Nogo-A-Δ20/EphA4 interaction antagonizes apoptosis of neural stem cells by integrating p38 and JNK MAPK signaling

Jun-Ling Wang1 · Wei-Guang Chen2 · Jia-Jia Zhang3 · Chao-Jin Xu2

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Abstract
Nogo-A protein consists of two main extracellular domains: Nogo-66 (rat amino acid [aa] 1019–1083) and Nogo-A-Δ20 (extracellular, active 180 amino acid Nogo-A region), which serve as strong inhibitors of axon regeneration in the adult CNS (Central Nervous System). Although receptors S1PR2 and HSPGs have been identified as Nogo-A-Δ20 binding proteins, it remains at present elusive whether other receptors directly interacting with Nogo-A-Δ20 exist, and decrease cell death. On the other hand, the key roles of EphA4 in the regulation of glioblastoma, axon regeneration and NSCs (Neural Stem Cells) proliferation or differentiation are well understood, but little is known the relationship between EphA4 and Nogo-A-Δ20 in NSCs apoptosis. Thus, we aim to determine whether Nogo-A-Δ20 can bind to EphA4 and affect survival of NSCs. Here, we discover that EphA4, belonging to a member of erythropoietin-producing hepatocellular (Eph) receptors family, could be acting as a high affinity ligand for Nogo-A-Δ20. Trans-membrane protein of EphA4 is needed for Nogo-A-Δ20-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-Δ20 and EphA4 residues Gln-390, Asn-425, Pro-426 might play critical roles in Nogo-A-Δ20/EphA4 binding via molecular docking.

Keywords Nogo-A-δ20 · EphA4 · Apoptosis · p38 and JNK · Neural stem cells

Abbreviations
NSCs Neural stem cells
DHC Dehydrocorydaline chloride
SP SP600125
Rhy Rhynchophylline
GADPH Glyceraldehyde-3-phosphate dehydrogenase
CNS Central nervous system
SCI Control group: Con. spinal cord injury

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 (Spanevello et al. 2013; Xu et al. 2015; Depaepe et al. 2005; Aoki et al. 2004; Joly et al. 2014). The Eph family consists of 14–16 receptor members, including EphA and EphB, based on their sequence similarity and binding affinities (Darling and Lamb 2019; Lisabeth et al. 2013). With regard to Eph receptors, EphA4 has the unique property to bind to either
the ephrins A or ephrins B ligands, respectively (Bowden et al. 2009). 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 (Lamberto et al. 2014). One hand, deregulations of EphA4 expression are implicated in diverse cancers, including leukemia, prostate and glioblastoma (Lamberto et al. 2014). On the other hand, genetic deletion of EphA4 can promote axonal regeneration and functional recovery after spinal cord injury (Yiu and He 2006; Goldshmit et al. 2004). Interestingly, EphA4 is also expressed on NSCs and involved in NSCs differentiation, proliferation, migration and apoptosis (Khodosevich et al. 2011; Furne et al. 2009; Hara et al. 2010). To our knowledge, only Aβ and SORLA have been reported to binding with EphA4 receptor tyrosine kinase and attenuate cognitive memory deficits in neurodegenerative animal models (Huang et al. 2017; Vargas et al. 2014) (Table 1).

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-Δ20’ or ‘NiG-Δ20’ as well as a 66-amino-acid residues extracellular domain (Nogo-66; rat amino-acid 1019–1083) (Oertle et al. 2003; Mi et al. 2012). The domains are recognized to display strong inhibitory activity for axon growth (Huebner et al. 2011; Oertle et al. 2003). In addition, Nogo-66

Table 1 Key resources

| Regent                                    | Source                  | Catalogue numbers |
|-------------------------------------------|-------------------------|-------------------|
| β-actin antibody                          | Affinity Biosciences    | AF7018            |
| EphA4 antibody                            | Affinity Biosciences    | AF5496            |
| His-Tag Mouse Monoclonal Antibody          | Affinity Biosciences    | T0009             |
| NF-κB p65 antibody                         | Sigma Aldrich           | SAB4502609        |
| GADPH antibody                             | Sigma Aldrich           | G8795             |
| Caspase 8 antibody                         | Proteintech Group       | 66093-1-Ag        |
| α-tubulin antibody                         | Cell Signaling Technology | #2144      |
| ERK1/2 antibody                            | Cell Signaling Technology | 137F5             |
| Phospho-p44/42 MAPK                        | Cell Signaling Technology | 4370S            |
| A563                                       | BD Biosciences          | No. 612238        |
| A620                                       | Gift from Dr. Wei-Lin, Jin | sc-2003    |
| Protein A/G PLUS-Agarose                   | Santa Cruz              |                  |
| Phospho-p44/42 MAPK (Erk1/2)               | Cell Signaling Technology | 4370S            |
| Phospho-SAPK/JNK                           | Cell Signaling Technology | 9255S            |
| SAPK/JNK Antibody                         | Cell Signaling Technology | 9252S            |
| p38MAPK Antibody                          | Cell Signaling Technology | 9212S            |
| Phospho-p38 MAPK                           | Cell Signaling Technology | 4511S            |
| PARP (46D11) Rabbit mAb                    | Cell Signaling Technology | 9532S            |
| p53 (1C12) antibody                        | Cell Signaling Technology | #2542S           |
| GFAP antibody                              | Cell Signaling Technology | 80,788           |
| Caspase-3 Antibody                         | Cell Signaling Technology | #9662            |
| HRP-conjugated goat anti-mouse IgG         | Beyotime Biotechnology  | A0216             |
| HRP-conjugated goat anti-rabbit IgG        | Beyotime Biotechnology  | A0208             |
| PageRuler™ Plus Prestained Protein Ladder  | No: 26619               | Thermo Fisher     |
| Dual Color Prestained Protein Marker       | Epizyme Biotechnology   | No: W102          |
| Cell lysis buffer                          | Beyotime Biotechnology  | P0013             |
| Coomassie Blue Fast Staining Solution      | Beyotime Biotechnology  | P0017             |
| IPTG                                       | Beyotime Biotechnology  | ST098-5 g         |
| kanamycin                                  | Beyotime Biotechnology  | ST102             |
| Ni-NTA agarose                             | Qiagen Inc., Valencia   |                  |
| In situ apoptosis detection (TUNEL) kit     | Roche, Switzerland      |                  |
| Dehydrocorydaline chloride                 | MedChem Express company  | HY-N0674A         |
| SP600125                                   | MedChem Express company  | HY-12041          |
| Rhynchophylline (Rhy)                      | Baoji Herbest Bio-Tech  |                  |
bonds receptor NgR1 and PIRB and activates RhoA signaling pathway (Ramasamy et al. 2014; Schwab and E.). Recently, ADGRB1 has been identified as a new receptor for Nogo myelin-associated inhibitory proteins via using CRISPR screening (Chong et al. 2018). On one hand, Nogo-A-Δ20 binds to S1PR2, which represses synaptic plasticity and stabilizes neuronal circuits (Kempf et al. 2014). On the other hand, Nogo-A-Δ20 interplaying with HSPGs but not S1PR2 on the cell surface accelerates the migration and adhesion of neuroblasts (Kempf et al. 2014). Nevertheless, there is still an incomplete understanding of Nogo-A-Δ20 receptors.

Our previous experiments demonstrated that NSCs allogeneic transplantation have been successful strategies for experimental treatment of spinal cord injury ( SCI) (Xu et al. 2011, 2010). However, the extracellular inflammatory microenvironment at injury sites leads to massive cell death for NSCs grafts, which is considered to be associated to NSCs apoptosis (Wang et al. 2014). In addition, EphA4 receptors, a novel dependence receptor, could induce cellular apoptosis in the absence of ephrin ligands (Furne et al. 2009). MAPK pathway is involved in intracellular signaling cascades of EphA4 receptor (Fukai et al. 2008; Miao et al. 2001; Aoki et al. 2004). At the lesion site, factors such as NogoA and Eph receptors are thought to be detrimental to functional recovery after SCI (Liebscher et al. 2005; Goldsmith et al. 2006). Therefore, we employ cell models to simulate the microenvironment in vivo of transplanted NSCs and investigate whether NogoA binding to EphA4 receptor affects NSCs apoptosis in vitro by MAPK signaling.

Our study highlights a critical role of trans-membrane EphA4 as novel receptor for Nogo-A-Δ20-mediated inhibition of NSCs apoptosis. Our research results may be beneficial in future research to evaluate the anti-apoptotic roles of Nogo-A-Δ20/EphA4 interaction on neuron survival in progressive neurodegenerative diseases, including Huntington’s disease and Amyotrophic lateral sclerosis (Wang and Xu 2020).

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). The target of the antibody (Nogo-A), 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), Polyvinylidenefluoride (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), GFAP antibody (#80,788) and Caspase-3 Antibody (#9662) were from Cell Signaling Technology. Mouse monoclonal antibody GADPH (G8795) was from sigma Aldrich. Caspase 8 antibody (66093-1-lg) 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) SD rats (supplied by Chinese Academy of Sciences) according to the method described previously by our group (Zhang et al. 2016; Xu et al. 2011). All NSCs used in the experiments were between passages two to three. NSCs growing in EGF/bFGF supplemented medium.

Immunofluorescence Assay

We used a standard procedure for immunofluorescence staining of NSCs spheres in vitro as described previously by our group (Zhang et al. 2016; Wang et al. 2018). 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-Δ20 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 Mlul 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 μg/mL kanamycin at a shaking (~250 rpm) to saturation, were diluted 1:25 in 250 mL fresh LB in a 1L 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-Δ20 protein purification**

Purification of recombinant proteins was performed as described previously (Xue et al. 2016; Bartlow et al. 2011). 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 µm 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**

NSCs were treated with 300 nM Nogo-A-Δ20 and 10 μM Rhy (EphA4 inhibitor) for 48 h. 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 (Antrobus and Borner 2011). Briefly, NSCs were permeabilized with Lysis Buffer (50 mM Tris-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 × g rpm, 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.

**Ni2+ pull-down assay**

The assay was performed as described previously with slight modification (Zhang and Sarge 2008; Mi et al. 2012). Briefly, NSCs treated with 0.5 mM His-Nogo-A-Δ20 were lysed in 0.5% CHAPS (Posch 2014) (with superior solubilization power for membrane proteins) (Sigma-Aldrich) extract buffer (PBS (PH = 7.4), 1 mM EDTA, 5 mM NaF, 1×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 (Xu et al. 2019; Wang et al. 2018). 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 (Yeung and Stanley 2009) 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 (Wang et al. 2020). Then peroxidase reaction was visualized with an enhanced chemiluminescence (ECL; Beyotime Biotechnology) reagents and exposure times were only about 5 ~ 10 s to ensure a linear detection response. ECL detections were done with an accumulation signal mode. Images were acquired on the Clinx imager (www.clinx.cn). Quantification on densitometry of
protein bands using ImageJ software (National Institutes of Health).

**EphA4 siRNA transfection**

Primary NSCs were seeded at a density of $2 \times 10^6$ cells/ml in 6-well plates and cultured for 12 h, after which they were transfected with siRNAs-EphA4 and negative control siRNAs (Genepharma, Shanghai, China) using Lipofectamine™ 2000 Transfection Reagent (Catalog number:11668019) following the manufacturer’s instructions.

**Molecular docking analysis**

3D diagrams of Nogo-A-Δ20 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-Δ20 is through SWISS-MODEL software (Wang et al. 2020).

**Statistical analysis**

Experiments were repeated at triple times independently. All data were reported as Mean ± 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 between two populations were tested by Student’s t-test. 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-Δ20-EphA4 on NSCs.**

To evaluate whether EphA4 can be identified a new receptor for Nogo-A-Δ20 in regulating cell death, we examined the interactions by immunofluorescence and co-immunoprecipitation tests from NSCs samples. As one of active regions of Nogo-A, Nogo-A-Δ20 can strongly inhibit axon outgrowth (Oertle et al. 2003). To investigate a possible direct interacting of Nogo-A-Δ20 to EphA4, we first used an immunofluorescence test. NSCs staining with A563 and EphA4 antibodies. The data indicate that Nogo-A-Δ20 with EphA4 can be simultaneously expressed on the surface of NSCs and merge together labelled in yellow and all NSCs positive for Nogo-A and EphA4. (Fig. 1a and b). Meanwhile, expression of Nogo-A-Δ20 or EphA4 on NSCS can also be identified by Western blotting (Fig. 1c, d). Additionally, GFAP (glial fibrillary acidic protein)-positive marker (Ahmed et al. 2012) and housekeeping marker β-actin were used to prove the nature of NSCs samples (Fig. 1e, f). In addition, the relationship between the location of Nogo-A and Nogo-A-Δ20 is shown in Fig. 1g.

As shown in Fig. 2, Nogo-A-Δ20 was expressed in E. coli. BL21 (DE3), which be purified with different imidazole concentrations and analyzed by SDS-PAGE (Fig. 2a, b). The expression of Nogo-A-Δ20 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-Δ20 recombinant proteins were expressed in E. coli. BL21 (DE3) and successfully purified.

To further confirm Nogo-A-Δ20 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. 1g), but not by a control rabbit IgG. In addition, endogenous EphA4 protein was pulled down with His-tagged Nogo-A-Δ20 (Fig. 3b). Taken together, these results demonstrate that Nogo-A-Δ20 could bind to EphA4 in NSCs surface.

**NSCs apoptosis are counteracted by interactions of Nogo-A-Δ20 and EphA4**

Nogo-A can influence survival, proliferation, migration, and differentiation of NSCs, which was reviewed by our group (Xu et al. 2015). However, the effects of Nogo-A-Δ20 interacting with EphA4 on NSCs apoptosis have not been studied yet. To explore the function of Nogo-A-Δ20- EphA4 interplay in NSCs, we performed TUNEL staining (Fig. 4a–f). To our surprise, Nogo-A-Δ20 can decrease the number of TUNEL-labeled apoptotic NSCs at 300 nM and be attenuated by EphA4 inhibitor of Rhy (10 μM). The data indicate that Nogo-A-Δ20 plays an anti-apoptotic role on NSCs via interacting with EphA4 (#P < 0.05; ##P < 0.01) (Fig. 4g). To further confirm our observation, we conducted western blot analysis in the NSCs exposure to Nogo-A-Δ20 for 48 h, and we found the levels of several classical pro-apoptotic proteins, including full-length caspase 8 (0.55-fold reduction), caspase 3 (0.72-fold reduction), p53 (0.85-fold reduction) as well as cleaved-PARP (0.81-fold reduction) were apparently decreased, compared to control (Fig. 5a). Interestingly, in Nogo-A-Δ20 plus Rhy groups, the previous proteins were up-regulated again to some extent comparable to Nogo-A-Δ20 group (caspase 8, 1.8-fold upregulation; caspase 3, 2.7-fold upregulation; p53, 2.6-fold upregulation; cleaved-PARP, 2.1-fold upregulation) (*P < 0.05; **P < 0.01)
Fig. 5b–f). These results strongly demonstrate that Nogo-A-Δ20 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 non-neuronal cell death, in contrast, antisense oligonucleotides to GAPDH prevent cell death (Sawa et al. 1997; Saunders et al. 1999; Ishitani et al. 1996, 2003; Colell et al. 2009). Consistent with these studies, we also found decreased level of GAPDH (0.85-fold reduction) mediated the markedly anti-apoptotic effect in Nogo-A-Δ20 treatment of NSCs for 48 h, compared to control (***P < 0.01; **P < 0.01) (Fig. 5e).

Roles of MAPKs in antagonizing apoptosis of NSCs during Nogo-A-Δ20 and EphA4 binding

Previous studies have indicated that regulation of EphA4 signaling is related to MAPK pathway (Shu et al. 2016; Fukai et al. 2008). 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 (Fig. 6a), 30 min...
(Fig. 6b) and 1 h (Fig. 6c) to validate. As illustrated in Fig. 6e–f, phosphorylation of p38 was substantially decreased (0.52-fold reduction), but phosphorylation of JNK was significantly increased (1.68-fold upregulation) comparable to control after Nogo-A-Δ20 treatment for 5 mins (**P < 0.01). However, phosphorylation of ERK1/2 was not altered at 5 mins treatment. In addition, phosphorylation level of p38 was markedly enhanced (1.53-fold upregulation) and phosphorylation JNK was significantly reduced (0.56-fold reduction) after treatment for 5 mins with 10 μM Rhy comparing with Nogo-A-Δ20 group (##P < 0.01) (Fig. 6a). Interestingly, we also observed almost consistent results at 30 mins (Fig. 6i-j) and 1 h (Fig. 6m-n). These data indicate that inhibition of p38 MAPK and JNK signaling are necessary for Nogo-A-Δ20-EphA4 interaction-induced anti-apoptosis in NSCs.

Additionally, western blotting results showed that the levels of EphA4 were increased (1.62-fold upregulation) comparable to control after Nogo-A-Δ20 treatment for 5 mins (Fig. 6g, k, o) (**P < 0.01) and Rhy treatment alleviated the increase of EphA4 protein level (0.45-fold reduction) comparable to Nogo-A-Δ20 group (##P < 0.01) (Fig. 6g, k, o). The data hints that Nogo-A-Δ20 interacts not only with EphA4, but also increases EphA4 protein expression with some unknown mechanisms.

The p38 MAPK and JNK signaling are necessary for Nogo-A-Δ20-EphA4 in inhibition of NSCs apoptosis.

p38 MAPK and JNK as a major mediator of cell apoptosis and survival (Xu et al. 2012; Gururajan et al. 2005; Sui et al. 2014; Wada and Penninger 2004). To further confirm our findings and determine precise regulatory roles of p38 MAPK and JNK pathway in Nogo-A-Δ20-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-Δ20 for 48 h. As illustrated in Fig. 7a, treatment with 10 μM DHC notably antagonized anti-apoptotic roles of NSCs induced by Nogo-A-Δ20. The protein expression levels of pro-apoptotic full-length caspase 8 (1.84-fold upregulation), cleaved PARP (1.89-fold upregulation), caspase 3 (1.85-fold upregulation) as well as p53 (4.13-fold upregulation) were obviously elevated compared with Nogo-A-Δ20.
Next, we further examined whether inhibition of JNK singling induced apoptosis resistance in NSCs. As described in Figs. 8a–e, 10 μM JNK inhibitor SP600125 substantially raised pro-apoptotic proteins level including full-length caspase 8 (1.73-fold upregulation), cleaved PARP (2.58-fold upregulation), caspase 3 (1.55-fold upregulation) and p53 (1.38-fold upregulation) as against Nogo-A-Δ20 group (*P < 0.05; **P < 0.01). These findings strongly support that Nogo-A-Δ20/EphA4 interplay antagonizes NSCs apoptosis through raising phosphorylation of JNK.

Because Rhy has a multitude of activities and targets, thus, EphA4 knockdown experiments also were performed and confirm whether EphA4 is required for the anti-apoptosis effects of Nogo-A-Δ20 in NSCs (Fig. S2A-E). To investigate whether EphA4 is necessary for NSCs survival when NSCs treatment with Nogo-A-Δ20, we first verify EphA4 siRNAs knockdown efficacy through blotting (Fig. S2 A, C). Next, the primary NSCs were transfected with a ctl-small interfering RNA (sictl) or EphA4 siRNA for 48 h, then Nogo-A-Δ20 treatment for another 24 h. The results show that cleaved-PARP and caspase 3 level could be enhanced by ~1.8 fold in Nogo-A-Δ20 + EphA4 siRNA groups, compared to Nogo-A-Δ20 + sictl treatment (Fig. S2 B, D, and E) (**P < 0.01). Nevertheless, to determine whether SP and DHC treatment affect the apoptosis in NSCs, our supplementary experiments showed that SP did not significantly influence the expressions of pro-apoptotic proteins like caspase 8, cleaved-PARP and p53 (Fig. S3 A, C-E) and DHC also did not work against caspase 8 and cleaved-PARP (Fig. S3 B, F-G), whereas DHC raised the p53 expression level, compared to control group (*P < 0.05) (Fig. S3 B, H). Overall, based on EphA4 knockdown and JNK inhibitor (SP) as well as p38MAPK activator (DHC) experiments, our research
support the previous conclusion that EphA4 is required for Nogo-A-Δ20 mediating downstream anti-apoptosis effects in neural stem cells.

**Interactions of Nogo-A-Δ20 with EphA4 by molecular docking studies**

The extracellular region of EphA4 consists of ephrin ligand-binding domain (LBD), cysteine-rich domain, and 2 fibronectin type III domains (Fn1 and Fn2). LBD binds to the receptor-binding domain (RBD) of ephrins (Xu et al. 2013). Thus, to analysis the binding model between Nogo-A-Δ20 and ectodomain of EphA4 (Fig. S1), we performed molecular docking via ZDOCK server. As shown in Fig. 8a, the interaction between Nogo-A-Δ20 (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-Δ20 with the residues of Gln-390 and Asn-425 at EphA4 ectodomain (Fig. 8a). Intriguingly, we observed that proline residues at position 194 (magenta) of Nogo-A-Δ20 and lysine at position 205 (red) of Nogo-A-Δ20 asparagine at 391 (magenta) of EphA4 form isopeptide bond. The prior 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. 

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**Fig. 5** 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. 6 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 5 min (a), 30 min (b) and 1 h (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 5 min treatment (d, e, f, g), at 30 min treatment (h, i, j, k) and at 1 h treatment (l, m, n). The means ± 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.

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 (Tagami et al. 2000). However, the function of extraneuronal Nogo-A-Δ20 signaling in apoptosis of NSCs is not known. Here we describe a novel Nogo-A-Δ20-interacting protein belonging to Eph family, EphA4 and demonstrates their functional significance of binding in NSCs apoptosis. In addition, MAPK pathways are involved in these processes of NSCs apoptosis, during Nogo-A-Δ20/ EphA4 binding on NSCs.

Currently, abnormal increased activity of p38 MAPK 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 (Noberrini et al. 2008; Murai et al. 2003; Gu et al. 2018). 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 (Fu et al. 2014). Hence, Rhy was used as antagonist of EphA4 in our research. Consistent with preceding evidence, we found that 10 μM Rhy could block Nogo-A-Δ20 anchoring to EphA4 and facilities 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 (Pearson et al. 2001; Chang and Karin 2001; Morrison 2012). More recently, it has been reported that EphA receptor as regulators is linked to downstream signaling effectors such as MAPKs (Miao et al. 2001; Aoki et al. 2004). Hence, we hypothesized that Nogo-A-Δ20/EphA4 interactions may mediate regulation of cell apoptosis by MAPKs cascade singling. In this manuscript, 300 nM Nogo-A-Δ20 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 (Fig. 4). The results preliminary prove that Rhy, a potent antagonist of EphA4, can block binding between Nogo-A-Δ20 and EphA4, which finally lead to cell death. Then, we detected whether pro-apoptotic proteins were also altered in Nogo-A-Δ20 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 (Fig. 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-Δ20/ EphA4 interaction (Fig. 6). In contrast, total ERK and p-ERK maintain unchanging. The data hint that ERK pathway is not responsible for Nogo-A-Δ20 preventing apoptosis occurrence via binding with EphA4. Perhaps ERK pathway activation are always involved in the proliferation and differentiation of NSCs (Shu et al. 2016).

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 (Sarkar et al. 2002; Park et al. 2002; Deschesnes et al. 2001), on the other hand, p38-MAPK cascades enhance cell survival and growth (Liu et al. 2001). Our results show that p38-MAPK activity is reduced while NSCs exposed to Nogo-A-Δ20 for 48 h followed by decreased NSCs apoptosis. Nevertheless, activation of p38-MAPK activity by DHC attenuates inhibition of apoptosis induced by Nogo-A-Δ20 (Fig. 7). These data therefore indicate that the downregulation of p38-MAPK pathway is required for anti-apoptotic effects mediated by Nogo-A-Δ20/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 (Wada and Penninger; Dhanasekaran and Reddy 2017). In addition, JNKs have been shown to be involved in promoting apoptosis by nuclear signaling and mitochondrial-mediated intrinsic apoptotic pathways (Dhanasekaran and Reddy 2008). Likewise, our results indicate that reduction of JNK-MAPK activity by SP600125 (a selective inhibitor of JNK) enhance apoptotic NSCs (Fig. 9). Thus, these data suggest that upregulation of JNK activity are involved in inhibition of apoptosis for Nogo-A-Δ20 binding to EphA4 on NSCs. Taken together, our data demonstrate that p38-MAPK signaling and JNK-MAPK participating in apoptotic signaling pathway are

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**Fig. 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 Figs 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-Δ20 group. Data were normalized to that of Control.
various and complicated, the outcomes are dependent on stimuli and cell types.

Although our results suggest that p38 and JNK MAPK pathways are correlated with preventing apoptosis, it is not clear that whether Nogo-A-Δ20-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-Δ20 accompanied by alteration of p38 and JNK MAPK activity (Fig. 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-Δ20/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 (Wagner and Nebreda 2009).

Conclusions

In the present studies, we identified EphA4 as functional receptors for the Nogo-A active domain, Nogo-A-Δ20 (rat amino acid 544–725). We found that Nogo-A-Δ20 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 prediction show that amino acid residues Lys-205, Ile-190, Pro-194...
Fig. 9 Schematic diagram of the mechanism of Nogo-A-Δ20 with EphA4 cooperate to decrease 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: 2.081 Å) 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.
in Nogo-A-Δ20 and EphA4 residues Gln-390, Asn-425, Pro-426 may be pivotal binding sites for Nogo-A-Δ20/EphA4 interaction. Thus, our current results propose a novel mechanism by which Nogo-A-Δ20 reduces NSCs apoptotic signals via interacting with EphA4.

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Author contributions J-LW: Formal analysis, writing original draft. W-GC: formal analysis. C-JX: formal analysis, writing—original draft. J-JZ performed partial WB.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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