaoling Zhou, and Feng Zhang performed the experiments, collected and analyzed the data. Linyan Huang, Bing Gua and Jiangang Shen were responsible for the conception and design and assembly of data, data analyses and interpretation. Zhaoli Hu wrote the manuscript. Suhua Qi and Linyan Huang provided support for the analysis and critical revision of the manuscript. Suhua Qi provided the funds. All authors have read and approved the final manuscript.

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Consent for publication

Not applicable

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Figures

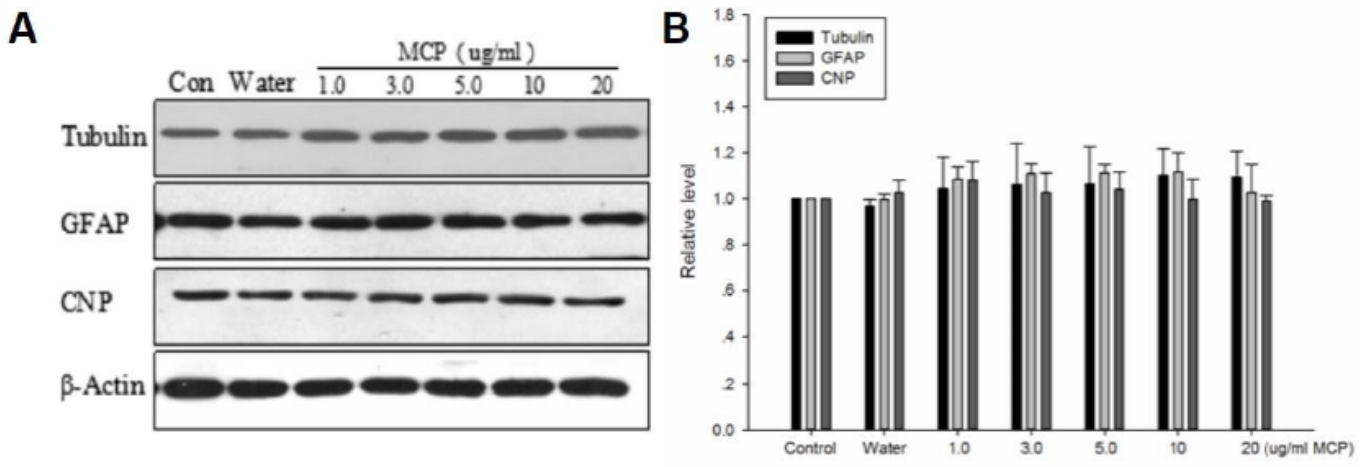

Figure 1

MCPs have no effects on the differentiation of C17.2-NSCs under physiological conditions. a Representative image of western blots for TUJ1, GFAP and CNP from indicated group. β-actin was used for signal normalization. b Data are presented as the relative density of TUJ1, GFAP and CNP compared with that of β-actin. Data were given as mean ± SD. The data were obtained from no less than three independent cells in each experimental group.

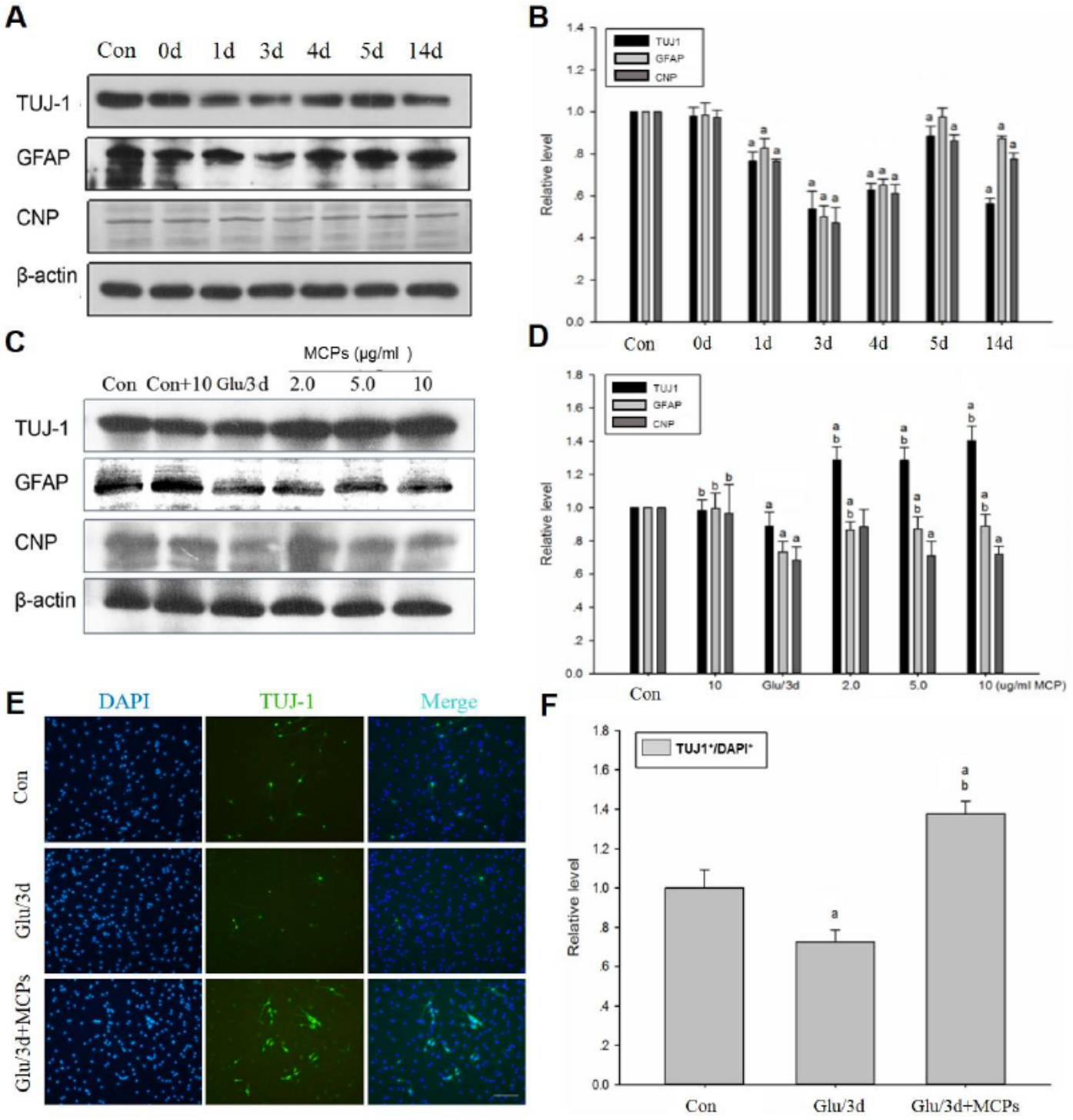

Figure 2

MCPs can promote the neuronal differentiation of C17.2-NSCs under pathological conditions. a C17.2-NSCs were treated with glutamic acid and then perfused of different periods (0d, 1d, 3d, 5d, 7d, 14d). Representative images of western blots for TUJ1, GFAP and CNP from indicated group. b Data were presented as the relative density of TUJ1, GFAP and CNP compared with that of β-actin. c C17.2-NSCs were treated with different concentration of MCP (2.0, 5.0, 10.0μg/ml) after glutamic acid stimulation

reperfusion of 3d (Glu/3d+MCP group). Representative images of western blots for TUJ1, GFAP and CNP from indicated group. d Data were presented as the relative density of TUJ1, GFAP and CNP compared with that of β-actin. e The immunofluorescent staining of TUJ1 (green) in the Glu/3d and Glu/3d+MCP group. DAPI was used to stain the cell nucleus (blue). Scale bar, 100 μm. f Quantification the proportion of TUJ-1+ cells in DAPI+ cells. Data were given as mean ± SD. Data represent the mean of at least three independent experiments ± SD. aP<0.05 vs. control, bP<0.05 vs. Glu/3d group.

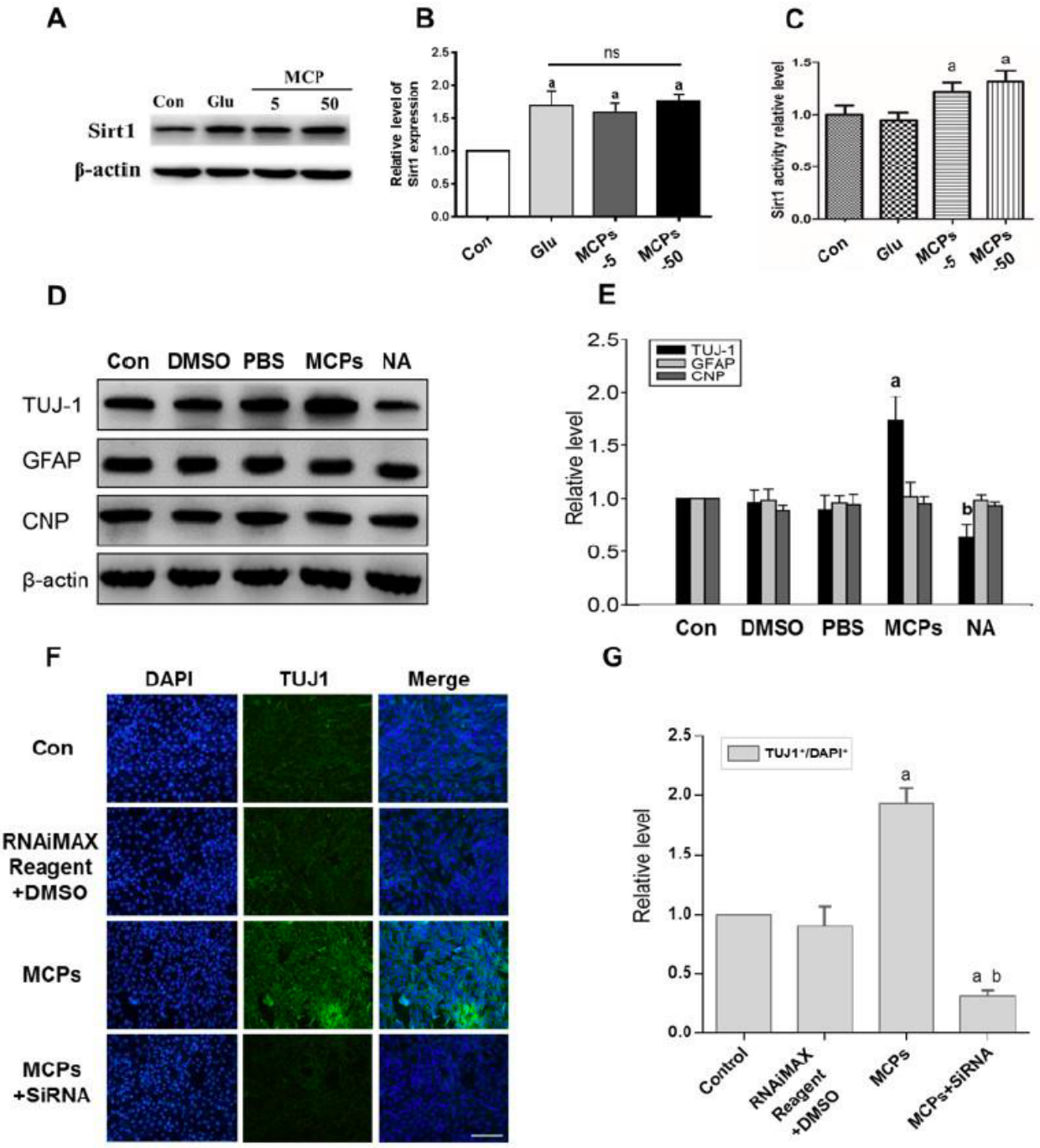

Figure 3

Activation of SIRT1 inducing by MCPs is responsible for the neuronal differentiation of C17.2-NSCs. a Represent western immunoblots of SIRT1 from indicated group. b Data were presented as the relative density of SIRT1 compared with that of β-actin from indicated group. c Activity of SIRT1 in C17.2-NSC differentiation by SIRT1 activity assay. d Representative images of western blots for TUJ1, GFAP and CNP from indicated group. e Data were presented as the relative density of TUJ1, GFAP and CNP compared with that of β-actin. f Representative immunofluorescence staining of TUJ1 (green) and DAPI (blue) in C17.2-NSCs from indicated group. Scale bar, 100 μm. g Quantitative analysis showing the percentage of TUJ1+ cells in indicated group. Data were given as mean ± SD. The data were obtained from no less than three independent cells in each experimental group. ap< 0.05 vs. Con, bp< 0.05 vs. MCPs group.

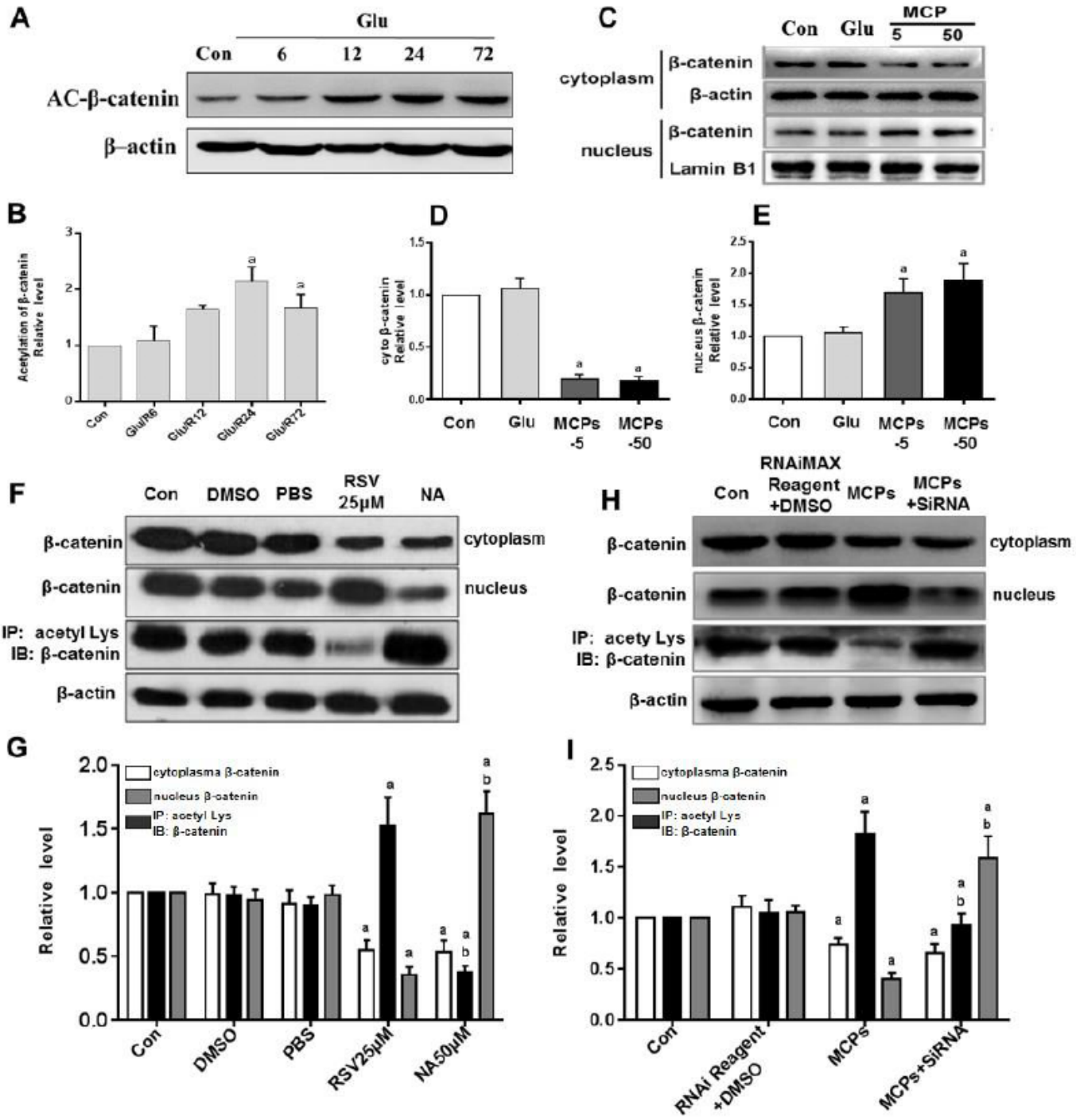

Figure 4

MCPs-induced deacetylations via SIRT1 promotes nuclear accumulation of β-catenin in C17.2-NSCs. a Represent western immunoblots of Ac-β-catenin after glutamic acid stimulation reperfusion of indicated time point. b Quantitative analysis of the expression of Ac-β-catenin compared with that of β-actin from indicated group. c Fractionated extracts analysis. Represent western immunoblots of β-catenin in nuclear and cytoplasmic from indicated group. d-e statistical analysis for β-catenin nuclear accumulation. β-actin

or LaminB1 was used for signal normalization. f, h Represent western immunoblots of the nuclear accumulation of β-catenin and Ac-β-catenin (IP: acety Lys,IB: β-catenin) from indicated group in glutamate-treated C17.2-NSCs. g Quantitative analysis of the nuclear accumulation of β-catenin and Ac-β-catenin treatment with RSV or NA (f). i Quantitative analysis of the nuclear accumulation of β-catenin and Ac-β-catenin treatment with MCPs or SIRT1-siRNA (h). Data represent the mean of at least three independent experiments ± SD. ap< 0.05 vs. Con, bp< 0.05 vs. RSV25μM or MCPs group.

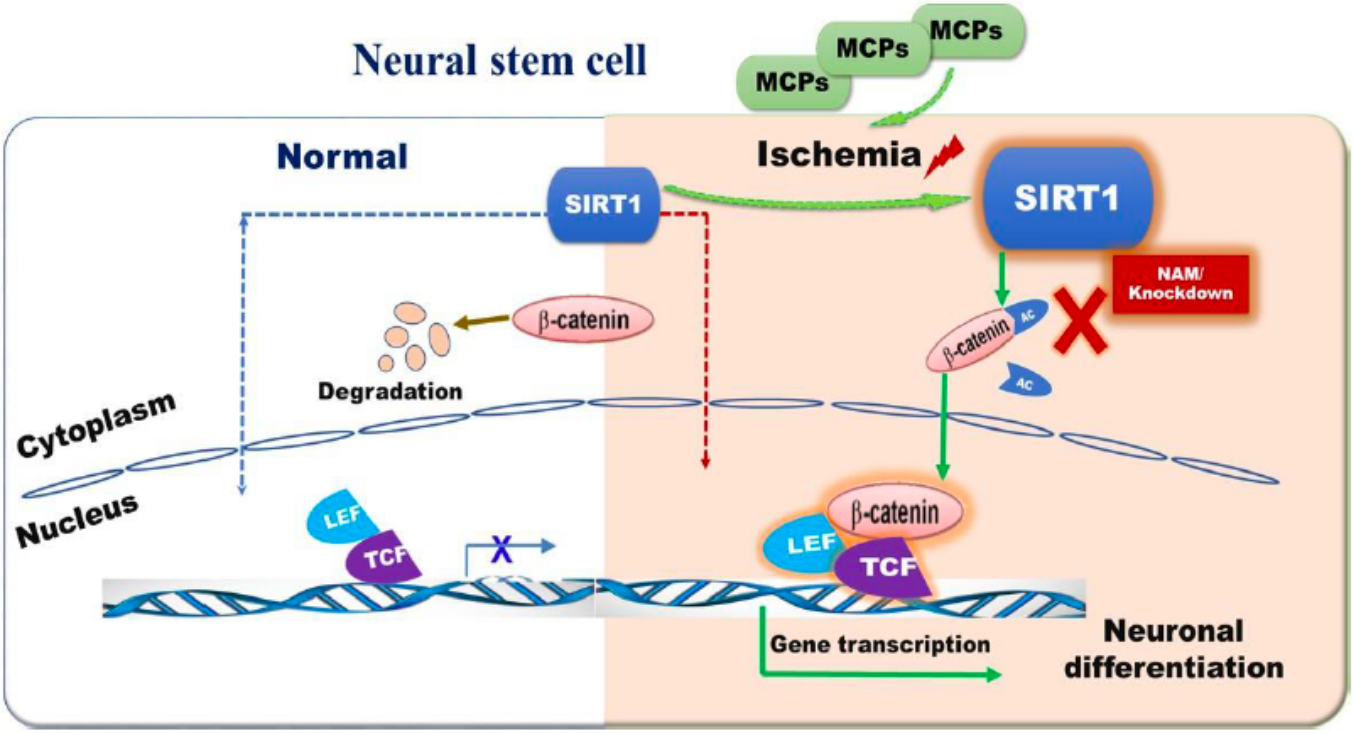

Figure 5

The mechanism graph of the regulatory network of MCPs/ SIRT1/β-catenin in the neuronal differentiation of NSCs under glutamate-induced injury.