Regular Article

Neuroprotection by B355252 against Glutamate-Induced Cytotoxicity in Murine Hippocampal HT-22 Cells Is Associated with Activation of ERK3 Signaling Pathway

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Glutamate differentially affects the levels extracellular signal-regulated kinase (ERK)1/2 and ERK3 and the protective effect of B355252, an aryl thiophene compound, 4-chloro-*N*-(naphthalen-1-ylmethyl)-5-(3-(piperazin-1-yl)phenoxy)thiophene-2-sulfonamide, is associated with suppression of ERK1/2. The objectives of this study were to further investigate the impact of B355252 on ERK3 and its downstream signaling pathways affected by glutamate exposure in the mouse hippocampal HT-22 neuronal cells. Murine hippocampal HT22 cells were incubated with glutamate and treated with B355252. Cell viability was assessed, protein levels of pERK3, ERK3, mitogen-activated protein kinase-activated protein kinase-5 (MAPKAPK-5), steroid receptor coactivator 3 (SRC-3), p-S6 and S6 were measured using Western blotting, and immunoreactivity of p-S6 was determined by immunocytochemistry. The results reveal that glutamate markedly diminished the protein levels of p-ERK3 and its downstream targets MK-5 and SRC-3 and increased p-S6, an indicator for mechanistic target of rapamycin (mTOR) activation. Conversely, treatment with B355252 protected the cells from glutamate-induced damage and prevented the glutamate-caused declines of p-ERK3, MK-5 and SRC-3 and increase of p-S6. Our study demonstrates that one of the mechanisms that glutamate mediates its cytotoxicity is through suppression of ERK3 and that B355252 rescues the cells from glutamate toxicity by reverting ERK3 level.

Key words extracellular signal-regulated kinase (ERK)3; MK-5; B355252; glutamate; cytotoxicity; mechanistic target of rapamycin (mTOR)

INTRODUCTION

Glutamate, the most abundant endogenous excitatory neurotransmitter in brain, plays a crucial role in neuronal tissue damage in neurodegenerative diseases. Under normal physiological condition, glutamate regulates memory, learning, cognitive, emotional, endocrine and other visceral functions.[1] However, under pathological conditions, when excessive release of glutamate or impaired uptake of glutamate occurs, the sharp increased glutamate in extracellular space will bind to glutamate ionotropic receptors including α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) and kinate. This results in a massive influx of calcium into cells, which subsequently catalyzes cell signaling cascades, induces mitochondrial dysfunction, increases formation of reactive oxygen species (ROS), and eventually leads to cell death.[2,3] This phenomenon is termed as glutamate excitotoxicity. Glutamate excitotoxicity are involved in the pathogenesis of almost all neurological diseases, including cerebral hemorrhage, cerebral ischemia, stroke, cerebral trauma, brain tumors, and neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD). Huntington's disease and behavioral diseases such as attention deficit hyperactivity disorder (ADHD).[4,5] Unfortunately, current therapeutic approaches for these devastating diseases are scarce, with only limited and non-sustainable benefits. Thus, there is an urgent need for innovative and effective therapeutic interventions to improve treatment outcomes for these disorders.

Mitogen-activated protein kinases (MAPKs) regulate mitosis, metabolism, gene expression, proliferation, growth and death.[6] MAPKs can be activated by a variety of factors, including growth factors, serum hormones, cytokines, and related stress responses (UV irradiation, hypoxia, and heat shock).[7] Extracellular regulated protein kinases (ERK) is one of the main members of MAPK family. ERK1 and 2 have about 84% amino acid homology and most of the same functions, so they were called ERK1/2.[8] ERK1/2 mediated cellular signal transduction pathway may be a common pathway of multiple stimuli, which is closely related to the survival of neurons. Phosphorylation activates ERK1/2[9] and then the activated ERK1/2 translocate into the nucleus, acting on transcription factors to promote the transcription and expression of certain genes and participate in a variety of biological responses.[6] Previous study has shown that inhibition of glutamate-caused activation of ERK1/2 protects cells from glutamate cytotoxicity.[10] The results suggest that ERK1/2 play a detrimental role in glutamate toxicity.[11,12]

ERK3, also known as MAPK6, is a new member of MAPK subfamily.[13] It is regulated by cytokines *via* c-Jun-mediated transcription. ERK3 modulates the expressions of steroid receptor coactivator 3 (SRC-3) and vascular endothelial growth

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factor receptor 2 (VEGFR2), thereby promoting cell migration, proliferation and angiogenesis.[14] ERK3 has been shown to promote lung cancer cell invasiveness by regulating site-specific phosphorylation of SRC-3.[15] However, there are no reports on ERK3 signaling pathway in neuronal cells.

Arylthiophene compound, 4-chloro-*N*-(naphthalen-1-ylmethyl)-5-(3-(piperazin-1-yl)phenoxy)thiophene-2-sulfonamide (aka B355252) was first synthesized by Dr. A.L. Williams in Biomanufacturing Research Institute and Technology Enterprise (BRITE) at North Carolina Central University in 2010.[16] This compound was initially used to potentiate the neurite growth when applied together with nerve growth factor (NGF) in PC12 cell derived NS-1 cells.[16] Later, it is demonstrated that B355252 protects murine hippocampal neuronal HT-22 cells against glutamate mediated cytotoxicity, 6-hydroxydopamine induced cell death, and cobalt chloride induced chemical hypoxic damage.[17] To date, the known mechanisms of B355252 action include inhibition of calcium influx into the cell, reduction of ROS formation, stabilization of mitochondrial membrane potential, decreases of apoptosis inducing factor (AIF) nuclear translocation, BAX formation, caspase-3 activation, and limitation of autophagy induction.[17,18] Interestingly, B355252 differentially affects MAPK expression. While B355252 completely ablated the glutamate-caused ERK1/2 activation, it blocked glutamate-induced inhibition of ERK3.[17] The objectives this study were to further investigate the impact of B355252 on ERK3 and its downstream signaling pathways affected by glutamate exposure in HT-22 cells and to compare its effect with glutamate NMDA receptor blocker MK-801.

MATERIALS AND METHODS

Chemicals Dulbecco's modified Eagles medium (DMEM; ATCC, Manassas, VA, U.S.A.), phosphate buffered saline (PBS), fetal bovine serum (FBS), Trypsin-Versene mixture (containing 0.05% porcine trypsin and ethylenediaminetetraacetic acid (EDTA)), and L-glutamine solution were purchased from HyClone Laboratories (GE Healthcare Life Sciences, Logan, UT, U.S.A.). Penicillin (10000 U/mL, equivalent of 6250 μg/mL) and streptomycin (10000 μg/mL) stock solution was obtained from Lonza (Lonza, Basel, Switzerland). Glutamate was purchased from MP (MP Biomedicals, LLC., Santa Ana, CA, U.S.A.). MK-801 was obtained from Sigma (St. Louis, MO, U.S.A.), and B355252 was synthesized in BRITE at North Carolina Central University by Williams *et al.*[16] The chemical structure of B355252 has been previously published.[16]

Cell Culture Murine hippocampal neuronal HT-22 cells were routinely maintained in a DMEM medium, which was supplemented with 10% FBS and penicillin (62.5 μg/mL)/streptomycin (100 μg/mL) at 37 °C in 5% CO_2 and 85% humidity. Cell culture media were changed once every 2 d.

Glutamate Dose Response Actively growing cells were seeded at 1.5×10^4 cells/well of 96-well culture plates and incubated for 24h. Cells were then treated with DMEM media containing multiple concentrations of glutamate, 0.5–8.0 mM, to cause cytotoxicity. The cells were incubated with glutamate at 37 °C for 18h prior to assessing cell viability using a resazurin assay as described below. A concentration of 2 mM glutamate reduced the cell viability to about 50% and was used for subsequent experiments.

B355252 Dose Response HT-22 cells (1.5×10^4) were plated in 96-well plates and incubated for 24 h. B355252 was dissolved in dimethyl sulfoxide (DMSO) as stock solution (10 mM). The cell cultures were pre-incubated with various concentration of B355252 (1.25–20 μM) for 2 h and followed by the addition of 2 mM glutamate at 37 °C for 18 h. The cells were then harvested and viability was assessed by resazurin assay. Glutamate NMDA receptor blocker MK-801 (10–80 μM) was also used as a positive control.

Cell Viability Assay Cell viability was assessed using resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) assay. The live cells were measured in a PheraStar multipurpose plate reader (BMG Labtech, Ortenberg, Germany) at 540 nm excitation /590 nm emission. Percent of viable cells were compared among the experimental groups.

Western Blotting HT-22 cells were seeded in dishes (7.5 cm) and pre-treated for 2 h with B352552 or MK-801 before glutamate exposure for 18 h. The cells were lysed and centrifuged at $20817 \times \boldsymbol{g}$ for 15 min at 4 °C and the supernatants were collected as total protein. Equal amount of protein (30 μg) in each group were loaded onto 4–12% NuPAGE sodium dodecyl sulfate (SDS) polyacrylamide gels (Invitrogen, Waltham, MA, U.S.A.), separated *via* electrophoresis, and electrotransferred onto polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Burlington, MA, U.S.A.). The membranes were blocked with blocking buffer at room temperature for 1h and then incubated with antibodies against p-ERK3 (S189, product # PA5-38485, ThermoFisher, MA, U.S.A.) ERK3 (product # 4067, Cell Signaling Technology, Danvers, MA, U.S.A.), anti-SRC-3 (product #2126), anti-MAPKAPK-5 (product # 7419), anti-Phospho-S6 (product # 4858), and anti-S6 (product #2317), all at 1 : 1000 dilution overnight at 4 °C and secondary antibodies for 1h at room temperature sequentially. The blots were scanned to LI-COR Biosciences Odyssey Infrared Fluorescent scanner (Lincoln, NE, U.S.A.). Protein band fluorescent intensities were measured and presented as a ratio of target protein over loading control protein band intensity. Beta-actin (1 : 5000 dilution, Abcam, Cambridge, U.K.) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1 : 1000, ThermoFisher) was used as a loading control.

Immunofluorescence HT-22 cells (2000) were plated in 8-well chamber slides overnight and treated with B355252 (2.5 μM) and glutamate (2 mM) for 1 h. The cells were incubated with anti-phospho S6 ribosomal protein (Ser235/236, product # 4858, Cell Signaling Technology) and Alexa Fluor 647 conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody (Life Technology). Nuclear DNA was visualized using a 4′-6-diamidino-2-phenylindole (DAPI) mounting solution (Vector Laboratories).

Data Analysis One-way ANOVA followed by Tukey's test was used for parametric data and Kruskal–Wallis followed by Mann–Whitney's *U* test were used for non-parametric data. Statistical significance was set at p-value of <0.05. The values are expressed as mean ± standard deviation (S.D.).

RESULTS

Cytotoxicity of Glutamate on HT-22 Cells To find an adequate dosage for glutamate toxicity, HT-22 cells were in-

cubated with 0.5, 1.0, 2.0, 4.0, and 8.0 mM of glutamate for 18 h. As shown in Fig. 1, glutamate caused cell death in a dose dependent manner. No decline of cell viability was observed at the glutamate concentration of 0.5 mM. The cell viability significantly reduced to 80% when glutamate was increased to 1.0 mM compared to the control ($p < 0.001$). The viabilities were further decreased to 50 and 20% with glutamate concentrations increased to 2 and 4 mM ($p < 0.001$), respectively. The viability was diminished to 5% of the control value with 8 mM of glutamate ($p < 0.001$). Calculation of EC_{50} showed that the glutamate dosage to induce 50% of cell death is 1.892 mM (Fig. 1). For convenience of calculation, we selected 2 mM for subsequent experiments.

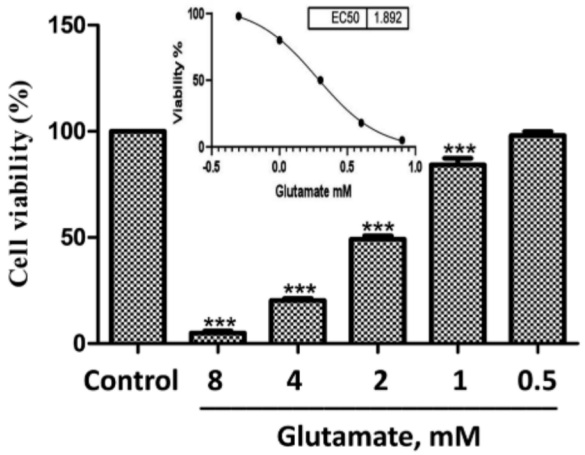

Fig. 1. Glutamate Toxicity in HT-22 Cells

Cell viability was assessed after 18h of glutamate exposure using resazurin assay. Values are represented as means ± S.D. ***$p < 0.001$ *vs.* Control. The glutamate concentration in x-axis of the inserted EC 50 curve is in log scale.

B355252 Ameliorated Glutamate Cytotoxicity To determine the efficacy of B355252 on glutamate-induced cell death, five dosages (1.25, 2.5, 5.0, 10, and 20 μM) of B355252 were examined. The cells were treated with B355252 for 2 h prior to 2 mM glutamate exposure. The cell viability was assessed by resazurin assay after 18 h of glutamate addition. As shown in Fig. 2, cells in Control group reach to about 70–80% confluence and attached to the plate. The cell density significantly dropped and many cells detached from the plate and displayed as shrunken cells after glutamate incubation for 18h (Fig. 2A). Co-treatment of B355252 or MK-801 brought the cell morphology and density back to close to the Control. As summarized in Fig. 2B, glutamate reduced the cell viability to 50% of the control ($p < 0.001$ *vs.* Control). Treatment with 1.25 μM of B355252 prevented the glutamate-caused decline of cell viability ($p < 0.01$ *vs.* Glutamate). The cell viability further increased to 118% when 2.5 μM of B355252 was applied ($p < 0.001$ *vs.* Glutamate). The cell viability slightly declined to 90% of control when the dosage of B355252 increased to 5.0 μM ($p < 0.01$ *vs.* Glutamate). High concentration of B355252 (10 and 20 μM) resulted in significant reduction of cell viability to less than 10% of the control ($p < 0.001$ *vs.* Control). Because the final DMSO concentrations in 10 and 20 μM of B355252 were 1 and 2%, respectively, the significant decreases of cell viability at the high concentrations of B355252 could be caused by the combination of glutamate and DMSO, or the combination of all three components. Nonetheless, because 2.5 μM of B355252 provided the best efficacy against glutamate toxicity, this dose was chosen for subsequent B355252 experiments. The efficacy of MK-801 against glutamate toxicity was also assessed following the same

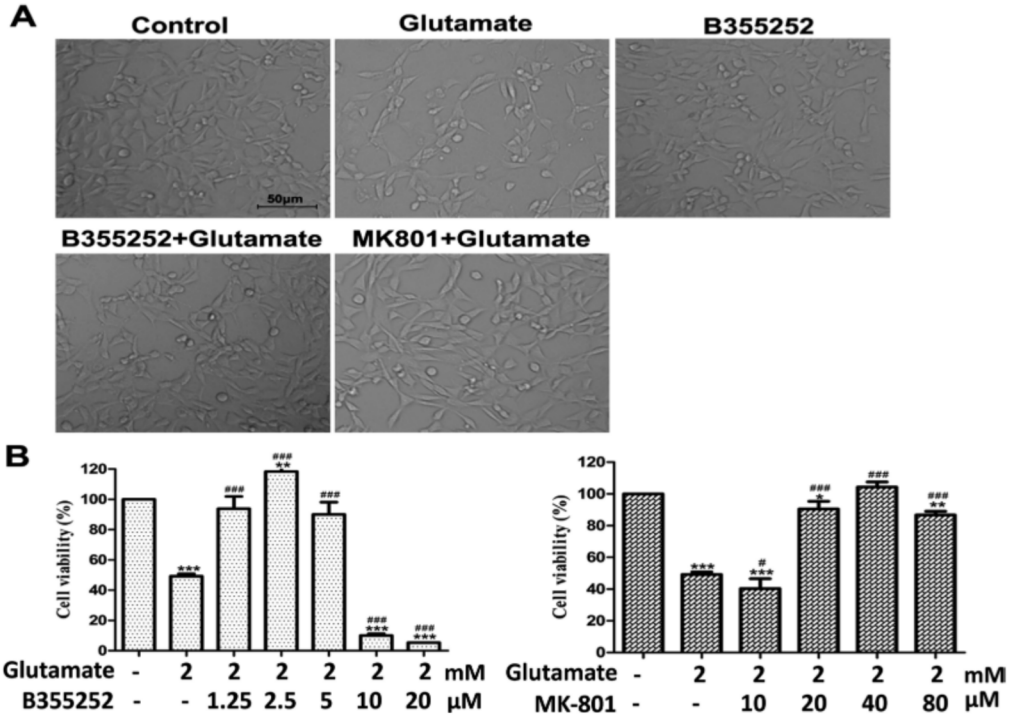

Fig. 2. Protective Effects of B355252 and MK-801 against Glutamate Toxicity

(A) Representative photomicrograms presenting cell morphology observed by light microscope in different experimental groups. (B) Summarized bar graphs showing the viability in glutamate exposed and B355252 (left penal) or MK-801 (right panel) treated HT-22 cells. Values are means ± S.D. as a percent (%). **$p < 0.01$, ***$p < 0.001$ *vs.* Control and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs.* Glutamate.

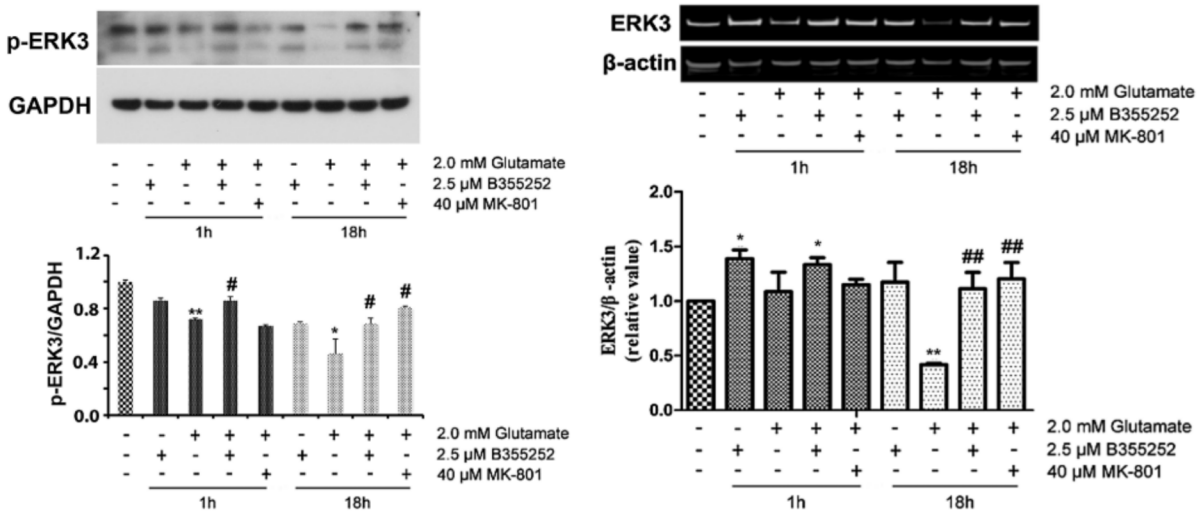

Fig. 3. B355252 Prevented Glutamate-Induced Reduction of ERK3

HT-22 cells were treated with 2.0 mM glutamate in the presence or absence of either B355252 (2.5 μM) or MK-801 (40 μM) for 1h or 18h. The cell extracts were prepared for Western blotting using anti-p-ERK3 and anti-ERK3 antibodies. GAPDH and β-actin were used as loading controls. Upper panels, representative western blots; lower panels, summarized bar graphs showing p-ERK3 and ERK3 relative band densities. P-ERK3 antibody detected 2 bands around 100 KD and both were used for band density measurement. p-ERK3 and ERK3 protein band densities were measured and presented as ratios relative to GAPDH and β-actin. Data are presented as means ±S.D. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Control and $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* Glutamate alone.

treatment regime as B355252. Treatment with 10 μM MK-801 failed to rescue cells from glutamate-induced damage. When MK-801 dosage increased to 20, 40, and 80 μM, the cell viabilities were recovered to close to 100% of the control. These data suggest that B355252 is equally, if not more, efficacious in preventing glutamate cytotoxicity. Because 40 μM MK-801 yielded the best protective effect, this dose was used for subsequence MK-801 experiments.

B355252 Prevented Glutamate-Caused Suppression of ERK3 In previous studies, we found that glutamate increased the phosphorylation of ERK1/2 while B355252 significantly prevented the surge of ERK1/2 in HT-22cells.[17] To further determine the effect of B355252 on ERK3 expression, we co-treated HT-22 cell with 2.5 μM of B355252 and 2.0 mM of glutamate. MK-801 (40 μM) was used as a positive control. The cells were harvested after 1h, and 18h and glutamate exposure, lysed with lysing buffer, and performed Western blot with anti-p-ERK3 and anti-ERK3 antibody. As shown in Fig. 3, p-ERK3 significantly decreased in glutamate treatment group at both 1 and 18 h. Co-treatment of glutamate with B355252 prevented the fall of p-ERK3 at 1 and 18 h, while MK-801 only prevent the glutamate induced fall at 18 h. Similarly, glutamate significantly reduced ERK3 by 58% compared to control cells ($p<0.01$). Pretreatment with B355252 completely reversed the glutamate-induced decline of ERK3. MK-801 was equally effective in restoring the ERK3 level. These results suggest that both B355252 and MK-801 are efficacious in preventing glutamate-induced suppression of ERK3 signaling.

B355252 Increased MAPKAPK-5 Protein in Glutamate Exposed HT-22 Cells MAPK-activated protein kinase-5 (MAPKAPK-5 or MK-5) is the only identified ERK3 substrate.[19] To examine whether MAPKAPK-5 is involved in neuronal cell apoptosis triggered by glutamate, protein levels of MAPKAPK-5 in glutamate, B355252 and MK-801treated HT-22 cells were analyzed by Western blot. As shown in Fig. 4, B355252 *per se* or in combination with glutamate moder-

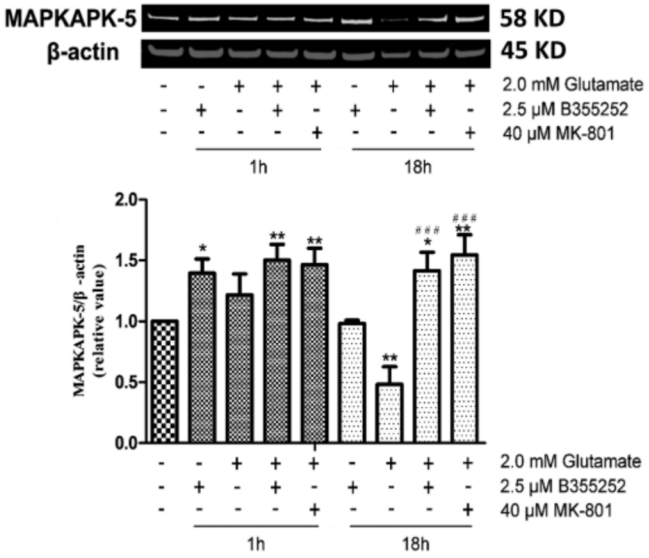

Fig. 4. B355252 Reversed Glutamate-Induced Reduction of MAPKAPK-5

HT-22 cells were treated with 2.5 mM glutamate in the presence or absence of B355252 or MK-801 for 1 or 18 h. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Control and $^{\#\#\#}p<0.001$ *vs.* Glutamate.

ately increased MAPKAPK-5 protein when compared with control HT-22 cells at 1 h after glutamate exposure. MK-801 enhanced MAPKAPK-5 to the same extent as B355252 did at 1 h. Glutamate, however, did not seem to have a significant influence in MAPKAPK-5 after 1 h treatment. Rather, it had a much delayed effect. Thus, after 18 h of glutamate exposure, MAPKAPK-5 levels dramatically decreased to about 50% of control value ($p<0.01$). Co-treatment of glutamate-exposed cells with either B355252 or MK-801completely reversed and increased the levels of MAPKAPK-5 ($p<0.001$ *vs.* Glutamate and $p<0.05$ *vs.* Control). The changes of MAPKAPK-5 in the experimental groups followed the same pattern as ERK3.

B355252 Corrected Glutamate-Caused Suppression of

SRC-3 SRC-3 interacts with several nuclear transcription factors to regulate the gene expression. SRC-3 has been used as a therapeutic target for cancer treatment.[20] SRC-3 is a downstream target of ERK3.[15] To determine whether B355252 pretreatment protected glutamate-induced cytotoxicity through promoting the ERK3/SRC-3 signaling pathways, we measured SRC-3 protein levels. As demonstrated in Fig. 5, B355252 treatment *per se* mildly increased SRC-3 content at 1 h. However, at this time-point, glutamate treatment did not affect the SRC-3 level. Treatment of B355252 or MK-801 to glutamate exposed cells moderately increased the SRC-3 level at 1 h ($p<0.05$). At 18 h, glutamate exposed cells had much lower level of SRC-3 (48% of control value, $p<0.01$) and treatment with B355252 or MK-801 abolished the glutamate suppressive effect and further increased the SRC-3 level when compared with control or glutamate group. Therefore, our data suggest that the protective effect of B355252 on glutamate-induced cytotoxicity may involve activation of ERK3/SRC-3 signaling.

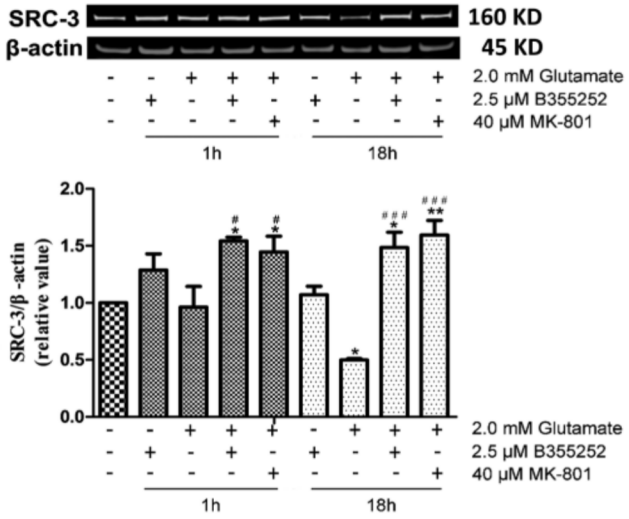

Fig. 5. B355252 Blocked Glutamate-Induced Decrease of SRC-3

Data are presented as means ± S.D. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Control and $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ *vs.* Glutamate.

B355252 Partially Ameliorated Glutamate-Induced p-S6 Increase Phospho-S6 is an important indicator of mechanistic target of rapamycin (mTOR) activation, which has been reported to mediate development of a variety of neurological diseases.[21] To determine whether the protective effect of B355252 involves suppression of mTOR signaling, we detected p-S6 and S6 levels using specific antibodies. As shown in Fig. 6, after 1 h of treatment, B355252 *per se* induced a significant surge of p-S6, glutamate further increased the level of p-S6. Treatment with B355252 or MK-801 partially reduced the p-S6 level to the value of B355252 treatment alone. After 18 h, the p-S6 levels in all groups were lower compared to those at 1 h. However, the highest p-S6 level was seen in glutamate treatment group. Treatment with B355252, but not MK-801, to glutamate exposed cells mildly reduced the p-S6 ($p<0.05$). The S6 level was not altered by glutamate at 1h; however, it increased at 18 h in glutamate exposed cells ($p<0.01$ *vs.* Control). Both B35252 and MK-801 reduced the glutamate-caused elevation of S6 ($p<0.05$ *vs.* Glutamate). Results from p-S6 immunocytochemistry (Fig. 7) support the observation of p-S6 protein electrophoresis. As shown, non-treated control cells had very little p-S6 positive staining. Immunoreactivity of p-S6 was increased by glutamate ($p<0.01$ and suppressed by B355252 and MK-801 treatments ($p<0.05$).

DISCUSSION

Our results have demonstrated that glutamate-induced neuronal cell death was associated with suppression of ERK3 and its downstream target proteins including MAPKAPK-5 and SRC-3. B355252 rescued the cells from glutamate toxicity by preventing the glutamate caused decreases of ERK3, MAPKAPK-5, and SRC-3. In addition, glutamate increased the protein levels of p-S6 while B355252 ameliorated the increase.

It is well known that glutamate causes neuronal cell death.

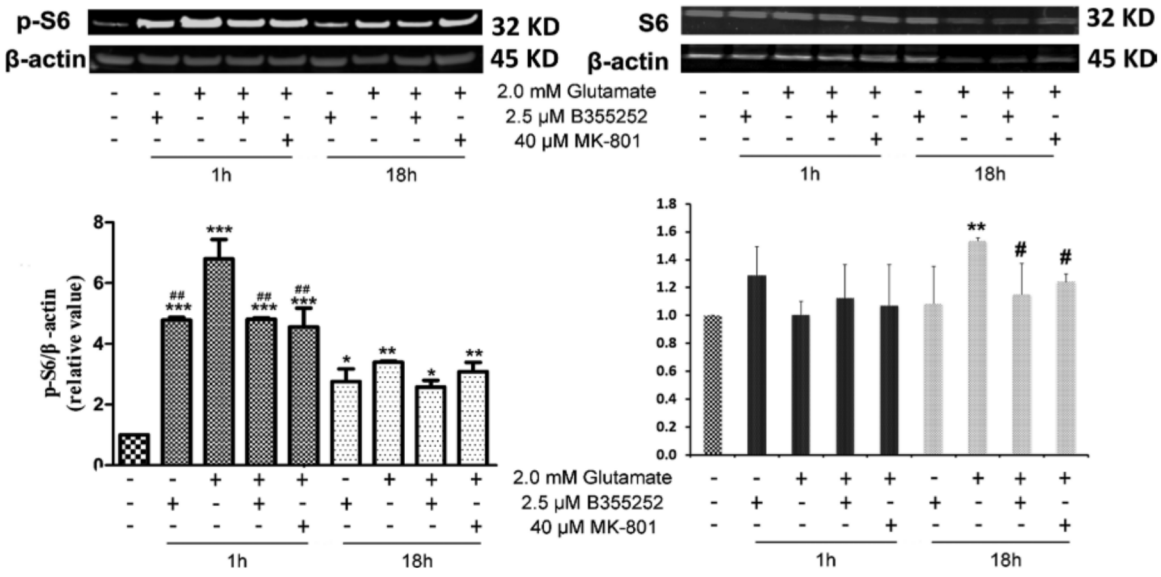

Fig. 6. Glutamate Increased and B355252 Ameliorated p-S6 Content

Both p-S6 and S6 were detected using specific antibodies. P-S6 and S6 band densities were presented as ratios of targeting band to beta-actin. Data are presented as means ± S.D. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* Control and $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* Glutamate.

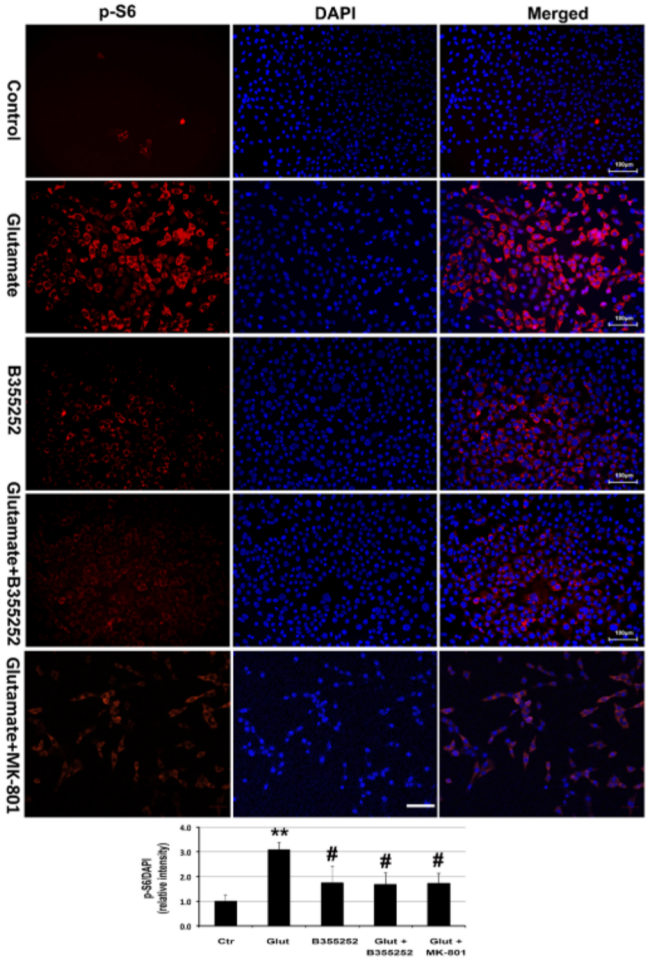

Fig. 7. Immunocytochemistry of p-S6

The cells were stained with anti-p-S6 antibody (red color) and counter stained with DAPI to mark nuclei (blue color). Magnification, 40×. Scale bar = 100 μm). Bar graph presents the relative fluorescent intensity of p-S6 normalized by DAPI fluorescent intensity. Control value was adjusted as 1. $^{**}p<0.01$ *vs.* Control, $^{\#}p<0.05$ *vs.* Glutamate. (Color figure can be accessed in the online version.)

In this study, we used various dosages of glutamate and found 2 mM induced close to 50% of cell death after 18 h of glutamate exposure. Dose titration experiment revealed that B355252 at the concentration of 2.5 μM provided the best protective effects against glutamate toxicity. Further increases of the B355252 concentration to over 10 μM significantly reduced the cell viability, suggesting that at high dosage, B355252 is toxic to the HT-22 cells. This toxicity is likely ascribed to B355252 *per se* because we have observed in a parallel study that HT22 cell viability suddenly dropped to less than 20% of the control value when B355252 concentration increased to 10 μM.[19] B355252 has been shown to potentiate nerve growth factor (NGF)-stimulated neurite outgrowth.[16] Our previous studies have revealed protective effects of B355252 in glutamate neurotoxicity model, 6-OHDA-induced model of Parkinson's disease (PD) and $CoCl_2$-induced damage in the murine hippocampal cell line HT-22. The mechanisms of B355252 involve reducing the production of intracellular ROS and suppressing the activation of ERK1/2.[17]

ERK3 is a member of MAPK family proteins. Unlike ERK1/2, which has been shown to be activated through phosphorylation by glutamate exposure and inhibited by B355252, the role of ERK3 in glutamate cytotoxicity and B355252-mediated neuorprotection is unknown.[12,23] ERK3 expression is upregulated in cancer tissues such as breast cancer, gastric cancer, and skin cancer.[20,24,25] Upregulation of ERK3 is associated with tumor invasion and metastasis, suggesting that ERK3 promotes tumor invasiveness.[15] Another study reported that the downregulation of ERK3 inhibits the migration and proliferation of human umbilical vein endothelial cells (HUVECs) cells, suggesting that ERK3 regulates tubule formation by the endothelial cells.[14] However, the function of ERK3 in neural tissue remains largely unknown. In the current study, we examined ERK3 signaling pathway in glutamate and B355252 treated HT-22 cells. Our results demonstrated that glutamate significantly suppressed the p-ERK3 and ERK3 protein levels, and treatment with B355252 brought the both back to control levels. It is not known whether B355252 is unique in its ability to ameliorate the effects of glutamate, or would any compound that sufficiently raises ERK3 levels be able to overcome glutamate's effects. Unfortunately, there has been no other published study reported the influence of glutamate on ERK3 except one of our own study. Nonetheless, our results suggest that suppression of ERK3 is associated with cell death and prevention of ERK3 decline is linked with cell survival in neurons treated with toxic glutamate.

ERK3 regulates the expression of MAPKAPK-5, SRC-3 and mTOR/p-S6. MAPKAPK-5, also described as p38-regulated/activated protein kinase (PRAK), was originally discovered in 1998, as a novel serine/threonine kinase that could be phosphorylated and activated by p38 MAPK and/or ERK3 and ERK4.[26] MAPKAPK-5 has been found to be ubiquitously expressed in humans with main expression in cardiac, skeletal, pancreatic and lung tissues. In resting cells, the protein is mainly found in the nucleus, but can translocate between the nucleus and the cytoplasm.[27] MAPKAPK-5 is involved in cell migration, proliferation, invasion, angiogenesis, transcription, cancer, cytoskeletal remodeling, tumorigenesis, anxiety control and body movement.[28–30] SRC-3, a member of p160 steroid receptor coactivator family, regulate target gene expression by interacting with several receptors localized in the nucleus.[21,31–34]

SRC-3 plays a critical role in host defense against bacterial infections and the development of the central nervous system (CNS).[35,36] In this study, we measured MAPKAPK-5 and SRC-3 protein levels using specific antibodies. As expected, the changes of MAPKAPK-5 and SRC-3 followed the same trend as ERK3. Therefore, both two proteins decreased after 18h of glutamate exposure and B355252 treatment was able to block the glutamate-caused suppressions of MAPKAPK-5 and SRC-3. These data, together with changes in ERK3 suggest that glutamate suppresses ERK3 signaling pathway and B355252 blocks the suppression effect of glutamate on ERK3 signaling. ERK3 suppresses mTORC1. Downregulation of ERK3 activates mTORC1 and overexpression of EKR3 inhibited cell proliferation through inactivation of mTORC1.[37] MTOR, as part of the mTORC1 complex, is a key regulator of various cellular functions, such as protein synthesis, cell growth, division, and survival. Upon activation, mTOR regulates initiation factor 4E-binding protein (4E-BP1) and 70 kDa S6 ribosomal protein kinase (p70S6K1) at T389, and the latter subsequently phosphorylates the ribosomal S6 protein (S6). p-S6 has commonly been used as a marker of mTOR pathway

activity.[38] In the present study, we measured the levels of p-S6 as an index for mTOR pathways activation and the results demonstrated that the p-S6 was significantly elevated by glutamate exposure. Unexpectedly, B355252 treatment *per se* also increased the p-S6 content though to a lesser extent than that in glutamate treatment for unknown reasons. Nonetheless, what is important is that B355252 significantly reduced the glutamate-induced elevation of p-S6. It is interesting that although the amount of ERK3 protein for the combination for glutamate and B355252 or MK-801 after 1h does not significantly change, the SRC-3 and p-S6 were altered significantly at 1h by B355252 and MK-801 treatment compared with glutamate. This is probably because SRC-3, and p-S6 are also regulated by other upstream factors in addition to ERK3. For example, the ubiquitin ligase carboxyl terminus of HSP70-interacting protein (CHIP) and Sirt3 have been shown to upregulate SRC-3 and mTOR regulates p-S6. We speculate that B355252 and MK-801 may affect the levels of SRC-3 and p-S6 at 1h by regulating other upstream regulators as well.

Immunocytochemistry further verified this finding. These results suggested that the release of glutamate-inhibited ERK3 signaling pathway is a fundamental mechanism through which B355252 protects against glutamate-induced neuronal cell death. Recently, using *in vitro* blood–brain barrier (BBB) model, we found B355252 could pass through the BBB (Pokharel et al, unpublished data). In the follow up study, we will exam whether this compound could pass through the BBB in animal.

CONCLUSION

In summary, glutamate-caused neuronal cell death is associated with suppression of ERK3 and its downstream MAPKAPK-5 and SRC-3 pathways. B355252 protected the cells from glutamate toxicity and prevented the glutamate-caused declines in ERK3, MAPKAPK-5 and SRC-3. Furthermore, glutamate activated mTOR signaling as reflected by increased level of p-S6 and B355252 lowered the elevation. It is concluded that one of the mechanisms that glutamate mediates its cytotoxicity is through suppression of ERK3 and that B355252 rescues the cells from glutamate toxicity by reverting ERK3 level. However, we acknowledge that the results presented here are still preliminary and a solid conclusion for loss of function of ERK3 can only be validated by small interfering RNA (siRNA), short hairpin RNA (shRNA) or gene knockout in future experiments.

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Conflict of Interest The authors declare no conflict of interest.

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