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Promotion of Momordica Charantia polysaccharides on neural stem cell proliferation by increasing SIRT1 activity after cerebral ischemia/reperfusion in rats

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ABSTRACT

The deacetylase SIRT1 has been reported to play a critical role in regulating neurogenesis, which may be an adaptive processes contributing to recovery after stroke. Our previous work showed that the antioxidant capacity of Momordica charantia polysaccharides (MCPs) could protect against cerebral ischemia/reperfusion (I/R) after stroke. However, whether the protective effect of MCPs on I/R injury is related to neural stem cell (NSC) proliferation remains unclear. In the present study, we designed invivo and invitro experiments to elucidate the underlying mechanisms by which MCPs promote endogenous NSC proliferation during cerebral I/R. Invivo results showed that MCPs rescued the memory and learning abilities of rats after I/R damage and enhanced NSC proliferation in the rat subventricular zone (SVZ) and subgrannular zone (SGZ) during I/R. Invitro experiments demonstrated that MCPs could stimulate the proliferation of C17.2 cells under oxygen-glucose deprivation (OGD) conditions. Further studies revealed that the proliferation-promoting mechanism of MCPs relied on increasing the activity of SIRT1, decreasing the level of acetylation of β-catenin in the cytoplasm, and then triggering the translocation of β-catenin into the nucleus. These data provide experimental evidence that the up-regulation of SIRT1 activity by MCPs led to an increased cytoplasmic deacetylation of β-catenin, which promoted translocation of β-catenin to the nucleus to participate in the signaling pathway involved in NSC proliferation. The present study reveals that MCPs function as a therapeutic drug to promote stroke recovery by increasing the activity of SIRT1, decreasing the level of acetylated β-catenin, promoting the nuclear translocation of β-catenin and thereby increasing endogenous NSC proliferation.

1. Introduction

Ischemic stroke is a leading cause of permanent disability worldwide. To date, tissue-plasminogen activator(t-PA) thrombolysis and mechanical thrombectomy are the sole proven and clinically definitive therapies for ischemic stroke, but only 3 % of patients' experience substantially benefit from thrombolytic therapy, largely due to the narrow therapeutic time window (4.5 h after stroke onset) and serious intracranial hemorrhagic complications (Saver et al., 2016; Schwamm et al., 2013). Exogenously transplanted mesenchymal stem cells (MSCs) or neural stem cells (NSCs) can improve functional recovery after stroke (Marei et al., 2018). To overcome the limitations of neuroprotective

Abbreviations: MCPs, momordica charantia polysaccharides; NSC, neural stem cell; t-PA, tissue-plasminogen activator; SIRT1, silent information regulator 2 homolog 1; SVZ, subventricular zone; SGZ, subgranular zone; PI3K, phosphatidylinositol-3-kinase; CNS, central nervous system; MCAO, middle cerebral artery occlusion; OGD, oxygen and glucose deprivation.

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strategies, neurorestorative therapies that can help to increase endogenous brain repair and improve stroke outcomes are being considered. Since spontaneous compensatory NSC proliferation is largely limited and insufficient for functional recovery after stroke(Ruan et al., 2015; Zhu et al., 2018), new strategies to achieve post-ischemic brain repair via the promotion of endogenous NSC proliferation are required.

The restoration of neurological function through replacement of damaged or lost cells is of great importance in the ischemic brain. It is thought that neurogenesis includes NSC proliferation and differentiation, with new neuron migration to the ischemic region. NSCs produce neurons throughout life in two regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus(Taupin and Gage, 2002). Ischemic stroke can activate adult NSCs, enhancing NSC proliferation in animal models and in patients with stroke (Eriksson et al., 1998; Reynolds and Weiss, 1992). Recently, multiple advances have been made in understanding the mechanisms of NSC proliferation after ischemic stroke. Researchers have focused on exploring the cellular signaling pathways related to NSC proliferation. Numerous pathways, including the phosphatidylinositol-3- kinase (PI3K) pathway (Koh and Lo, 2015), the Notch pathway (Engler et al., 2018), the Sonic hedgehog pathway (Hyun et al., 2013), and the Wnt/β-catenin pathway (Sanderson et al., 2013), can regulate endogenous neurogenesis. The activation of all of these pathways can promote the proliferation of NSCs, and drugs that can enhance NSC proliferation in stroke by affecting these pathways are being investigated.

As an obligate component of stem cell division and differentiation, the Wnt/β-catenin signaling pathway plays many pivotal roles in the central nervous system and participates in a variety of functions, including NSC proliferation and neuronal differentiation, development, and maturation. Recent evidence indicates that the Wnt/β-catenin pathway is involved in NSC proliferation in the adult brain (Aizman et al., 2013; Kalladka and Muir, 2014; Kuwabara et al., 2009). Shruster et al. demonstrated that Wnt/β-catenin signaling can induce NSC proliferation and improve neurological function after focal ischemic injury in a mouse model (Koh and Park, 2017). As a multiple structural/functional protein, β-catenin can mediate cell-cell adhesion and act as a key mediator of the canonical Wnt pathway (Cheng et al., 2020).

Sirtuins (SIRTs), or silent information regulator 2 (SIR2) proteins, are a family of proteins that regulate genetic information. There are seven sirtuins (SIRT1-SIRT7) in mammals that exhibit not only functional diversity but also different cellular localizations (SIRT1 and 2 in the cytoplasm, SIRT1, 2, 6 and 7 in the nucleus and mainly SIRT3, 4 and 5 in mitochondria). SIRT1 is the best-known sirtuin and is involved in many biological and pathological processes, including cell senescence, apoptosis, autophagy, oxidative stress, inflammation, and DNA damage repair (Guan et al., 2019; Rada et al., 2018; Shi et al., 2018; Singh et al., 2017; Testai et al., 2020; Willert and Nusse, 1998). SIRT1 is expressed widely in the brain and has important physiological roles and endogenous neuroprotective effects during brain ischemic injury(Wang et al., 2018). SIRT1 regulates multiple signaling pathways, including the MAPK, NF-κB, JAK/STAT, PI3K/Akt, and Notch pathways (Bai et al., 2015, 2018; Erkasap et al., 2017; Fan et al., 2018; Herskovits and Guarente, 2014; Liu et al., 2019). Among these pathways linked to nerve regeneration, the Wnt/β-catenin signal pathway has an especially important role. For example, SIRT1 can promote nuclear translocation of β-catenin and activate the Wnt/β-catenin signaling pathway involved in the regeneration of stem cells (Lévy et al., 2004; Simic et al., 2013). As a required component of stem cell division and differentiation, the Wnt/β-catenin signaling pathway plays pivotal roles in the CNS (Clevers et al., 2014; Sato et al., 2004). β-catenin deacetylation is considered the key feature in the activation of the canonical Wnt signaling pathway (Zhang et al., 2011). As a downstream target of SIRT1, β-catenin was verified to be deacetylated by SIRT1, which leads to its nuclear accumulation (Zhou et al., 2016). These observations imply that the effects of SIRT1 on neurogenesis are related to SIRT1-mediated β-catenin deacetylation and Wnt/β-catenin pathway activation. SIRT1 exerts its biological effects mainly by deacetylating various targets, with its deacetylation activity changing dynamically under different pathological conditions (Li et al., 2013; Michishita et al., 2005). Hence, the modulation of the deacetylation activity of SIRT1 via pharmacological compounds can have beneficial effects on NSC proliferation during ischemic stroke.

Momordica charantia (M. charantia, also known as bitter melon) is a classic vegetable crop in Asia. M. charantia is widely used in China to treat cardiovascular diseases, diabetes and cancer. These pharmacological effects of M. charantia depend on its various bioactive components, with its polysaccharides receiving much attention. Furthermore, M. charantia polysaccharides (MCPs) exert multiple pharmacological effects, including hypoglycemic, cholesterol-reducing, antioxidant, antitumor, and anti-obesity effects (Chan et al., 2005; Jayasooriya et al., 2000; Nerurkar and Ray, 2010; Shan et al., 2012). In our previous project using HPLC-MS/MS, the bioactive ingredients of MCPs were identified, including galacturonic acid, glucose, galactose, rhamnose, xylose, and mannose (Gong et al., 2015). Our further investigation showed that MCPs have neuroprotective roles in cerebral ischemia/reperfusion (I/R) via their antioxidant effects and their ability to inhibit JNK3 signaling cascades (Duan et al., 2015). However, whether MCPs affect stroke-induced NSC proliferation is unknown.

Here, we observed the pharmacological role of MCPs in improving the learning and memory abilities of rats subjected to intraluminal middle cerebral artery occlusion (MCAO). Next, we assessed if MCPs play a role in the proliferation of NSCs through in vivo and in vitro experiments. We sought to determine whether the increased level of the NSC proliferation mediated with MCPs is due to up-regulation of SIRT1 activity in cerebral I/R. Additionally, we aimed to further investigate the mechanisms by which SIRT1 regulates subcellular distribution of β-catenin in the proliferation of NSCs. Overall, the present study helps us to understand the underlying mechanism of MCPs in NSC proliferation and provides a basis for applying MCPs as a neurorestorative drug in treating cerebral ischemic stroke.

2. Results

2.1. MCPs rescued the memory and learning abilities of MCAO rats

To investigate the effects of MCPs on the spatial learning and memory deficits in MCAO rats, Morris water maze tests were used. The Morris water maze, which assesses the escape latency to the platform, the path efficiency to the first entry onto the platform, the number of platform area crossings and the swimming trajectories, is a test of the spatial learning of rodents that relies on the use of distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform. We conducted parallel Morris water maze tests of Control group, MCAO group and MCAO + MCPs group.

As shown in Fig. 1 (A–D), compared with the Control rats, the MCAO rats exhibited significantly increased escape latencies to platform (Fig. 1A) and reduced path efficiencies to first entry onto the platform (Fig. 1B), times across the platform (Fig. 1C) and swimming trajectories at the spatial probe phase (Fig. 1D). These results suggested that MCAO decreased the memory and learning abilities of the rats.

The animals were subjected to intragastric administration of MCPs (200 mg/kg) after MCAO once a day for 7 days. Compared with the MCAO rats, the MCAO + MCPs rats exhibited significantly reduced escape latencies to the platform (Fig. 1A) and increased path efficiencies to the first entry of the platform (Fig. 1B) and times across the platform (Fig. 1C) after I/R. Fig. 1D shows that the MCAO + MCPs group crossed more frequently than the MCAO group. These results suggested that MCPs rescued the memory and learning abilities of rats after I/R.

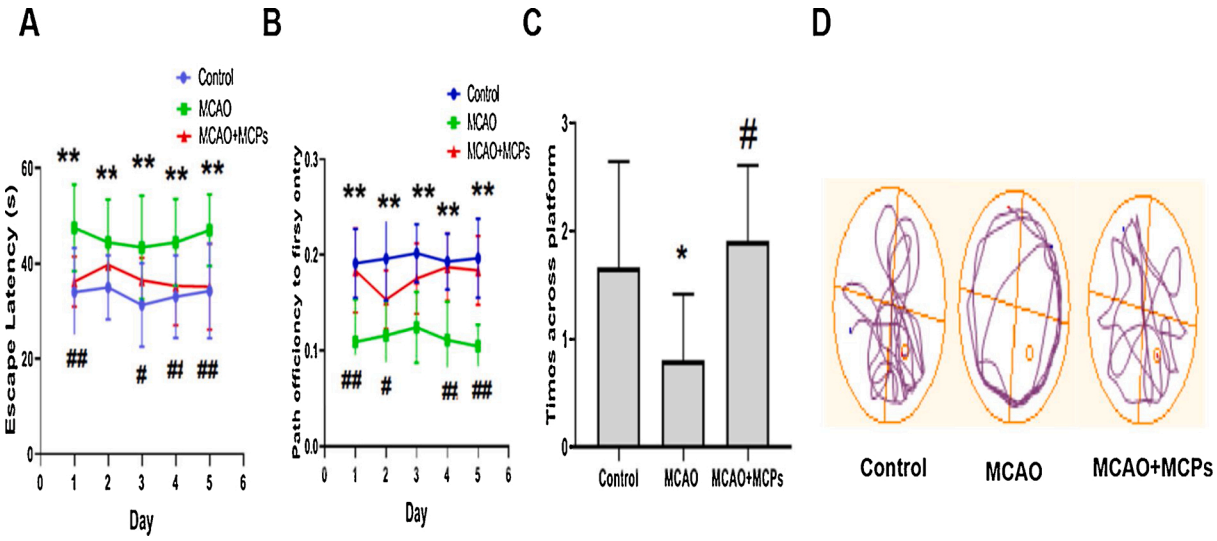


Fig. 1. MCPs ameliorated cognitive deficits in rats with MCAO. The animals were given intragastric administration with MCPs (200 mg/kg) for 7 days after MCAO. Morris water maze task: quantification of (A) the escape latency to the platform, (B) the path efficiency to the first entry of the platform, (C) times across the platform, and (D) representative swimming pattern. The data were expressed as means ± SD (n = 10–15). Control VS MCAO: $*P < 0.05$, $**P < 0.01$, MCAO VS MCAO + MCPs: $\#P < 0.05$, $\#\#P < 0.01$.

2.2. MCPs enhanced the proliferation of NSCs in vivo and in vitro

To analyze the effects of MCPs on the proliferation of NSCs in MCAO rats, we evaluated NSCs that were detected by Nestin immunofluorescence staining in the SVZ and SGZ. Immunofluorescence analysis showed that the MCAO + MCPs group exhibited enhanced NSC proliferation in the SVZ and SGZ compared with the MCAO group. (Fig. 2A, B).

To investigate whether MCPs enhance the proliferation of NSCs, we treated C17.2 cells with MCPs (0.5, 1.5, 2.5, 5, 8, 10, 20, or 25 μg/ml) under normal conditions. A CCK-8 kit was used to detect the cell proliferation in the different groups. We found that MCPs (0.5, 1.5, or 2.5 μg/ml) could increase the proliferation of C17.2 cells compared with water alone (Fig. 2C). We also used BrdU immunofluorescence staining

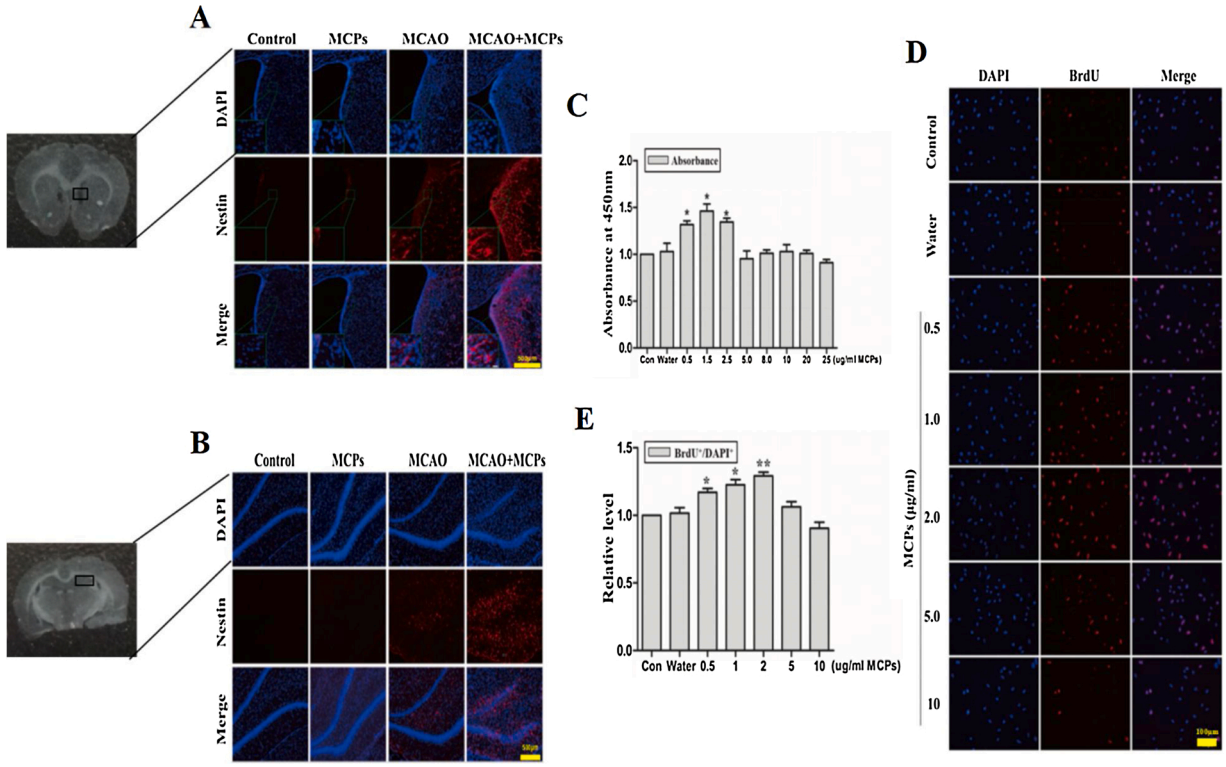


Fig. 2. MCPs regulated the NSC proliferation in vivo and in vitro. A,B: Immunostaining with anti-Nestin antibody and DAPI in SVZ(A) and SGZ(B) of MACO rats treated with MCPs (n = 5 rats), Scale bar = 500 μm. C: Cell proliferation was determined by CCK-8 assay and the number of C17.2 cells treated with MCPs was quantitatively analyzed (n = 3). D: Immunostaining with BrdU showed the effects of different concentrations of MCPs (water, 0.5, 1.0, 2.0, 5.0, 10.0 μg/ml) on C17.2 cells proliferation under normal condition, Scale bar = 100 μm.. E: Quantitative analysis of the number of $BrdU^+/DAPI^+$ C17.2 cells treated with MCPs. $*P < 0.05$, $**P < 0.01$ VS water.

to observe the effects of MCPs on the proliferation of NSCs.We treated C17.2 cells with MCPs (0.5, 1, 2, 5, or 10 μg/ml). We found that the groups treated with MCPs (0.5, 1, or 2 μg/ml) had more BrdU positive cells, indicating that MCPs (0.5, 1, or 2 μg/ml) could increase the proliferation of C17.2 cells compared with the Water group (Fig. 2D-E).

2.3. MCPs increased the activity of SIRT1 in vitro

To confirm the effect of MCPs on the proliferation of NSCs via SIRT1, the C17.2 cells were treated with MCPs (2.5, 5, 10 and 50 μg/ml) and/or EX527 after OGD6h/R24. The data showed that SIRT1 activity was increased in a dose-dependent manner in MCPs treatment group after OGD6h/R24 (Fig. 3A). We found that the expression level of SIRT1 was significantly increased after OGD treatment compared with Control group but MCPs treatment did not change the the expression level of SIRT1 after OGD/R (Fig. 3B, C). Accurate intracellular localization of protein is a critical part for their physiological function and regulation. SIRT1 was reported to be a nucleocytoplasmic shuttling protein (Charles et al., 2017). These findings suggested that nucleocytoplasmic shuttling of SIRT1 may play an important role in regulating NSC proliferation. Thus, we examined the effect of MCPs on the nucleocytoplasmic localization of SIRT1 in C17.2 cells. We found that MCPs promoted the shuttling of SIRT1 from the nucleus to the cytoplasm (Fig. 3B, C). In addition, to prove that MCPs enhance NSC proliferation via SIRT1, EX527, a potent and selective SIRT1 inhibitor, was used. First, we used a CCK-8 kit to detect the cell density in the different groups. We found that treatment with MCPs (5 μg/ml) increased the cell density compared with treatment with OGD alone, while EX527 decreased the cell density compared with MCPs (Fig. 3D). Second, we used BrdU to mark the proliferating cells. We found that the MCPs group (5 μg/ml) had more BrdU positive cells than the OGD group, while EX527 reduced the number of BrdU positive cells compared with MCPs (Fig. 3E, F). MCPs increased the proliferation of the C17.2 cells, and EX527 reversed the effect of MCPs on the responses to OGD, suggesting that MCPs and EX527 had opposing effects on SIRT1 deacetylase activity.

2.4. MCPs decreased the level of acetylated β-catenin and increased the accumulation of β-catenin in the nucleus in vitro

To elucidate if MCPs regulate the proliferation of NSCs by affecting

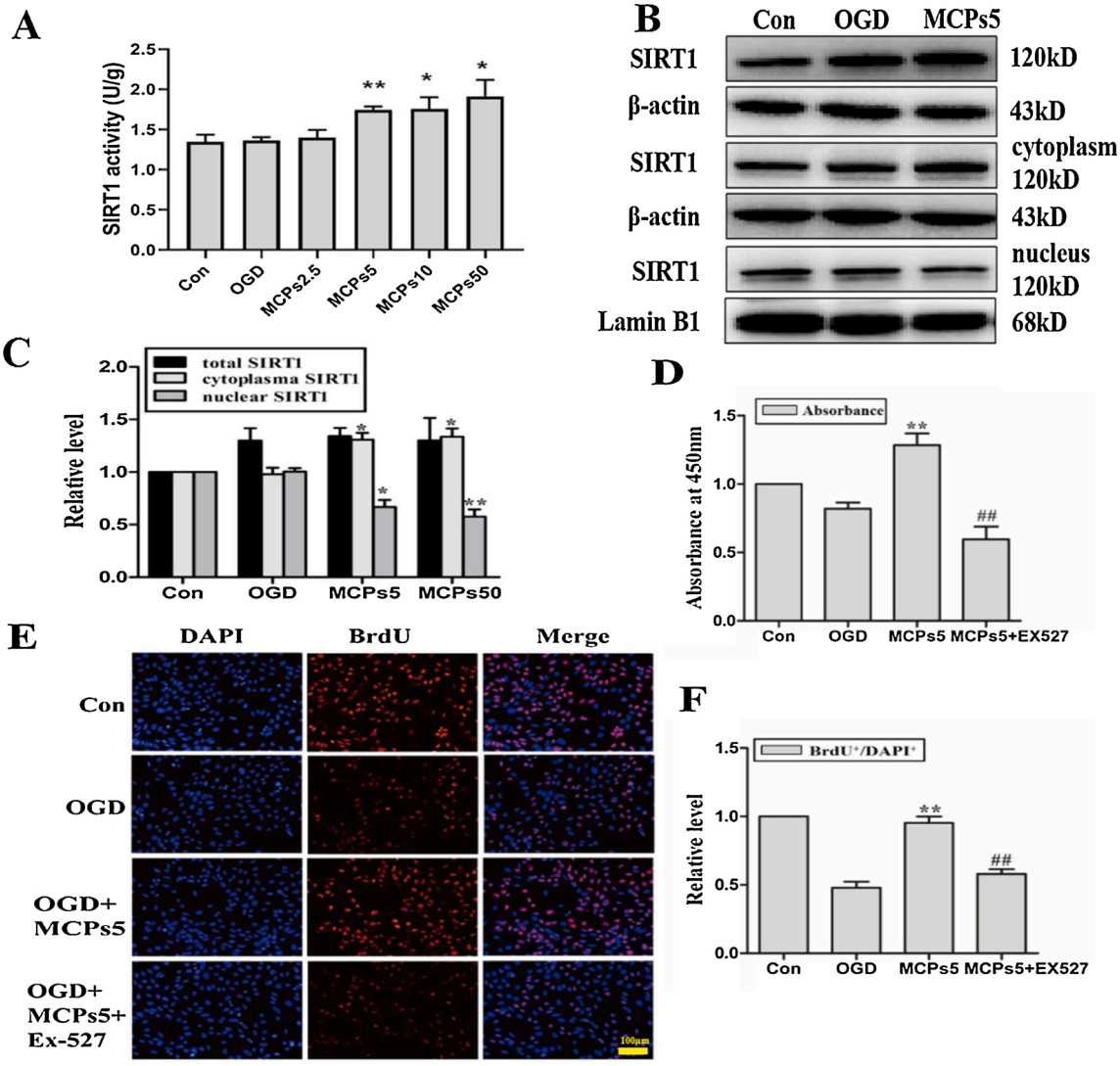


Fig. 3. The effects of MCPs on the SIRT1 activity and NSC proliferation under OGD condition. (A) C17.2 cells were treated with MCPs (2.5, 5, 10 and 50 μg/ml) under OGD for 6 h and reoxygenation for 24 h condition or without MCPs under normal condition for 24 h, the activities of SIRT1 were measured using SIRT1 activity assay kit (n = 3). (B, C) MCPs regulated nucleocytoplasmic distribution of SIRT1 under OGD condition (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ VS OGD. (D, E, F) MCPs promoted the proliferation of C17.2 cells under OGD condition. (D) Cell density was detected by CCK-8 assay. (n = 6) (E, F) Immunofluorescence analysis confirmed the effects of MCPs on the proliferation of C17.2 cells. (n = 3) $^{**}P < 0.01$ VS OGD, $\#\#P < 0.01$ VS MCPs5.

the posttranslational modification and subcellular distribution of β-catenin, the level of acetylated β-catenin in the cytoplasm and the accumulation of β-catenin in the nucleus under normal and OGD conditions were examined. The C17.2 cells were treated with MCPs (5 or 50 μg/ml) after OGD6h/R24 or without MCPs under normal conditions for 24 h. We extracted the cytoplasmic proteins from each group. The samples were immunoprecipitated with an anti-acetyl lysine antibody and then immunoblotted with an anti-β-catenin antibody. As shown in Fig. 4A, we found that MCPs decreased the level of acetylated β-catenin in the cytoplasm. It has been reported that deacetylating β-catenin can promote the nuclear translocation of β-catenin in mesenchymal stem cells (32). Therefore, we hypothesized that MCPs decreased the level of acetylated β-catenin in the cytoplasm and then promoted the nuclear translocation of β-catenin. To prove this hypothesis, we detected the expression levels of β-catenin in the cytoplasm and nucleus after MCPs treatment. As shown in Fig. 4A, B, MCPs decreased the expression of β-catenin in the cytoplasm and promoted the accumulation of β-catenin in the nucleus after OGD exposure. These results indicated the possibility that MCPs promoted the nuclear translocation of β-catenin by decreasing the level of acetylated β-catenin in the cytoplasm.

2.5. MCPs regulated the nucleocytoplasmic localization of β-catenin through SIRT1 deacetylase activity

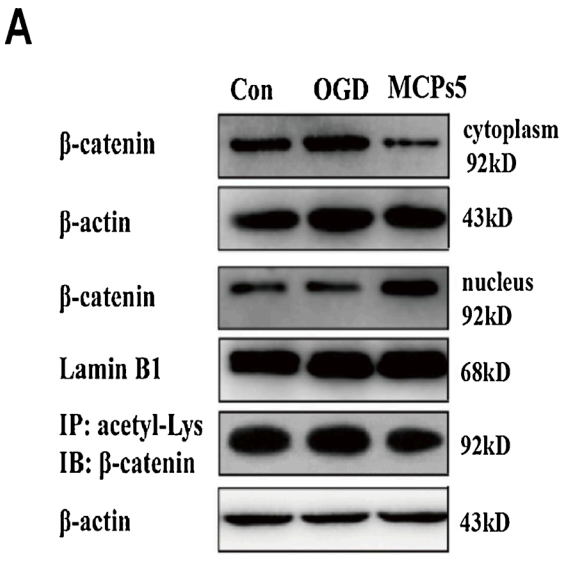
To elucidate if MCPs promoted the nuclear translocation of β-catenin by increasing the deacetylase activity of SIRT1, we used EX527, a potent and selective SIRT1 inhibitor, to reverse the effects of MCPs in stimulating SIRT1 activity under OGD conditions and the subcellular distribution of β-catenin. The levels of acetylated β-catenin in the cytoplasm and the accumulation of β-catenin in the nucleus were examined. The C17.2 cells were treated with MCPs (5 or 50 μg/ml) and/or EX527 (1 μmol/L) under OGD6h/R24 or without MCPs under normal conditions for 24 h. We extracted the cytoplasmic proteins from each group. The samples were immunoprecipitated with an anti-acetyl lysine antibody and then immunoblotted with an anti-β-catenin antibody. As shown in Fig. 5A, B, compared with those in the MCPs group, the effects of MCPs on decreasing the level of acetylated β-catenin in the cytoplasm were reversed in the MCPs + EX527 group. We also detected the expression levels of β-catenin in the cytoplasm and nucleus after MCPs + EX527 treatment. As shown in Fig. 5C, D, we found that the effects of MCPs on the promotion of the nuclear translocation of β-catenin after OGD were reversed in the MCPs + EX527 group.

3. Discussion

Previous studies have demonstrated that MCPs have ROS-scavenging and antioxidant capacities that contribute to their antitumor and hypoglycemic effects (Guan and Lingxiao, 2012; Liu et al., 2014) and their roles in protecting against cerebral ischemia-reperfusion injury(Gong et al., 2015). In this study, we reported for the first time that MCPs showed a neurorestorative effect by promoting the proliferation of NSCs both in vivo and in vitro. The potential molecular mechanism by which MCPs promote neuroproliferation is to act as a SIRT1 activator, increasing SIRT1 deacetylase activity, and subsequently causing decreased β-catenin acetylation, and enhancing the nuclear accumulation of β-catenin, thereby inducing endogenous NSC proliferation and resulting in improved neurological functional recovery after stroke.

It is generally accepted that ischemic stroke leads to severe neural impairment and learning and spatial memory defects. Previous studies have demonstrated that learning and memory function correspond with hippocampal neurogenesis (Winocur et al., 2006). In the present study, behavioral results indicated that rats treated with MCPs exhibited improved memory and learning abilities compared with normal SD rats (data not shown). Moreover, the behavioral results showed that MCPs partially reversed the MCAO-induced memory and learning deficits and improved cognitive functional recovery (Fig. 1 A-D). Previous studies by our coworkers have demonstrated that MCPs have neuroprotective roles in cerebral I/R via their antioxidant effects and their abilities to inhibit JNK3 signaling cascades(Gong et al., 2015). Hence, we considered that the effects of MCPs on both neuroprotection and NSC proliferation contributed to improving the learning ability and memory function of rats subjected to MCAO and that MCPs exerted a neurorestorative effect and improved stroke outcome. However, the mechanisms involved in the MCPs-induced proliferation of NSCs are not well established.

Given the narrow therapeutic time window for the treatment of acute ischemic stroke, promotion of post-stroke brain function repair is critical for stroke outcome. Elucidating the mechanisms underlying the activation of NSC self-renewal, proliferation and neurogenesis is crucial for developing new strategies to promote post-stroke functional brain repair. SIRT1 is a nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylase in mammalian cells (Blander and Guarente, 2004). SIRT1 can deacetylate numerous mammalian transcription factors, such as p53 and NF-kB, thus regulating cell differentiation, survival, stress resistance and metabolism through the deacetylation of target molecules (Imai and Guarente, 2014). Many experiments have demonstrated that SIRT1 is an important regulatory protein in



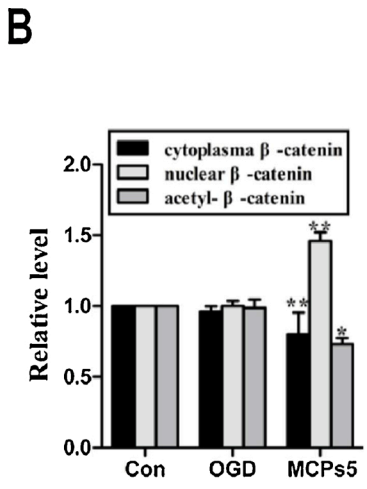


Fig. 4. The effects of MCPs on the acetylation and nucleocytoplasmic localization of β-catenin in C17.2 cells. (A) C17.2 cells were treated with MCPs (5 μg/ml) under OGD for 6 h and reoxygenation for 24 h condition or without MCPs under normal condition for 24 h. Expression of β-catenin was examined by Western blotting with anti-β-catenin antibody and acetylated β-catenin was detected by immunoprecipitation. (B) The statistical analysis of the levels of cytoplasma β-catenin, nuclear β-catenin and acetyl-β-catenin. (n = 3), *$P < 0.05$, ** $P < 0.01$ VS OGD.

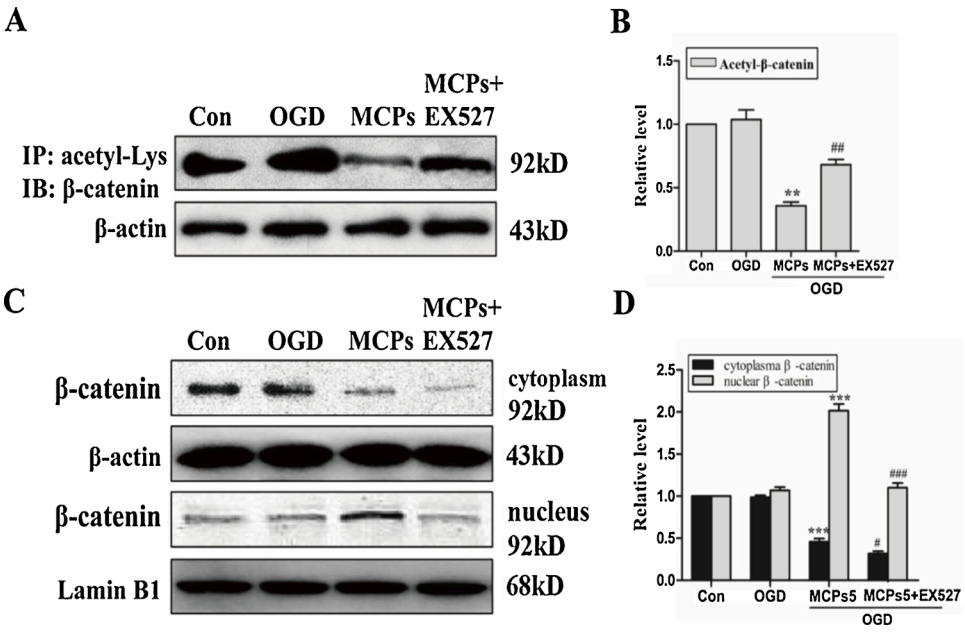


Fig. 5. MCPs regulated the nucleocytoplasmic localization of β-catenin by the SIRT1 deacetylase activity. (A, C) C17.2 cells were treated with MCPs (5 μg/ml) with/without EX527 (1 μmol/L) under OGD for 6 h and reoxygenation for 24 h condition or without MCPs under normal condition for 24 h. Expression of β-catenin was examined by Western blotting with anti-β-catenin antibody and acetylated β-catenin was detected by immunoprecipitation. (B, D) The statistical analysis of the levels of cytoplasma β-catenin, nuclear β-catenin and acetyl-β-catenin. The results were given as means ± SD. The data were obtained from no less than three independent experiments. (n = 3), $^{**}P<$ 0.01 VS OGD, $^{***}P<$ 0.001 VS OGD, $\#P<$ 0.05 VS MCPs5, $\#\#P<$ 0.01 VS MCPs5, $\#\#\#P<$ 0.001 VS MCPs5.

neurogenesis. Thus, Udomruk et al. found that sesamin increases the level of SIRT1 and plays a role in enhancing the effect of NGF on inducing neurogenesis, furthermore, these effects were blocked by the specific SIRT1 inhibitor JGB1741 (Udomruk et al., 2020). Sellner et al. found that pharmacological activation of SIRT1 improves adult neurogenesis and leads to enhanced performance of cx3cr1(-/-) mice in a hippocampus-dependent learning and memory task (Sellner et al., 2016). In this study, we evaluated the potential neuroprotective effects of MCPs on neurobehavioral outcomes. The findings provided evidence that MCPs might exert neuroprotection against I/R injury both in vitro and in vivo, possibly by promoting NSC proliferation. To further explore whether or not the effects of MCPs described here were related to SIRT1, we conducted invitro experiments indicating that MCPs could increase SIRT1 activity in a dose-dependent manner (Fig. 3A). These results demonstrate that MCPs could indeed promote NSC proliferation. Considering the effects of MCPs on SIRT1 activity and NSC proliferation, we speculated that MCPs promote NSC proliferation by increasing the deacetylase activity of SIRT1.

It has been reported that salvianolic acid B, magnolol, melatonin, and others can play neuroprotective roles in ischemia/reperfusion injuries by increasing SIRT1 expression, and that these effects can be reversed by EX527, a specific SIRT1 inhibitor (Kou et al., 2017; Lv et al., 2015; Wang et al., 2020; Yang et al., 2015). Additionally, overexpression of SIRT1 leads to increases in neurite length, whereas the SIRT1 inhibitor EX527 has the opposite effect (Lee et al., 2018). Therefore, we used EX527 in the present study to inhibit the activity of SIRT1 and explored the mechanism of the effects of MCPs on NSC proliferation. We observed that EX527 could reduce the effects of MCPs on stimulation of SIRT1 activity and effectively prevent MCP-induced proliferation of C17.2 cells under OGD conditions, verifying that MCPs promoted NSC proliferation via stimulating SIRT1 activity (Fig. 3D-F). Our data suggested that MCPs do increase SIRT1 activity but do not alter the expression level of SIRT1 under OGD conditions (Fig. 3B-C), indicating that MCPs could increase SIRT1 activity by regulating the structure or subcellular distribution of SIRT1 rather than its expression. Although the underlying mechanism regarding how MCPs enhanced SIRT1 activity was not revealed in the present study, there is some evidence that several bioactive ingredients in plants known to be allosteric activators of SIRT1,such as resveratrol (RSV), fisetin, and luteolin, can all directly achieve an increase in SIRT1 activity without increasing the levels of SIRT1 protein in cells(Cao et al., 2015; Jin et al., 2014; Rafacho et al., 2015). Given their free radical scavenging activities, it is not surprising that all these plant bioactive ingredients exhibit potent anti-oxidative and anti-inflammatory properties, and consequently counteract cellular senescence in a SIRT1-dependent manner. A recent study revealed that RSV improved oxidative stress and mitochondrial dysfunction in myocardial tissue or cardiomyocytes, mostly through SIRT1-mediated PGC-1α deacetylation (Khanna et al., 2020). Our previous study about the protective effect of RSV demonstrated that RSV attenuates cerebral ischemia/reperfusion damage, which is mediated partly through SIRT1 activation (data not show). However, these findings differ somewhat from those obtained by Pacholec et al. suggesting that resveratrol was not a direct activator of SIRT1 (Pacholec et al., 2010). Because SIRT1 is an enzyme, mechanisms regulating its modification are diverse and complex, including altering its expression, its conformation after interacting with other protein partners, the availability of its substrates, or its posttranslational modification (phosphorylation, methylation, SUMOylation, and nitrosylation) (Revollo and Li, 2013). Therefore, we speculated that MCPs and other bioactive ingredients in plants could influence SIRT1 activity either by direct interaction with SIRT1 or by indirect regulation through other mediators. Certainly, further studies are needed to corroborate more detailed mechanisms for MCPs regulation on SIRT1 activity. However, we chose to omit them from this study and to focus instead on SIRT1′s impact on the regulation of specific downstream targets.

β-catenin is a downstream target of SIRT1 deacetylation, whose roles are regulated by SIRT1 activity(Zhou et al., 2015). Under physiological conditions, proper transport of a protein to its final cellular destination is known to be crucial for its function. Only in the nucleus does β-catenin exert its functions, and deacetylation of β-catenin promotes its accumulation in the nucleus (Bai et al., 2014; Ma et al., 2017). Thus, the appropriate localization of β-catenin is crucial. Studies have characterized the mechanism by which the subcellular localization of β-catenin is regulated via nuclear-cytoplasmic shuttling (Winocur et al., 2006). In this study, we found that treatment with MCPs significantly decreased the β-catenin levels in the cytoplasm and increased its nuclear accumulation.

Therefore, we speculated that MCPs promoted neuroproliferation by increaseing SIRT1 deacetylase activity, which decreased the level of acetylated β-catenin. This decreased β-catenin acetylation enhanced the accumulation of β-catenin in the nucleus, leading to endogenous NSC proliferation after stroke. To verify this speculation, we observed changes in the deacetylation of β-catenin in the cytoplasm and the

nuclear translocation of β-catenin after treatment with MCPs or MCPs + EX527 under OGD conditions. We found that MCPs increased SIRT1 activity in a dose-dependent manner and then, as a result, increased the deacetylation of β-catenin in the cytoplasm. Additionally, we found that MCPs triggered the accumulation of β-catenin in the nucleus (Fig. 4A-B). We found that MCPs increased the deacetylation of β-catenin in the cytoplasm and triggered the accumulation of β-catenin in the nucleus. But EX527 could reverse all these effects of MCPs (Fig. 5A-D). These findings verified our speculation that MCPs decreased the level of acetylated β-catenin and enhanced the accumulation of β-catenin in the nucleus by increasing the deacetylase activity of SIRT1 (Fig. 6).

Adult neurogenesis plays a crucial role in functional recovery after stroke. Understanding the mechanisms by which SIRT1 regulates neurogenesis may aid the improvement of efficient therapies for renewal after stroke. Drugs targeting SIRT1 may emerge as a promising approach to facilitate brain repair processes. In this experimental study, we observed that MCPs could regulate SIRT1 activity in vitro. Additionally, our results showed that MCPs stimulated the proliferation of C17.2 cells under OGD conditions and enhanced the proliferation of NSCs in the SVZ and SGZ of rats during ischemia/reperfusion. We found that MCPs increased the activity of SIRT1, increased the deacetylation of β-catenin and triggered its translocation into the nucleus after OGD. Additionally, EX527, a selective SIRT1 inhibitor, reversed the effects of MCPs, which suggested that MCPs promoted NSC proliferation by regulating SIRT1 deacetylase activity. These data provided experimental evidence that MCPs function as a therapeutic drug to promote stroke recovery by improving the endogenous mechanisms of neurogenesis.

4. Materials and methods

4.1. Antibody and reagents

The following primary antibodies from Abcam Biotechnology, Inc. were used: anti-SIRT1 antibody (19A7AB4) (ab110304), anti-Nestin antibody (2Q178) (ab6142), goat anti-rat IgG H&L antibody (ab150157), anti-β-catenin antibody (ab32572), and anti-acetyl Lysine (ab22550) antibody. Monoclonal anti-Lamin B1 (#13,435) antibody was obtained from Cell Signaling Biotechnology, Inc. Anti-BrdU antibody (B35138), labeled donkey anti-rabbit IgG antibody, donkey anti-mouse IgG (H + L) (A-31570), goat anti-rabbit IgG (H + L) (A-21429) were purchased from Life technologies. Mouse anti-β-actin monoclonal antibody (TA-09) was bought from ZSGB-Bio Co., Ltd. The secondary antibodies used in our experiment were goat anti-mouse IgG and goat anti-rabbit IgG, which were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bromodeoxyuridine (BrdU) were obtained from Sigma-Aldrich. EX527 was acquired from BioVision Technologies, Inc.

4.2. Cultured neural stem cells

C17.2 cells, a multipotent 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). The C17.2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10 % fetal bovine serum (FBS; Gibco) and 2 mM L-glutamine (Sigma-Aldrich) at 37 °C in humidified incubators with a 5 % CO_2 atmosphere. In the BrdU experiment, MCPs were added to the C17.2 cells for 24 h, and then, the immunofluorescence was observed after 8 h of BrdU labeling. In the CCK-8 (Solarbio) experiment, MCPs were added to the C17.2 cells for 24 h, and then, we added 10 μl CCK-8 to each well of 96-well plates, and incubated the plates for 0.5 hours–4 hours. Finally, we detected the absorbance at 450 nm.

4.3. Oxygen and glucose deprivation (OGD)

To mimic cerebral ischemia/reperfusion in vitro, we conducted an OGD experiment. Briefly, the cells were moved into an incubator with a 5 % CO_2 and 1.2 % O_2 atmosphere and cultured in no-glucose DMEM for 6 h. Then, the cells were moved back to normoxic incubators and cultured in normal medium for 24 h.

4.4. Cellular immunofluorescence staining

The cells were fixed with 4 % paraformaldehyde solution for 20 min at room temperature (RT) after washing with PBS. For BrdU detection, the cells were subjected to acid hydrolysis using 2 N HCl for 30 min at RT. Then, after blocking with a buffer (PBS with 10 % goat serum and 0.5 % Triton X-100) for 2 h at RT, the cells were incubated with the

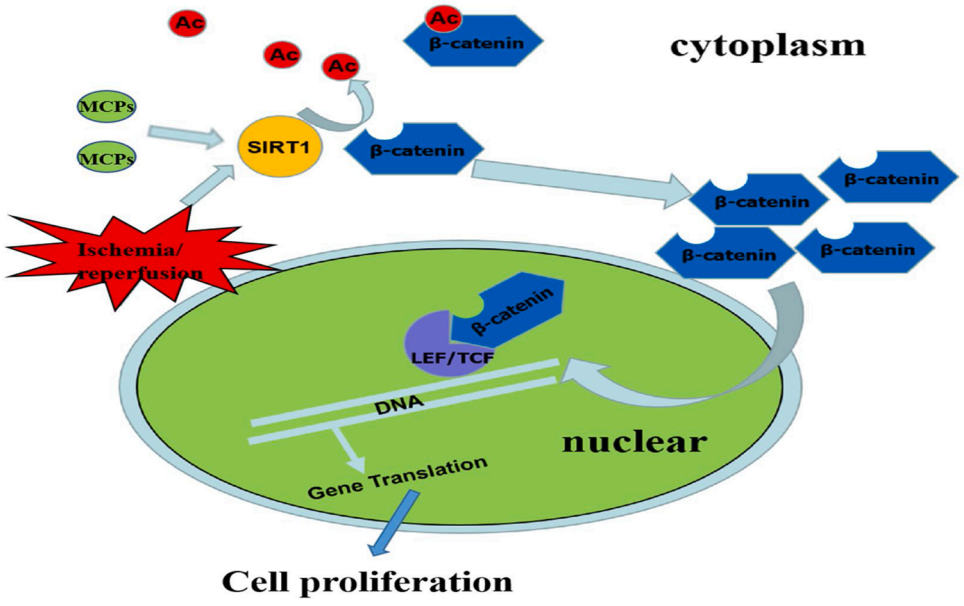


Fig. 6. The possible molecular mechanism for MCPs promoting the proliferation of neural stem cells. MCPs act as a SIRT1 activator to increase SIRT1 enzymatic activity and decrease the level of acetylation of β-catenin, which enhances the accumulation of β-catenin in the nucleus, consequently leading to the endogenous NSC proliferation.

primary antibody, namely, anti-BrdU (diluted 1:200), overnight at 4 °C. Fluorescent secondary antibodies were incubated with the cells for 2 h at RT, and DAPI (10 mg/mL) was added 30 min before the end of the incubation. Images of 10 randomly selected fields of view were captured under a fluorescence microscope and analyzed by Image-Pro Plus 6.0 software to calculate the percentage of positive cells.

4.5. Animal model of ischemia and reperfusion

A total of 80 adult male Sprague Dawley rats weighing 240~280 g were provided by Shanghai Experimental Animal Center, Chinese Academy of Science. All the animal protocols were in accordance with the institutional guidelines, and the experimental procedures were approved by the Animal Ethics Committee of Xuzhou Medical University (Approval ID: SCXK (SU) 2010-0003, 25 October 2010). The rats were maintained in standard cages in a controlled environment (temperature: 24 ± 1 °C; relative humidity: 50~60 %; light period: 06:00~18:00) and given access to food and water ad libitum. The rats were used in the study after three days of acclimatization. The middle cerebral artery occlusion (MCAO) stroke model was established as described previously (Wei et al., 2018).

4.6. Administration of drugs

EX527 was dissolved in DMSO and then diluted with DMEM before use. MCPs were extracted from MC with water extraction by sonication and alcohol precipitation, followed by the elimination of proteins and starch(Hong-Man et al., 2011). The contents of the MCPs were analyzed by gas chromatography (GC) of acetylated aldononitriles and high-performance liquid chromatography (HPLC). The animals were subjected to intragastric administration of MCPs (200 mg/kg) after MCAO once a day for 7 days. For invitro experiments, cells were incubated with MCPs (5, 50 μg/ml) or EX527 (1 μmol/L) for 30 min before OGD/R.

4.7. Morris water maze (MWM) tests

Seven days after cerebral I/R induction, MWM tests were conducted to evaluate learning and memory performance via a spatial probe test and a hidden platform test. The MWM apparatus was a pool (150 cm in diameter, 60 cm in height) filled with water (22 ± 0.5 °C) to a depth of 35 cm. The apparatus was divided into four quadrants, and an invisible platform (10 cm in diameter) was submerged 1.5 cm below the water surface in one quadrant. All the rats were trained to find the platform for 5 consecutive days (90 s/day), and then, the learning performance was assessed by measuring the time (escape latency) required to find the platform and the path efficiency to the first entry onto the platform for 5 days. During the 5 days, the animals were subjected to intragastric administration of MCPs (200 mg/kg) once a day. On the 6th day, the platform described above was removed, and the rats underwent a probe trial. The number of times the rats crossed the platform area (times across the platform) and the swimming trajectory at the spatial probe phase (swimming trajectory) were observed. The escape latency, path efficiency to first entry, times across the platform, and swimming trajectory were recorded by a video tracking system.

4.8. Sample preparation

The cells were washed 3 times with cold PBS and allowed to incubate with 300~400 μL of RLN lysis buffer (containing 50 mM Tris−HCl, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5 % Nonidet P-40, 1 mM Na_3VO_4, 1 mM p-nitrophenyl phosphate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin) for 20 min before collection. The mixture was then centrifuged at 3000 *g* for 10 min at 4 °C to isolate the supernatant, which contained the cytoplasmic proteins. The pellet was washed by adding 400 μL of RLN lysate and centrifuged at 3000 *g* for 10 min at 4 °C. All of the supernatant was discarded. Then, 100 μL of RIPA lysis buffer (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 phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin) was added, mixed and allowed to stand for 10 min. Then, after 3 ultrasonic treatments for 5 s at intervals of 5 s and centrifugation at 12,000 *g* for 20 min at 4 °C, the supernatant was extracted as the desired nuclear protein.

4.9. Immunoprecipitation

The protein samples were preincubated for 1 h with 20 μl of protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) at 4 °C and centrifuged to remove the proteins that adhered nonspecifically to protein A. The supernatants were incubated with 1−2 μg of primary antibodies for 4 h or overnight at 4 °C. Protein A was added to the tube for another 2 h of incubation. The samples were centrifuged at 10,000 *g* for 2 min at 4 °C, and the pellets were washed three times with immunoprecipitation buffer. The bound proteins were eluted by boiling at 100 °C for 5 min in SDS-PAGE loading buffer and then isolated by centrifugation. The supernatants were used for immunoblot analysis.

4.10. Assessment of SIRT1 activity

SIRT1 activity was measured using the SIRT1 activity assay kit (Cell SIRT1 Colorimetry Assay Kit) (GenMed Scientifics Inc., U.S.A.) following the manufacturer's instructions.

4.11. Immunoblotting

The proteins were separated on polyacrylamide gels and then electrotransferred to polyvinylidene fluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking in Tris-buffered saline with 0.1 % Tween-20 (TBST) and 3 % bovine serum albumin for 3 h, the membranes were incubated with primary antibodies in TBST containing 3 % bovine serum albumin overnight at 4 °C. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies in TBST for 1 h at RT. After washing, the membranes were developed with the Immobilon ECL Ultra Western HRP Substrate (Merck KGaA) by chemiluminescence and imaged by a digital imaging system (Bio-Rad Laboratories, Inc). The density of the bands on the membrane was analyzed with ImageJ analysis software (Softonic International S.A.).

4.12. Immunofluorescence staining of brain slices of the SVZ and SGZ

The rats were deeply anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and then transcardially perfused with normal saline followed by ice-cold 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were quickly removed and fixed with the same fixation solution overnight at 4 °C. The postfixed brains were immersed in 30 % sucrose solution for dehydration, and coronal frozen sections (20 μm thick) were prepared with a microtome.

After washing with 0.01 M PBS, the sections were incubated with blocking buffer (PBS with 10 % goat serum and 0.5 % Triton X-100) for 2 h at RT. The sections were incubated with the primary antibody (anti-Nestin, diluted 1:300) overnight. After washing with PBS, the appropriate fluorescent secondary antibodies were added to the sections and incubated for 2 h at RT. DAPI (10 mg/mL) was added 30 min before the end of the incubation period. The sections were observed after washing with PBS, and images were captured with a Carl Zeiss Axio Observer Z1 fluorescent imaging system. Finally, the images were analyzed by Image-Pro Plus 6.0 software.

Data analysis and statistics

The values were expressed as the means ± S.D. The statistical analysis of the results was carried out by Student's *t*-test or one-way analysis of variance, followed by Duncan's new multiple range method or the Newman-Keuls test. *P*-values < 0.05 were considered significant.

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CRediT authorship contribution statement

Juyun Ma: Conceptualization, Methodology, Writing - original draft. **Haidi Fan:** Conceptualization, Methodology, Writing - original draft. **Heng Cai:** Methodology. **Zhaoli Hu:** Writing - review & editing, Visualization. **Xiaoling Zhou:** Methodology. **Fengying Li:** Methodology. **Hansen Chen:** Methodology. **Jiangang Shen:** Conceptualization, Project administration. **Suhua Qi:** Conceptualization, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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