Nogo-A- Δ 20/EphA4 interaction antagonizes apoptosis of neural stem cells by integrating p38 and JNK MAPK signaling

junling wang

Wenzhou Medical University

weiguang chen

Wenzhou Medical University

chaojin xu (✉ doctordoctor79@163.com)

Wenzhou Medical University

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Abstract

Background: Nogo-A protein consists of two main extracellular domains: Nogo-66 (rat amino acid [aa] 1019–1083) and Nogo-A- Δ 20 (extracellular, active 180 amino acid Nogo-A region), which serves as strong inhibitors of axon regeneration in the adult CNS. Although receptors S1PR2 and HSPGs have been identified as Nogo-A- Δ 20 binding proteins, it remains at present elusive whether other receptors directly interacting with Nogo-A- Δ 20 exist, and affect cell death.

Methods: In pursuit of NSCs, we isolated stem cells from fetal brain (embryonic day 17; E17) Sprague–Dawley rats. In order to whether Nogo-A- Δ 20 binds to EphA4, immunofluorescence, tunel assays, co-immunoprecipitation, pull down, and molecular docking technologies were used.

Results: Here, we discover that EphA4 could be acted as a high affinity ligand for Nogo-A- Δ 20. Trans-membrane protein of EphA4 is needed for Nogo-A- Δ 20–triggered inhibition of NSCs apoptosis, which are mediated by balancing p38 inactivation and JNK MAPK pathway activation. Finally, we predict at the atomic level that essential residues Lys-205, Ile-190, Pro-194 in Nogo-A- Δ 20 and EphA4 residues Gln-390, Asn-425, Pro-426 might play critical roles in Nogo-A- Δ 20/EphA4 binding via molecular docking.

Conclusion: The results of the present study show that EphA4 is a novel high-affinity receptor for Nogo-A- Δ 20 and Nogo-A- Δ 20/ EphA4 interaction mediates NSCs survival by integrating p38 and JNK MAPK signaling.

Introduction

Eph (erythropoietin-producing hepatocellular) tyrosine kinase receptors (RTKs) and its ligands, ephrins (Eph receptor interacting proteins) are all membrane-bound proteins, which are referred to as the key modulators in axonal guidance, CNS injury and NSCs development [2, 9, 23, 47, 57]. The Eph family consists of 14–16 receptor members, including EphA and EphB, based on their sequence similarity and binding affinities [8, 30]. With regard to Eph receptors, EphA4 has the unique property to bind to either the ephrins A or ephrins B ligands, respectively [4]. Furthermore, EphA4 demonstrates the functional complexities among different signaling molecules. For instance, EphA4 receptor could be activated via all ephrin ligands, containing five glycosylphosphatidylinositol (GPI) -anchored of ephrin-As and three transmembrane segment ephrin-Bs [29]. Besides, deregulations of EphA4 expression are implicated in diverse cancers, including leukemia, prostate and glioblastoma [29]. Interestingly, EphA4 is also expressed on NSCs and involved in NSCs differentiation, proliferation, migration and apoptosis [15, 18, 27]. To our knowledge, only A β and SORLA have been reported to binding with EphA4 receptor tyrosine kinase and attenuate cognitive memory deficits in neurodegenerative animal models [19, 50].

Over the past 20 years, Nogo-A has emerged as a major myelin-associated growth inhibitors for axon regeneration in the adult CNS. Nogo-A with 1163 aa (amino acid) in rat contains a short Nogo-A-active region (rat amino acid 544–725; a component of Amino-Nogo) known as ‘Nogo-A- Δ 20’ or ‘NiG- Δ 20’ as well as a 66-amino-acid residues extracellular domain (Nogo-66; rat amino-acid 1019–1083) [32, 37]. The

domains are recognized to display strong inhibitory activity for axon growth [20, 37]. In addition, Nogo-66 binds receptor NgR1 and PirB and activates RhoA signaling pathway[41, 45]. Recently, ADGRB1 has been identified as a new receptor for Nogo myelin-associated inhibitory proteins via using CRISPR screening [6]. One hand, Nogo-A- Δ 20 binds to S1PR2, which restrains synaptic plasticity and stabilize neuronal circuits [26]. On the other hand, Nogo-A- Δ 20 interplaying with HSPGs but not S1PR2 on the cell surface accelerates the migration and adhesion of neuroblasts [25]. However, it is not known if Nogo-A- Δ 20 can affect apoptotic responses of NSCs by interacting with EphA4.

In the present studies, we identified EphA4 as functional receptors for the Nogo-A active domain, Nogo-A- Δ 20 (rat amino acid 544–725). We found that Nogo-A- Δ 20 inhibits EphA4 receptor-mediated apoptotic cell death of NSCs through attenuating p38 and activating JNK MAPK signaling. However, ERK pathway is not involved in these processes. In addition, molecular docking predication show that amino acid residues Lys-205, Ile-190, Pro-194 in Nogo-A- Δ 20 and EphA4 residues Gln-390, Asn-425, Pro-426 may be pivotal binding sites for Nogo-A- Δ 20/EphA4 interaction. Thus, our current results propose a novel mechanism by which Nogo-A- Δ 20 affects NSCs apoptotic cell signaling via interacting with EphA4.

Materials And Methods

Materials

PageRuler™ Plus Prestained Protein Ladder (No: 26619) was from Thermo Fisher Scientific Biotechnology. Dual Color Prestained Protein Marker (No: WJ102) was from Epizyme Biotechnology. A563 (No. 612238) was used from BD Biosciences (PharMingen, San Diego, CA, USA). Home-made rabbit polyclonal antibody A620 was a generous gift from Dr. Wei-Lin, Jin. Beta actin antibody (AF7018), EphA4 (AF5496), His-Tag Mouse Monoclonal Antibody (T0009) were from Affinity Biosciences. HRP-conjugated goat anti-mouse IgG (A0216), HRP-conjugated goat anti-rabbit IgG (A0208), Rat IgG (A7031), Cell lysis buffer for Western and IP (P0013), Polyvinylidene fluoride (PVDF; FFP24), BeyoECL Star chemiluminescent substrate (No: P0018AS), Coomassie Blue Fast Staining Solution (P0017), IPTG (ST098-5 g) and kanamycin (ST102) were from Beyotime Biotechnology. Protein A/G PLUS-Agarose (sc-2003) was from Santa Cruz Biotechnology. P44/42 MAPK (Erk1/2) (137F5) rabbit mAb (4695S), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (4370S), Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb (9255S), SAPK/JNK Antibody (9252S), p38MAPK Antibody (9212S), Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (4511S), PARP (46D11) Rabbit mAb (9532S), p53 (1C12) antibody (#2524S), Caspase-3 Antibody (#9662) were from Cell Signaling Technology. Mouse monoclonal antibody GADPH (G8795) was from sigma Aldrich. Caspase 8 antibody (66093-1-Ig) was Proteintech Group. Methanol (CAS NO: 67-56-1; Changshu Hongsheng Fine Chemical Co., Ltd; China). Imidazole (CAS NO: 288-32-4; Sinopharm Chemical Reagent Co., Ltd). Ni-NTA agarose (Qiagen Inc., Valencia, CA). In situ apoptosis detection (TUNEL) kit (Roche, Switzerland) was used. The p38 MAPK activation Dehydrocorydaline chloride (DHC; HY-N0674A) and JNK inhibitor SP600125 (SP; HY-12041) were supplied by MedChem Express company. Rhynchophylline (Rhy) was from Baoji Herbest Bio-Tech Co., Ltd.

Neural stem cells (NSCs) were obtained from the fetal brain (embryonic day 17; E17) Sprague–Dawley rats (supplied by Chinese Academy of Sciences) according to the method described previously by our group. All NSCs used in the experiments were between passages two to three.

Immunofluorescence Assay

We used a standard procedure for immunofluorescence staining of NSCs spheres *in vitro* as described previously by our group [54, 63]. Fluorescent images were obtained using a fluorescence microscope (Olympus BX53; Olympus, Tokyo, Japan).

Cell Growth And Medium

The E. coli BL21 (DE3) was used for cloning experiment and protein expression. The plasmid Pet30a (+) was used for amplification of the Nogo-A- Δ 20 gene. Next, the complete target gene (rat aa544 ~ 725) and 6xHis gene sequences were designed, synthesized subcloned into Nde I and HindIII sites of Pet30a (+) vector for E. coli expression. Following confirmation of the inserted sequences by MluI and HindIII digestion, picked positive plasmids were transformed into E.coli BL21 (DE3) for expression of the His-tagged fusion proteins. Overnight cultures (~ 16 h) in Luria–Bertani (LB) containing 50 ug/mL kanamycin at a shaking (~ 250 rpm) to saturation, were diluted 1:25 in 250 mL fresh LB in a 1 L flask. When OD600 value was approximately 0.6 ~ 0.8, cells were induced using 1 mM IPTG at 37 °C with shaking 180 rpm for 4 h. Cells pellets were collected by centrifugation (1, 2000 rpm for 30 min at 4 °C) and stored at -30 °C prior to lysis.

His-Nogo-A- Δ 20 Protein Purification

Purification of recombinant proteins was performed as described previously [3, 61]. Briefly, cell pellets were suspended in 25 mL of lysis buffer (PBS, pH 8.0, with 20 mM imidazole). The bacterial cell pellets were sonicated at 30% amplitude for 30 min on ice, cell debris was pelleted at 1, 2000 rpm for 30 min at 4 °C and supernatants were passed through a 0.45 μ m filter prior to column loading. The filtered supernatants was purified by affinity chromatography using recharged Ni-NTA Sepharose (Qiagen) column (5 ml) at a rate of 0.5 ml/min and refolded when dialyzed in PBS using Slide-A-Lyzer (Pierce) according to the manufacturer's synopsis. The purity of purified His-tagged proteins was verified by SDS-PAGE. Finally, the recombinant proteins were concentrated using Amicon ultrafiltration cartridges with a MWCO of 10 kDa (Millipore, Bedford, MA, USA) and stored in PBS at -80 °C until use. The concentrations of concentrated proteins were determined by BCA protein quantitation kit.

TUNEL Assay

Apoptotic NSCs were detected by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining method by following the manufacturer's instructions of In Situ Cell Death Detection kit (Roche). Finally, stained cells were captured under a fluorescence microscope (Olympus BX53; Olympus, Tokyo, Japan) and cellular apoptosis was quantified using ImageJ software (National Institutes of Health).

Co-immunoprecipitation

The assay followed previous described method with slight modification [1]. Briefly, NSCs were permeabilized with Lysis Buffer (50mMTirs-HCl pH 7.5, 150 mM NaCl, 2 mM CaCl₂, 10% glycerol, 0.02% NP-40) supplemented with protease inhibitors for 30 min at 4 °C. After centrifugation for 30 min at 13.000 x g, 100 µg cell supernatant was incubated with Rat IgG or rabbit-A620 overnight at 4 °C to form the immunocomplex. Transfer the lysate and immunocomplex solution to the tube containing the pre-washed Protein A/G PLUS-Agarose. The protein-A/G beads were then removed by centrifugation. The mixture was incubated under rotary agitation for 3 h at 4 °C. Finally, washed five times with lysis buffer on ice, then mixed with 2xloading buffer and assayed by IB analyses.

His Pull-down Assay

The assay was performed as described previously with slight modification [32, 64]. Briefly, NSCs treated with 0.5 mM His-Nogo-A-Δ20 were lysed in 0.5% CHAPS [40] (with superior solubilization power for membrane proteins) (Sigma-Aldrich) extract buffer (PBS (PH = 7.4), 1 mM EDTA, 5 mM NaF, 1x protease inhibitor cocktail (Roche), 1 mM PMSF), and then cell lysates and purified His tagged protein were incubated with Ni-NTA resin beads with gentle agitation at 4 °C for 2 h. The resin was successively washed at room temperature with four different washing buffers, containing 5 mM, 25 mM, 50 mM and 100 mM imidazole (each wash once) in 0.5% CHAPS extract buffer. Then, 300 µl extract buffer with 250 mM imidazole were added to the beads and protein eluted by slowly shaking at RT for 30 min. The eluate was processed for Western blot with anti-His antibody and A620 antibody.

Western Blot Analysis

Lysate Proteins of NSCs were obtained using RIPA lysis buffer (Beyotime Biotechnology). 20 µg NSCs lysate proteins and purified His-Nogo-A-Δ20 protein (amino acid peptide 564–749, ~ 23KD) were separated on 5 ~ 10% Tris–glycine SDS–PAGE gels and transferred onto nitrocellulose or PVDF membranes, according to the methods established by us [54, 58]. The total protein concentration was measured by Detergent Compatible Bradford Protein Assay Kit (No: P0006C; Beyotime Biotechnology). Additionally, after transfer and blocking with 5% non-fat dry milk, phospho antibody was firstly incubated with blot. Until the membrane had been exposed, then the blot was incubated in stripping buffer [62] at room temperature for 5 ~ 15 min with mild agitation, followed by two washes with TBST, and proceeded for another non-phosphorylated antibody detection according to the standard protocol developed by us

[56]. Images were acquired on the Cline imager (www.clinx.cn). Quantification on densitometry of protein bands using ImageJ software (National Institutes of Health).

Molecular Docking Analysis

3D diagrams of Nogo-A- Δ 20 protein and EphA4 protein interactions are plotted through ZDOCK and choose PyMOL software to view. According to the method described previously by us, the predication of PDB-structure of Nogo-A- Δ 20 is through SWISS-MODEL software [56].

Statistical analysis

Experiments were repeated at triple times independently. All data were reported as Mean $\pm$ SD. Statistical analysis was performed with GraphPad software (GraphPad Prism version 8.00, San Diego, CA). Experiments with three or more groups were subjected to a one-way ANOVA, followed by post hoc testing with Tukey LSD. Differences were considered significant when * $P < 0.05$ and # $P < 0.05$, highly as significant when ** $P < 0.01$ and ## $P < 0.01$.

Results

Characterization of interactions of Nogo-A- Δ 20-EphA4 on NSCs

As one of active regions of Nogo-A, Nogo-A- Δ 20 can strongly inhibit axon outgrowth [37]. The relationship between the location of Nogo-A and Nogo-A- Δ 20 is shown in Figure.2D. To investigate a possible direct interacting of Nogo-A- Δ 20 to EphA4, we first used an immunofluorescence test. NSCs staining with A620 [32] (provided by Dr. WeiLin, Jin) and EphA4 antibodies. The data indicate that Nogo-A- Δ 20 with EphA4 can be simultaneously expressed on the surface of NSCs and merge together labelled in yellow (Fig. 1A). Meanwhile, expression of Nogo-A- Δ 20 or EphA4 on NSCs can also be identified by Western blotting (Fig. 1B). To further confirm Nogo-A- Δ 20 physical association with EphA4, we used co-immunoprecipitation and His pull-down assays that also verify the direct interaction of two proteins. As illustrated in Fig. 3A, EphA4 was readily co-immunoprecipitated by A620, a rabbit antibody directed towards NogoA specific region (Fig. 2D), but not by a control rabbit IgG. In addition, endogenous EphA4 protein was pulled down with His-tagged Nogo-A- Δ 20 (Fig. 3B). Taken together, these results demonstrate that Nogo-A- Δ 20 binds to EphA4 and may regulate some physiological phenomenon of NSCs.

Finally, as shown in Fig. 2, Nogo-A- Δ 20 was expressed in E. coli. BL21 (DE3), which be purified with different concentrations of imidazole and analyzed by SDS-PAGE (Fig. 2A-B). The expression of Nogo-A- Δ 20 proteins were confirmed by western blot analysis using anti-His tag mouse monoclonal antibody. The protein bands at about 23 kDa were detected (Fig. 2C). After dialysis, ultrafiltration of 200 mM

elution, these proteins would be used in the experiments. These results indicate that His-tagged Nogo-A- Δ 20 recombinant proteins were expressed in *E. coli*. BL21 (DE3) and successfully purified.

NSCs apoptosis are counteracted by interactions of Nogo-A- Δ 20 and EphA4

Nogo-A can influence survival, proliferation, migration, and differentiation of NSCs, which was reviewed by our group [57]. However, the effects of Nogo-A- Δ 20 interacting with EphA4 on NSCs apoptosis have not been studied yet. To explore the function of Nogo-A- Δ 20-EphA4 interplay in NSCs, we performed TUNEL staining (Fig. 4A-B). To our surprise, Nogo-A- Δ 20 can decrease the number of TUNEL-labeled apoptotic NSCs at 300 nM and be attenuated by EphA4 inhibitor of Rhy (10 μ M). The data indicate that Nogo-A- Δ 20 plays an anti-apoptotic role on NSCs via interacting with EphA4. To further confirm our observation, we conducted western blot analysis in the NSCs exposure to Nogo-A- Δ 20 for 48 h, and we found the levels of several classical pro-apoptotic proteins, including full-length caspase 8, cleaved caspase 8, cleaved PARP, caspase 3 as well as p53 were markedly decreased, compared to control (* P < 0.05; ** P < 0.01) (Fig. 5A). Interestingly, in Nogo-A- Δ 20 plus Rhy groups, the previous proteins were significantly up-regulated again comparable to Nogo-A- Δ 20 group (# P < 0.05; ### P < 0.01) (Fig. 5B-F). These results strongly demonstrate that Nogo-A- Δ 20 contribute to cell survival via binding EphA4.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping protein is a highly conserved and commonly considered as a stable loading control contrasted to dynamic changes of other proteins expression. However, recent studies have shown that protein levels of GAPDH increase in related with neuronal and no neuronal cell death, in contrast, antisense oligonucleotides to GAPDH prevent cell death [7, 21, 22, 43, 44]. Consistent with these studies, we also found decreased level of GAPDH mediated the markedly anti-apoptotic effect in Nogo-A- Δ 20 treatment of NSCs for 48 h (** P < 0.01) (Fig. 5E).

Roles of MAPKs in antagonizing apoptosis of NSCs during Nogo-A- Δ 20 and EphA4 binding

Previous studies have indicated that regulation of EphA4 signaling is related to MAPK pathway [14, 46]. Accordingly, we further explored whether downstream signaling pathways of phosphorylation of ERK1/2, JNK and p38-MAP kinase (three MAPK modules) mediate anti-apoptotic roles for NSCs, then, we used Western blot assay at 5 min (Figure.6A), 30 min (Figure.6B) and 1 h (Figure.6C) to validate. As illustrated in Figure.6E-F, phosphorylation of p38 was substantially decreased, but phosphorylation of JNK was significantly increased comparable to control after Nogo-A- Δ 20 treatment for 5 min (** P < 0.01). However, phosphorylation of ERK1/2 was not altered at 5 min treatment. In addition, phosphorylation level of p38 was markedly enhanced and phosphorylation JNK was significantly reduced after treatment for 5 min with 10 μ M Rhy comparing with Nogo-A- Δ 20 group (# P < 0.05; ### P < 0.01). Interestingly, we also observed almost consistent results at 30 min (Figure.6I-J) and 1 h (Figure.6M-N). These data indicate that inhibition of p38 pathway and activity of JNK proteins enhancement are major players in Nogo-A- Δ 20/EphA4 interaction protecting NSCs from apoptosis.

Additionally, western blotting results showed that the levels of EphA4 were upregulated compared to control (Figure.6G, K, O) (** P < 0.01) and Rhy treatment alleviated the increase of EphA4 protein level

comparable to Nogo-A- Δ 20 group ($^{##}P < 0.01$) (Figure.6G, K, O). The data hints that Nogo-A- Δ 20 not only interacts with EphA4 but also affect EphA4 protein expression with some unknown mechanisms.

The p38 MAPK and JNK signaling are necessary for Nogo-A- Δ 20-EphA4 in inhibition of NSCs apoptosis

p38 MAPK and JNK as a major mediator of cell apoptosis and survival [17, 48, 52, 59]. To further confirm our findings and determine precise regulatory roles of p38 MAPK and JNK pathway in Nogo-A- Δ 20-EphA4 interaction-induced anti-apoptosis in NSCs, a potent p38 activator (Dehydrocorydaline chloride; DHC) and a specific inhibitor of JNK (SP600125) were added to NSCs cultures 3 h prior to treatment with Nogo-A- Δ 20 for 48 h. As illustrated in Figure.7A, treatment with 10 μ M DHC notably antagonized anti-apoptotic roles of NSCs induced by Nogo-A- Δ 20. The protein expression levels of pro-apoptotic full-length caspase 8, cleaved PARP, caspase 3 as well as p53 were obviously elevated compared with Nogo-A- Δ 20 group ($^{\#}P < 0.05$; $^{##}P < 0.01$) (Figure.7B-E). Therefore, these data show that inactivation of p38 MAPK (p-p38) as a potential mechanism participating in Nogo-A- Δ 20-mediated interaction with EphA4 against NSCs apoptosis.

Next, we further examined whether inhibition of JNK signaling induced apoptosis resistance in NSCs. As described in Figure.8A-E, 10 μ M JNK inhibitor SP600125 substantially raised pro-apoptotic proteins level including full-length caspase 8, cleaved PARP, caspase 3 and p53 as against Nogo-A- Δ 20 group ($^{\#}P < 0.05$; $^{##}P < 0.01$). These findings strongly support that Nogo-A- Δ 20/EphA4 interplay antagonizes NSCs apoptosis through raising phosphorylation of JNK.

Interactions of Nogo-A- Δ 20 with EphA4 by Molecular Docking Studies.

The extracellular region of EphA4 consists of ephrin ligand-binding domain (LBD), cysteine-rich domain, and fibronectin type III domain. LBD binds to the receptor-binding domain (RBD) of ephrins [60]. Thus, to analysis the binding model between Nogo-A- Δ 20 and ectodomain of EphA4 (Figure.S9), we performed molecular docking via ZDOCK server. As shown in Figure.9A, the interaction between Nogo-A- Δ 20 (green sticks) and EphA4 ectodomain (brown sticks) can be observed. Two hydrogen bonds were viewed between the residues of Lys-205 (bond length: ~ 3.2 Å) and Ile-190 (bond length: ~ 3.3 Å) in Nogo-A- Δ 20 with the residues of Gln-390 and Asn-425 at EphA4 ectodomain (Figure.9A). Intriguingly, we observed that proline residues at position 194 (magenta) of Nogo-A- Δ 20 and at 426 (red) of EphA4 directly bind. Moreover, lysine at position 205 (red) of Nogo-A- Δ 20 and asparagine at 391 (magenta) of EphA4 form isopeptide bond. The prior analysis data indicate that formation of proline-proline interaction and interchain isopeptide bonds [24, 28] may contribute to enhance stable affinity for protein-protein interactions. Based on the observations, the predicted results from atomic level demonstrate that binding to EphA4 by Nogo-A- Δ 20 may be attributable to its strong binding occurred in Nogo-A- Δ 20 residues Lys-205, Ile-190, Pro-194 and EphA4 residues Gln-390, Asn-425 as well as Pro-426.

Finally, we propose the graphic model in Figure.9B to illustrate the mechanisms how Nogo-A- Δ 20 bind to EphA4 attenuates NSCs apoptosis based on our results and other associated studies.

Discussion

Numerous evidences have shown that intraneuronal Nogo protein can interact with anti-apoptotic protein Bcl-2 or Bcl-xL and reduce their anti-apoptotic activity [49]. However, the function of extraneuronal Nogo-A- Δ 20 signaling in apoptosis of NSCs is not known. Here we describe a novel Nogo-A- Δ 20-interacting protein belonging to Eph family, EphA4 and demonstrates their functional significance of binding in NSCs apoptosis.

Currently, abnormal increased activity of EphA4 has been reported to implicate in different pathological processes of human diseases, including neurodegenerative diseases and cancers. Accordingly, novel small molecule inhibitors targeted EphA4, such as dimethylpyrrole derivatives, biologically active peptides (APY, KYL, VTM) and Nilotinib have been identified [16, 35, 36]. Unfortunately, lacking of adequate experiments and studies *in vivo* support antagonistic function of these inhibitors for EphA4. However, Nancy Y. Ipa *et.al.* reported that rhynchophylline (Rhy), a novel EphA4 inhibitor, could impede EphA4 signaling and improve Alzheimer's disease-associated synaptic dysfunctions in mouse models [13]. Hence, Rhy was used as antagonist of EphA4 in our research. Consistent with preceding evidence, we found that 10 μ M Rhy could block Nogo-A- Δ 20 anchoring to EphA4 and facilitates NSCs apoptosis at different time points.

The mitogen-activated protein kinases (MAPKs) pathway have been shown to exert an important role in transduction extracellular stimulation to intracellular targets. Besides, MAPK family members, including ERK1/2 (extracellular-signal-regulated kinases), JNK (Jun amino-terminal kinases), and p38/SAPKs (stress-activated protein kinases), participate in cell proliferation, survival, differentiation, and apoptosis or programmed cell death [5, 34, 39]. More recently, it has been reported that EphA receptor as regulators is linked to downstream signaling effectors such as MAPKs [2, 33]. Hence, we hypothesized that Nogo-A- Δ 20/EphA4 interactions may mediate regulation of cell apoptosis by MAPKs cascade singling. In this manuscript, 300 nM Nogo-A- Δ 20 treatment for 48 h markedly reduced the in number of TUNEL-labeled NSCs (marker of cell death), but apoptosis was significantly increased via adding Rhy into medium (Figure.4). The results preliminary prove that Rhy, a potent antagonist of EphA4, can block binding between Nogo-A- Δ 20 and EphA4, which finally lead to cell death. Then, we detected whether pro-apoptotic proteins were also altered in Nogo-A- Δ 20 treatment by western blot. Our data indicate that full-length caspase 8, cleaved caspase 8, cleaved PARP, caspase 3 as well as p53 were significantly reduced, compared with control. However, in Rhy intervention group, all above proteins level were be rescued (Figure.5). Next, we identified whether MAPKs participated in these process. Finally, we found that p38 and JNK MAPK singling are associated with NSCs survival-mediated by Nogo-A- Δ 20/ EphA4 interaction (Figure.6). In contrast, total ERK and p-ERK maintain unchanging. The data hint that ERK pathway is not responsible for Nogo-A- Δ 20 preventing apoptosis occurrence via binding with EphA4. Perhaps ERK pathway activation are always involved in the proliferation and differentiation of NSCs [46].

p38 MAPK is deemed stress responsive and has been involved in the regulation of cell apoptosis. However, given the roles of p38-MAPK in apoptosis are also diverse. One hand, there are even more

reports concerning a pro-apoptotic function of p38-MAPK pathway [10, 38, 42], on the other hand, p38-MAPK cascades enhance cell survival and growth [31]. Our results show that p38-MAPK activity is reduced while NSCs exposed to Nogo-A- Δ 20 for 48 h followed by decreased NSCs apoptosis. Nevertheless, activation of p38-MAPK activity by DHC attenuates inhibition of apoptosis induced by Nogo-A- Δ 20 (Figure.7). These data therefore indicate that the downregulation of p38-MAPK pathway is required for anti-apoptotic effects mediated by Nogo-A- Δ 20/EphA4 interaction. Similar to p38 MAPK, JNKs are ubiquitously expressed and JNK pathways control a series of cellular processes, such as cell growth, differentiation and apoptosis. Surprisingly, JNK signaling molecules also present multiple roles among apoptosis, including anti-apoptotic and pro-apoptotic function [12, 51]. In addition, JNKs have been shown to be involved in promoting apoptosis by nuclear signaling and mitochondrial-mediated intrinsic apoptotic pathways [11]. Likewise, our results indicate that reduction of JNK-MAPK activity by SP600125 (a selective inhibitor of JNK) enhance apoptotic NSCs (Figure.8). Thus, these data suggest that upregulation of JNK activity are involved in inhibition of apoptosis for Nogo-A- Δ 20 binding to EphA4 on NSCs. Taken together, our data demonstrate that p38-MAPK signaling and JNK-MAPK participating in apoptotic signaling pathway are various and complicated, the outcomes are dependent on stimuli and cell types.

Limitations Of The Study

Although our results suggest that p38 and JNK MAPK pathways are correlated with preventing apoptosis, it is not clear that whether Nogo-A- Δ 20-dependent stimuli causes phospho-signaling changes of EphA4 intracellular domain and recruits some downstream molecules to its phosphorylation sites. Further studies are needed to produce phospho-specific antibodies against EphA4 for detecting the phenotypic variation. Interestingly, we also found EphA4 expression was enhanced when NSCs treatment with Nogo-A- Δ 20 accompanied by alteration of p38 and JNK MAPK activity (Figure.6G, K, O). Therefore, we would determine whether modulating the level of p38 and JNK MAPK inside the cell can influence total protein expression of EphA4, and decide over life or death of NSCs soon. Subsequently, we only display parallel changing of p38 and JNK MAPK signaling during Nogo-A- Δ 20/EphA4 link, but whether cross-activation occurs between these pathways are needed to be furthermore tested, because cross-talks between MAPKs are involved in regulation of different cellular responses [53].

Conclusions And Future Directions

Our study highlights a critical role of trans-membrane EphA4 as novel receptor for Nogo-A- Δ 20-mediated inhibition of NSCs apoptosis. Nevertheless, the molecular mechanisms remain to be investigated. An attractive question for future research is to evaluate the anti-apoptotic roles of Nogo-A- Δ 20/EphA4 interaction on neuron survival in progressive neurodegenerative diseases, including Huntington's disease and Amyotrophic lateral sclerosis [55].

Abbreviations

Neural stem cells: NSCs; Dehydrocorydaline Chloride: DHC; SP600125: SP; Rhynchophylline: Rhy; Glyceraldehyde-3-phosphate dehydrogenase: GADPH; CNS: central nervous system.

Declarations

Acknowledgements

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Jun-Ling Wang: Formal analysis, writing original draft. **Wei-Guang Chen:** Formal analysis. **Chao-Jin Xu:** Formal analysis, Writing - original draft.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Figure Caption

Figure.S9 Overall diagram of interaction between Nogo-A- Δ 20 with EphA4

Cartoon structure of α -helix represents Nogo-A- Δ 20. Brown sticks indicate residues of binding sites located in EphA4 ecodomain. LBD: ephrins ligand-binding domain. RBD: receptor-binding domain of ephrins.

Figures

Fig.1

Figure 1

Co-expression of Nogo-A- Δ 20 and EphA4 in neural stem cells. (A) The monoclonal antibody (mAb) A563 against aa 563–627 (amino acids (aa) 563–627) of rat Nogo-A-NiR- Δ 20, which is the main inhibitory region of rat Nogo-A. Immunofluorescence staining demonstrate that NSCs spheres are both positive for A563 and EphA4. Co- expression regions are labeled in yellow in the merged images. Staining of Hoechst

33258 to show the nuclei. Scale bar 50 μ m. (B) Western blot analysis detecting Nogo-A- Δ 20 (~23 kDa) and EphA4 (~110 kDa) protein in membrane fractions of neurospheres.

Figure 2

Expression and purification of His-Nogo-A- Δ 20 protein. (A) Comassie blue-stained SDS-PAGE gel of 6xHis-tagged recombinant proteins. Marker, protein marker; induced by IPTG, cell lysate with 1mM IPTG induction for 4 h at 37°C; uninduced, cell lysate without induction. (B) Comparison of his6-Nogo-A- Δ 20

purifications using 50 mM, 100 mM and 200 mM imidazole loading conditions by SDS-PAGE and Comassie blue-staining. Marker, protein marker; 50 mM, 50 mM imidazole elution; 100 mM, 100 mM imidazole elution; 200 mM, 200mM imidazole elution. (C) Confirmation of the expression of His-Nogo-A- Δ 20 protein by western blot using the primary antibodies against His tag. His-tagged proteins were eluted with elution buffer containing 50 mM, 100 mM and 200 mM imidazole. (D) The diagram represents the three inhibitory regions of rat Nogo-A, which are Nogo-A NiR- Δ 2 (amino acids [aa] 59–172), NiR- Δ 20 (aa 544–725) and Nogo-66 (aa 1019–1083), respectively. The different antigen recognition sites can be recognized by various antibody.

Fig.3

Figure 3

Nogo-A-Δ20 Interacts with EphA4 in the NSCs' cellular state. (A) Co-immunoprecipitation assays between Nogo-A-Δ20 and EphA4 using lysate from NSCs. NSCs lysate were blotted with A620 antibody against amino-NogoA. (B) NSCs were incubated with 300nM Nogo-A-Δ20 for 48 h, and then were submitted to Ni²⁺ pull-down assay. Prey Protein, NSCs lysate; Bait Protein: purified His-Nogo-A-Δ20 protein. Ni-NTA

resin beads coupled with His-tagged recombinant protein was analyzed by immunoblotting with anti-His antibody. *Presented the specific bands of EphA4.

Figure 4

Cell-Surface EphA4 mediates Nogo-A-Δ20 inhibition of NSCs apoptosis. (A) NSCs were treated with 300nM Nogo-A-Δ20 and 10 μM Rhy (EphA4 inhibitor) for 48 h, determined by TUNEL staining. Scale bar 5μm. (B) Quantitative analysis of apoptotic NSCs revealed that a lower percentage of apoptotic cell with Nogo-A-Δ20 treatment group than Nogo-A-Δ20+Rhy. Representative data from three independent experiments are shown. **P < 0.01 vs control group, #P < 0.05 vs. Nogo-A-Δ20 group.

Fig.5

Figure 5

p38 MAPK inhibition and JNK activation are involved in regulation of NSCs apoptosis during Nogo-A-Δ20 acts at different time points. NSCs were treated with Nogo-A-Δ20 with Rhy or without Rhy for 5mins (A), 30mins (B) and 1h (C). After the indicated times, treated cells were lysed and subjected to Western blot analysis with different phosphorylations of MAPKs. Quantitation of the level of phosphorylations activity of MAPKs at 5mins treatment (D, E, F, G), at 30mins treatment (H, I, J, K) and at 1h treatment (L, M, O, N). The means $\pm$ SD from three separate experiments are shown. β -actin was used as a loading control for Western blotting and normalized the levels of protein. * $P < 0.05$ or ** $P < 0.01$ vs control group, # $P < 0.05$ or ### $P < 0.01$ vs. Nogo-A-Δ20 group. Data were normalized to that of Control.

Fig.6

Figure 6

Nogo-A-Δ20 prevents apoptosis of NSCs via EphA4. (A) Total NSCs proteins were extracted at 48 h after Nogo-A-Δ20 treated with Rhy or without Rhy (EphA4 inhibitor). Level of apoptosis-related proteins were detected by Western blotting. Equal loading control was confirmed by β-actin. (B-F) Data represent the means ± SD from three separate experiments. Each sample was normalized to β-actin. *P < 0.05 or **P <

0.01 vs control group, #P < 0.05 or ##P < 0.01 vs. Nogo-A-Δ20 group. Data were normalized to that of Control.

Fig.7

Figure 7

Inhibition of p38 MAPK mediates Nogo-A-Δ20 blocking apoptosis of NSCs via EphA4. (A) NSCs exposure to Nogo-A-Δ20 for 48 h with DHC or without DHC (p38 MAPK activator) treatment. Cell lysates were prepared and the expression level of pro-apoptosis proteins including caspase-8, PARP, caspase- 3 and

p53 were examined by Western blot analysis. β -actin was used to equivalent protein loading. (B-E) The figures are representative of 3 independent experiments, respectively. Target proteins were normalized to β -actin.*P < 0.05 or **P < 0.01 vs control group, #P < 0.05 or ##P < 0.01 vs. Nogo-A- Δ 20 group. Data were normalized to that of Control.

Fig.8

Figure 8

Activation of JNK pathway contributes to NSCs apoptosis during Nogo-A- Δ 20 binding EphA4. (A) Lysates from NSCs treated by Nogo-A- Δ 20 for 48 h with SP or without SP (JNK inhibitor) treatment were performed to western blot with apoptotic-related proteins. β -actin was selected as internal control. (B-E) Quantification by densitometric scans was presented by apoptotic-related antibody/ β -actin. Data were normalized to β -actin. n=3, Mean $\pm$ S.D. *P < 0.05 or **P < 0.01 vs control group, #P < 0.05 or ##P < 0.01 vs. Nogo-A- Δ 20 group. Data were normalized to that of Control.

A

B

Fig.9

Figure 9

Schematic diagram of the mechanism of Nogo-A-Δ20 with EphA4 cooperate to regulate NSCs apoptosis. (A) Molecular Docking conformation of Nogo-A-Δ20 in complex with EphA4 was performed using the ZDOCK server. Nogo-A-Δ20 is depicted as green sticks, whereas the Nogo-A-Δ20-binding extracellular domain of EphA4 is indicated with orange brown sticks and closing to purple β-sheet. The 3D structure of Nogo-A-Δ20 was predicted by us using SWISS-MODEL software and the human crystal structure of

EphA4 ectodomain (PDB ID: 4M4P; resolution: 2.081Å) was downloaded from website <http://www.rcsb.org/structure/4M4P> . The ZDOCK (version 3.0.2) with the default option were utilized for docking. According to the docking scores, the structure with the highest grade was subject to visual analysis with the software PyMoL software. (B) Working model of Nogo-A-Δ20 binding EphA4 inhibits NSCs apoptosis through coordinate inhibition of p38 and activation JNK MAPK pathways.

Supplementary Files

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- [Figure.S9.tif](#)