Regulation of Nogo-66 on apoptosis and its molecular mechanism

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Abstract

As is well know, Nogo-A play a crucial role on the inhibition of axonal regeneration in the adult central nervous system after traumatic injurices. In our previsous study,We have found that Nogo-P4,the active fragment of Nogo-66,can increases the Aβ secretion, but whether the Nogo-66 have the effect on aoptosis is not clear. Our results showed that Nogo-66 promoted cells aoptosis in human neuroblastoma SH-SY5Y cells by combine with NgR. Furthermore, it activated caspase3 and caspase9 protein expression. In addition, NEP1-40 and Y-27632 activated the PI3K/AKT signaling pathway,inhibiting the interaction between Bax and Bcl-2. Our data suggest that Nogo-66 increases aoptosis by activating caspase3 and caspase9 , which was affected by activating Nogo-66 receptor and downstream signaling molecules ROCK2 and phosphorylation of CRMP2.which may facilitate the onset and development of AD. The inhibition of NgR can improve apoptosis ,which is appears to be a good target for nerve disease therapy.

Keywords

Nogo-66, apoptosis, NEP1-40, Y-27632.

1. Introduction

Nogo-A has been identified as a key molecule that limits axon regeneration and is considered to be a major obstacle to nerve regeneration after injury in the adult mammalian central nervous system(CNS)1.Studies have revealed that Nogo-A interacted with the specific receptor complex on neurons to prevent neurite outgrowth,the receptor complex is include Nogo-66 receptor(NgR1) and p752.It was reported that LINGO-1,a nervous system-specific transmembrane protein,binds NgR1 and p75 which is an additional functional component of NgR1/p75 signaling complex2.The growth cone collapse domain of Nogo-A, Nogo-66, could specifically bind to NgR1 and exert its biological functions via activation of RhoA signaling3.

Rho kinase (ROCK) belongs to a family of serine/threonin kinases that are stimulated by G protein coupled receptor activation of the small plasma membrane bound GTP-binding proteins4,and Rho kinase could regulation cell death and survival4.The RhoA GTPase (Rho) signals through ROCK to control cytoskeletal dynamics and regulate neiron structure5.Based on the ubiquitous expression pattern across human,mouse and rat tissues,ROCK has been shown to be involved in a variety of fundamental cellular functions, such as contraction, adhesion, migration and proliferation6. Recently,evidence has accumulated suggesting that abnormal activation of ROCK plays an important role in various pathological conditions,including cerebral and coronary vasospasm, hypertension, vascular inflammation and remodeling, and arteriosclerosis7,8.

The PI3K/AKT signaling is a critical pathway in cell apoptosis9-11. Activated Akt can inhibit the release of cytochrome c and apoptosis factor, thereby inhibiting apoptosis, and promote the growth of cancer cells. The mechanisms underlying cancer are marked by complex aberrations that activate critical cellular signaling pathways in tumorigenesis.The phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling cascade is one of the most important intracellular pathways, which is frequently activated in diverse cancers12-14.

We have reported that Nogo-P4,the active fragment of Nogo-66,inhibited neurite outgrowth and increases the Aβ secretion by activation the NgR15.Hence, Nogo-66 receptor has been considered as

a plausible molecular target to Treat neurological diseases.However, the possibility that Nogo-66 may has effect on apoptosis has not yet been examined.Moreover,the detail mechanisms of apoptosis induced by Nogo-66 is not clear..he aim of this study,therefore,was to investigate whether Nogo-66 may have a promotive effect on apoptosis in SH-SY5Y cells. Furthermore,we examined the molecular pathways in an effort to elucidate the mechanisms by which Nogo-66 induced apoptosis.

2. Materials and Methods

2.1 Reagents

Human neuroblastoma SH-SY5Y cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA, CRL-2266). A caspase3 activity assay kit, caspase9 activity assay kit, reactive oxygen species (ROS) assay kit, cellular glutathione peroxidase (GPx) assay kit, lipid peroxidation MDA assay kit, Anti-RAGE antibody (Abcam, Cambridge, UK) PI3K antibody, P-PI3K antibody,AKT antibody, P-AKT antibody, NF-κB antibody, Bax antibody, β-actin antibody, and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Bcl-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), sheep anti-rabbit IgG H&L Dylight 488 and Northernlights anti-rabbit IgG NL 557 were purchased from R&D (R&D, USA).3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Sigma) , poly-L-lysine (PLL) (Sigma), fetal bovine serum (FBS) (Gibco).

2.2 Cell Culture

10 % FBS, and penicillin-streptomycin. SH-SY5Y cells were all maintained at 37 ℃ in a 95 % humidified incubator with 5 % CO_2 before the experiments.

2.3 Cell Viability Assay

96 wells were treated with 50 μl of PLL at 37 ℃ and 5 % CO_2 overnight ,and air drying after washed twice with PBS. 96 wells were plated with 1.0×10^5 / ml cell suspension density in a volume of 100 μl at 37 ℃ and 5 % CO2 for 24 h. The SH-SY5Y cells were randomly divided into four groups: (1) control cells, (2)control cells incubated for 24 h in DMEM without serum in the presence of Nogo-66, (3) control cells pretreated with NEP1-40 (2 μM) and then incubated for 24 h in DMEM without serum in the presence of Nogo-66, (4) control cells pretreated with Y-27632 (100 μM) and then incubated for 24 h in DMEM without serum in the presence of Nogo-66.

An MTT assay was used to evaluate the viability of the SH-SY5Y cells. Following treatment with antagonists and Nogo-66 as described above , 20 μl of MTT were added to each well and incubated for 4 h at 37 ℃. The absorbance was measured at 490 nm.

2.4 Annexin V-FITC and PI Staining

Cells were seeded in 12-well plates at 3.0×10^5 / ml for 16 h before attachment. Cells were treated different concentrations of EMMQ or vehicle control and incubated at 37 ℃ for 48 h. The collected cells were stained with 1 lL Annexin-V/FITC (20 lg/mL) and 1 lL of PI (50 lg/ mL) at room temperature for 30 min in the dark. Flow cytometer FACS CaliburTM (BD Bioscience) was used to analyze early and late phase of apoptosis using the FlowJo software (Tree Star, Ashland, OR).

2.5 Western Blot Analysis

Cells (13×103 cells/cm2) were plated on 12-well plates. The cells were collected by centrifugation at 1000 g for 5 min at 4 ℃. The supernatant was discarded, and cell pellets were sonicated for 15 s in ice-cold lysate buffer (20 mM Tris, pH 7.5; 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 1 % Triton X-100, 1 mM NaF, 1 mM Na3VO4, and 1:200 dilution of Protease Inhibitor Cocktail II). The cell lysate was centrifuged at 10,000g for 10 min at 4 ℃, and the supernatant was saved for analyses. Total protein was determined by bicinchoninic acid (BCA) method. For western blot analysis, the supernatant was added to an equal volume of loading buffer and heated to 95 ℃ for 8 min. Proteins were separated by SDS-PAGE on 12 % polyacrylamide gels and were transferred onto nitrocellulose

membranes. The membranes were incubated with primary antibodies (P-PI3K, PI3K, P-AKT,AKT, Bax, Bcl-2, ROCK2, CRMP2, p-CRMP2, and GAPDH) at 4 ℃, followed by the addition of goat anti-rabbit IgG andHRP-linked antibody (1:1000) and incubation for 2 h at room temperature. Images were collected, and the band density was analyzed using a Fluor-S MultiImager and Quantity One software (Bio-Rad, Hercules, CA). Each well was designated as an " n " of one, and experiments were repeated four times on separate days.

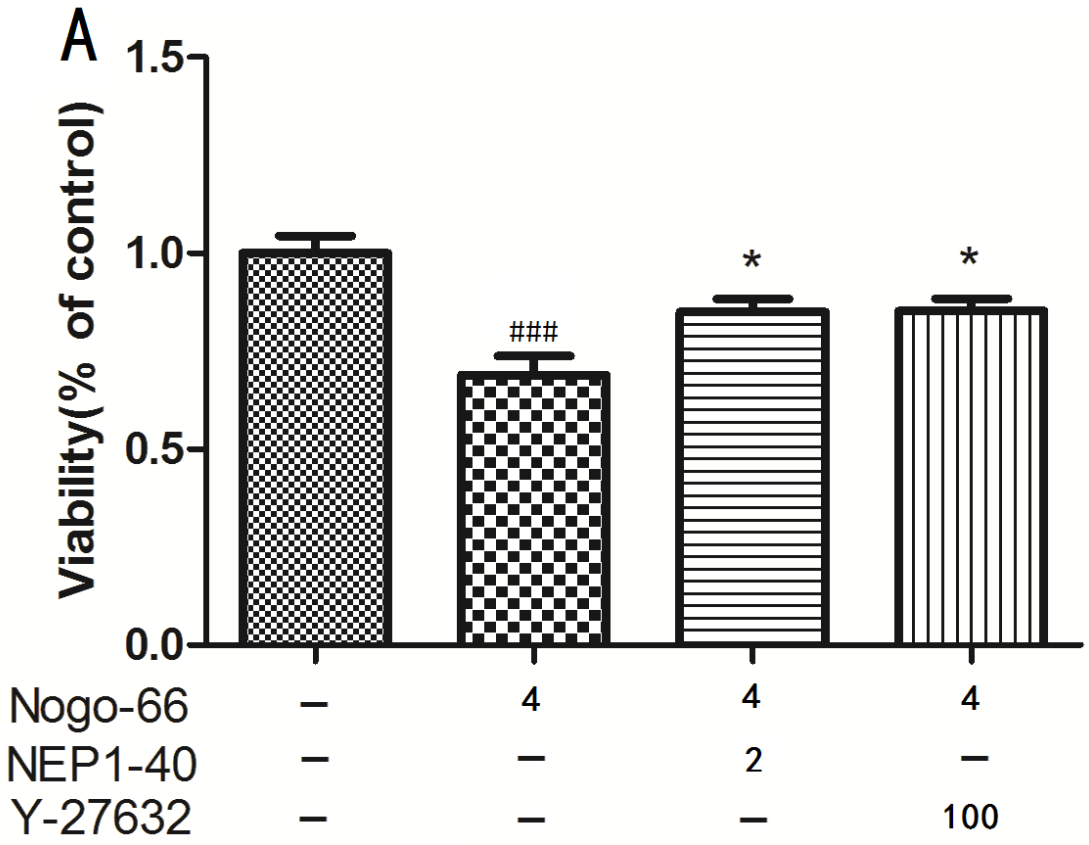
2.6 Data Analysis

All data are expressed as the mean ± SE. Analyses were performed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL). Comparisons between groups were analyzed by one-way ANOVA. The statistical significance was determined by Student's t-test for two groups.

3. Results

3.1 NEP1-40 and Y-27632 Increased Cell Viability Against Nogo-66-Induced Toxicity in SH-SY5Y Cells

The viability of the SH-SY5Y cells significantly decreased in the presence 4 μM Nogo-66 in DMEM. NEP1-40 and Y-27632 pretreatment for 2 h enhanced the cell viability of SH-SY5Y cells exposed to Nogo-66 (Fig. 1A). The Annexin V-FITC/PI double staining was used to evaluate the induced apoptosis. As the concentration reached 4 μM, cell populations of early and late apoptotic phase rose to 17.12 % in SH-SY5Y cells after 24 h treatment of Nogo-66.While pretreatment with NEP1-40 and Y-27632 for 2 h, cells apoptotic phase decline to 6 % (Fig. 1B). The results suggested that Nogo-66-treatment caused the apoptotic cell death and decrease the cell viabilities in SH-SY5Y cells.



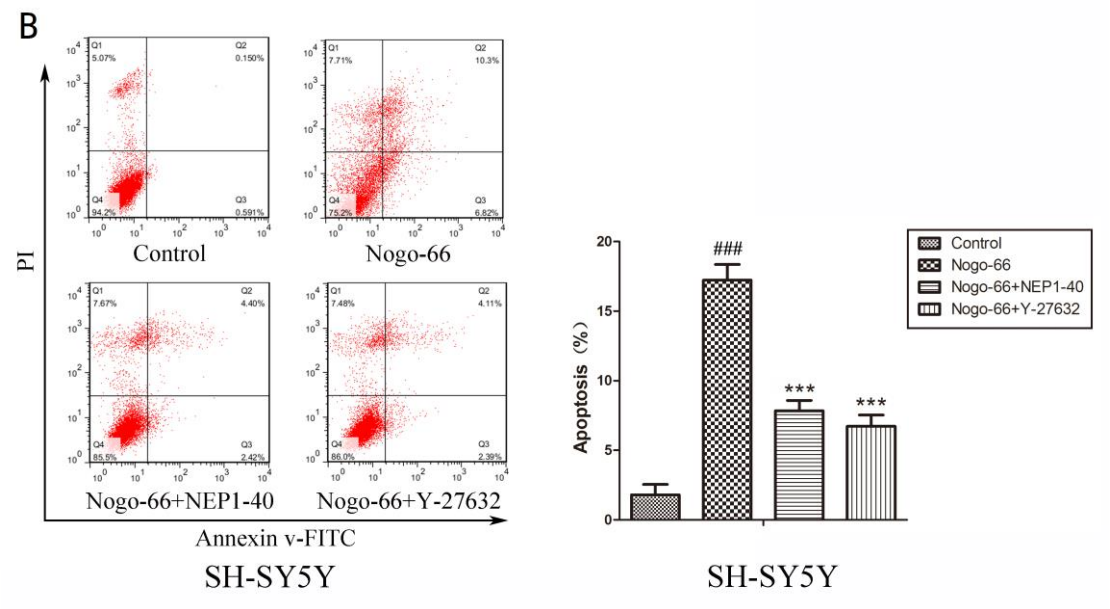


Fig. 1. (A)Quantitative MTT assessment of cell viability. Exposure to Nogo-66 decreased the viability of SH-SY5Y cells compared with the control group. NEP1-40 and Y-27632 application increased the viability of SH-SY5Y cells. (B) Apoptosis assay by double staining with Annexin V-FITC/PI in SH-SY5Y cells. $^{###}P<0.001$, the control vs the Nogo-66 group; $*P<0.05$, $***P<0.001$, the groups treated with NEP1-40 or Y-27632 and Nogo-66 vs the Nogo-66 treated group. Data presented are the mean OD values (±SD) of triplicate samples(n=6) .

3.2 Effects of Nogo-66 on Caspase3 and Caspase9 Activation in SH-SY5Y Cells

Mitochondrial dysfunction,caspase-dependent toxicity, and dysfunction of downstream signaling pathways documented critical apoptotic events during AD processes (Ghavami et al.2014). The cytosolic activity of caspase3 and caspase9 was detected as mitochondrial-related apoptotic molecular. The activities of both caspase3 and caspase9 increased in the Nogo-66 group, and antagonist (NEP1-40 and Y-27632) treatment significantly suppressed their activities (Fig. 2A,B).

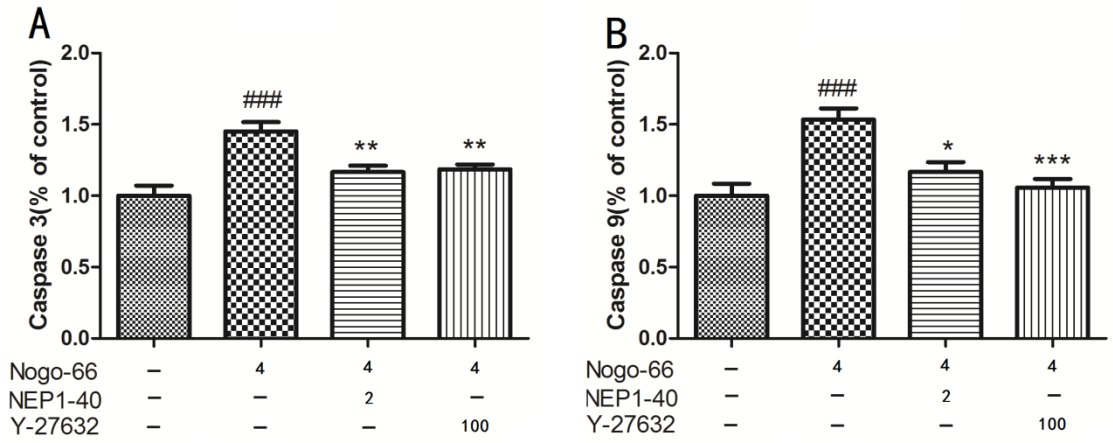


Fig 2. Effects of Nogo-66 on caspase3 and caspase9 activation.(A)The activity of caspase3 in SH-SY5Y cells following treatments.(B)The activity of caspase9 in SH-SY5Y cells following treatments . ###P<0.001, the control vs the Nogo-66 group; *P<0.05, **P<0.01, ***P<0.001, the groups treated with NEP1-40 or Y-27632 and Nogo-66 vs the Nogo-66 treated group. Data presented are the mean OD values (±SD) of triplicate samples (n=6).

3.3 Effects of Nogo-66 on PI3K/AKT,Bax,Bcl-1, ROCK2, CRMP2,P-CRMP2 Protein Expression

To explore potential intracellular signaling mechanisms responsible for apoptotic effects of Nogo-66-induced SH-SY5Y cell death, we assessed changes in PI3K/AKT activation based on phospho-specific antibodies in Western blots. Nogo-66 treatment significantly reduced the expression of PI3K and AKT phosphorylation. In contrast, NEP1-40 and Y-27632 significantly increased PI3K and AKT phosphorylation but had no effect on levels of total PI3K and AKT (Fig. 3A-C). Bax is an apoptosis-promoting protein that can activate a mitochondrial apoptotic cascade by binding to Bcl-2. Bax expression increased in the Nogo-66 group, whereas that of Bcl-2 decreased, NEP1-40 and Y-27632 reversed these phenomena (Fig. 3D-F).

Nogo-66 treatment significantly increased the expression of ROCK2 and phosphorylation of CRMP2. In contrast, NEP1-40 and Y-27632 significantly reduced expression of ROCK2 and phosphorylation of CRMP2 (Fig. 3G-I).

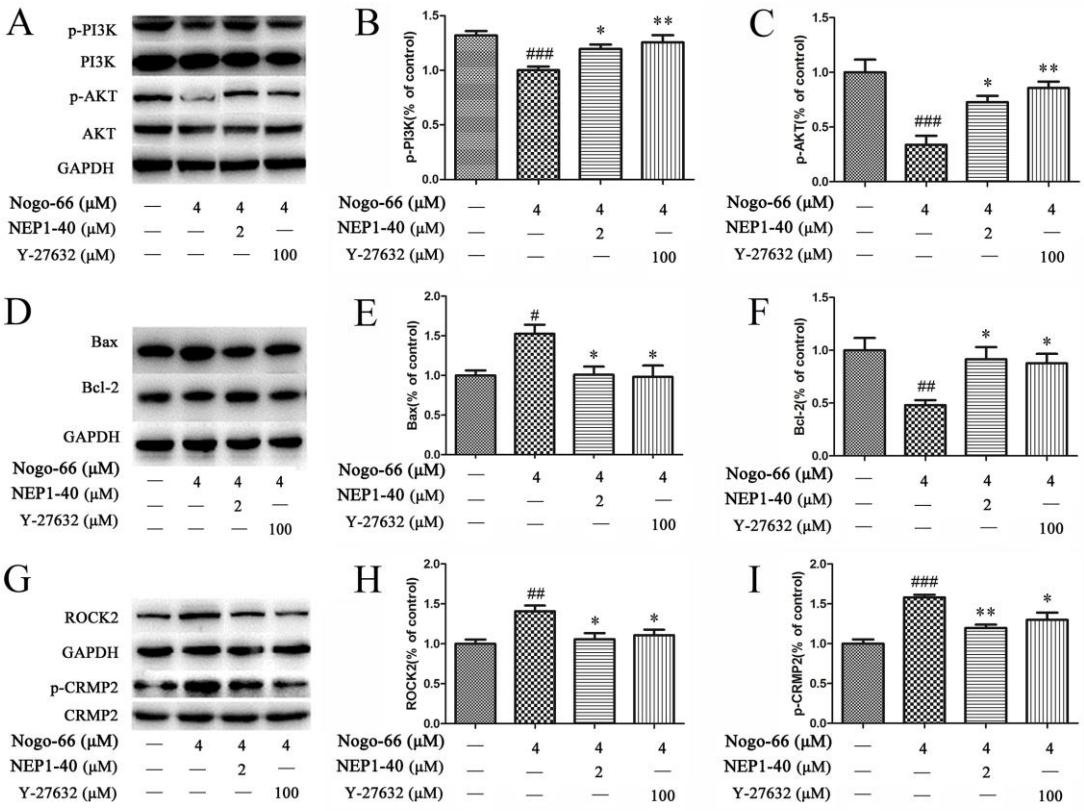


Fig.3.(A-I), Western blot analysis of the expression of phosphorylated and total levels of PI3K , phosphorylated and total levels of AKT,Bcl-2 ,Bax, ROCK2 protein and the phosphorylated and total levels of CRMP2 in SH-SY5Y cells.(A-C) Involvement of PI3K/AKT in the protective effects of NEP1-40 and Y-27632 against Nogo066-induced apoptosis. (D-F) Western blot analysis of SH-SY5Y for Bcl-2 and Bax. (G-I) Western blot analysis of the expression of the ROCK2 protein and the phosphorylated and total levels of CRMP2 in SH-SY5Y cells.sample loading was controlled by GAPDH protein quantification .The data are presented as the mean OD values (±SD) of triplicate samples. #P<0.05,##P<0.01,###P<0.001, the Nogo-66 treated group vs the control group; *P<0.05, **P<0.01,***P<0.001, the groups treated with NEP1-40 or Y-27632 and Nogo-66 vs the Nogo-66 treated group.

4. Discussion

In this study We investigated the potential role of Nogo-A in apoptosis after Nogo-66 binding with its receptor（NgR1）specificity .we reported that Nogo-66 specifically induced apoptosis by increasing

activity of caspase3 and caspase9 via PI3K/AKT pathway.

It is known to all that Nogo-66 exhibit neurite outgrowth inhibitor in various nerve cells in vitro,but its mechanism is not completely clear.The main signal transduction process involves binding of Nogo-66 to NgR on nerve cell membranes, activation of two downstream signaling molecules, ROCK and PKC,and subsequent exertion of neurite growth inhibition effect[16].It was shown that the common signaling mechanism for inhibition of neurite outgrowth is activation of endogenously bound RhoA via the receptor cluster Nogo Receptor(NgR),the low-affinity neurotrophin receptor, p75NTR, LINGO-1[2] and TROY[17].The growth cone collapse domain of Nogo-A, Nogo-66, could specifically bind to NgR1 and exert its biological functions via activation of RhoA signaling[3].In this study, we first time discussing the Nogo-66 could indeced apoptosis in vitro.Furthermore,we also tested the hypothesis that Nogo-66 indeced apoptosis via binding with NgR1.Our study showed that Nogo-66 would increase the positive effects on ROCK2 and p-CRMP2,then activating the activity of caspase3 and caspase9 by using SH-SY5Y cell lines. Nogo-66 and cell apoptosis are positively correlated to each other[13]. Thus, our data support the vies that Nogo-66 receptor is a therapeutic target for treatment of Neurological diseases.However, additional work is required to understand the internal effect associated with Nogo-66.Intrigued with this observation, our research objective was to explore the feasibility of Nogo-66 receptor to be an important target of reducing cell apoptosis.In this report,we offered several important insights into the changes on protein expression and enzyme activity.

Our results of the MTT assay and flow cytometer detection revealed that different concentrations of Nogo-66 induced apoptosis in SH-SY5Y cells .The results of the activation assay of caspase3 and caspase9 showed that Nogo-66 increased the activation assay of caspase3 and caspase9.The potential mechanism that Nogo-66- induced apoptosis has been explored.The Nogo-66-mediated apoptosis can be abrogated by the Rho kinase inhibitor and NgR antagonist,Y-27632 and NEP1-40.The acceleration effect on apoptosis by Nogo-66 was attenuated after cells were incubated with NgR antagonist NEP1-40.Similar results were obtained after pharmacologic inhibition of ROCK by Y-27632.The direct mechanism inducing these effects remains to be elucidated.It is reported that ROCK inhibition,Y-27632,potently supports survival of lesioned adult CNS neurons and additive effects on neurite outgrowth and regeneration[18].It has been reported that ROCK inhibitors have neuroprotective effective effects.Y-27632 is a potent ROCK inhibitor,and it was reported to protect retinal ganglion cells from serum deprivation or growth factor deprivation and axotomy-induced apoptosis[18].Studies also showed that ROCK inhibitor could rescue cells from apoptosis induced by ischemia injury[19].In addition,there is some evidence suggested that inhibition of ROCK could promote neu-rite outgrowth and reduce Aβ secretion induced by Nogo-P4 of the cultured cortical neurons[15].Furthermore, Rho signaling specifically controls dendrite branch length:increased Rho activity leads to branch shortening while decreased Rho activity leads to branch elongation[20].Others have shown that ROCK inhibition improves spatial learning and working memory in aged rats[21].

To determine the activations of downstream signally mechanisms which to mediate these effects, western blot was used to examine the downstream signalings.Western blot results showed that NEP1-40 and Y-27632,Nogo-66 receptor antagonists and ROCK inhibitors also up-regulation protern expression levels of p-PI3K and p-AKT dereased by Nogo-66,which was consistent with the findings above.A significant up-regulation of ROCK2 and p-CRMP2 of ROCK pathway and down-regulation of PI3K/AKT pathway was observed in SH-SY5Y cells treated with Nogo-66 compared to the control.PI3K/AKT pathways is one of signal transduction pathways most closely correlated with cell proliferation and apoptosis. Abnormal activation of PI3K /AKT signaling pathway induces abnormal cell proliferation and differentiation and promotes tumor cell growth. Phosphorylation of AKT generates p-AKT, which can further activate the mTOR pathway, increase the expression of p-mTOR and p-70S6K, and ultimately increase the proliferation and migration of tumor cells[10,22].

Taken together,these results indicate that Nogo-66 induced apoptosis by activation of NgR,ROCK2 and phosphorylation CRMP2. In our western blot analysis we found that Nogo-66 increased the

expressions of Bax and decreased the expressions of Bcl-2.We found that the cells treated with Y-27632 and NEP1-40 reversed the effect of Nogo-66 suggesting that apoptosis induced by Nogo-66 could be overcome by inhibiting Rho kinase and Nogo-66 receptor antagonist.We propose that the mechanism is the down stream signaling of Nogo-66 receptors activates the PI3K/AKT pathway and Bax/Bcl-2,a related pathway of apoptosis [23-25] ,after Nogo-66 interacting with its corresponding receptors.

In summary,our work demonstrates that Nogo-66 is able to signal apoptosis modifications through the activation of RhoA.Activation of RhoA causes an increase in ROCK-2 and phosphorylation of CRMP-2.In our study,this increase in ROCK-2 and phosphorylation of CRMP-2 rendered the enzyme activity of caspase3 and caspase9 to signal apoptosis.It suggests that ROCK appears to be a good target for related therapy[26-28].

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