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ORIGINAL PAPER

Inhibition of Retinal Ganglion Cell Axonal Outgrowth Through the Amino-Nogo-A Signaling Pathway

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Abstract Nogo-A is a myelin-derived inhibitor playing a pivotal role in the prevention of axonal regeneration. A functional domain of Nogo-A, Amino-Nogo, exerts an inhibitory effect on axonal regeneration, although the mechanism is unclear. The present study investigated the role of the Amino-Nogo–integrin signaling pathway in primary retinal ganglion cells (RGCs) with respect to axonal outgrowth, which is required for axonal regeneration. Immunohistochemistry showed that integrin αv, integrin α5 and FAK were widely expressed in the visual system. Thy-1 and GAP-43 immunofluorescence showed that axonal outgrowth of RGCs was promoted by Nogo-A siRNA and a peptide antagonist of the Nogo-66 functional domain of Nogo-A (Nep1–40), and inhibited by a recombinant rat Nogo-A-Fc chimeric protein (△20). Western blotting revealed increased integrin αv and p-FAK expression in Nogo-A siRNA group, decreased integrin αv expression in △20 group and decreased p-FAK expression in Nep1–40 group. Integrin α5 expression was not changed in any group. RhoA G-LISA showed that RhoA activation was inhibited by Nogo-A siRNA and △20, but increased by Nep1–40 treatment. These results suggest that Amino-Nogo inhibits RGC axonal outgrowth primarily through the integrin αv signaling pathway.

Keywords Amino-Nogo · Axonal outgrowth · Central nervous system · Integrin · Signaling pathway

Abbreviation

RGC	Retinal ganglion cell
CNS	Central nervous system
siRNA	Small interfering RNA
FAK	Focal adhesion kinase
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
ECL	Enhanced chemiluminescence
SD	Sprague–Dawley
PBS	Phosphate buffered saline
NC	Negative control
HRP	Horseradish peroxidase
GCL	Ganglion cell layer
INL	Inner nuclear layer

Introduction

Axonal regeneration after central nervous system (CNS) injury in adult mammals is difficult to induce, being prevented by multiple factors [1–4]. Nogo is a myelin-

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associated inhibitor that may be an important factor in axonal regeneration [5–7]. Nogo-A is the major Nogo isoform that plays an essential role in axonal regeneration. Nogo-A contains two functional domains: an amino-terminal domain (172 amino acids) and the Nogo-66 loop (located between two hydrophobic domains) [5, 7, 8]. The Nogo-66 domain both inhibits axonal regeneration and regulates axonal growth, guidance and CNS plasticity neurogenesis [9–17]. Conversely, the amino-terminal domain of Nogo-A (Amino-Nogo) only exerts an inhibitory effect on spinal nerve axonal regeneration [5, 18–20]. However, the role of Amino-Nogo in the optic nerve and the mechanism of by which Amino-Nogo inhibits CNS axonal regeneration have not been fully elucidated.

Integrins are heterodimeric cell-surface glycoproteins composed of 18 α and 8 β subunits that are non-covalently connected. Integrins bind ligands in the extracellular matrix and form adhesion complexes that couple to the actin cytoskeleton. These complexes are necessary for cellular expansion and axonal growth [21]. Amino-Nogo inhibits cell adhesion and axonal outgrowth by inhibiting integrins, and the effect of Amino-Nogo is related to different integrin subunits in axonal outgrowth [22, 23]. Integrins αv and α5, are widely expressed in the CNS [22], however, which integrin subunit is the main player in the Amino-Nogo–integrin signaling pathway operating in axonal outgrowth remains unknown.

The optic nerve is a special somatic sensory nerve that conducts visual impulses. The optic nerve originates in retinal ganglion cells (RGCs), and the axons of these cells comprise the optic nerve. To investigate the Amino-Nogo–integrin signaling pathway in the optic nerve, we examined the expression of Amino-Nogo–integrin signaling pathway-related proteins in the visual system, axonal outgrowth in primary RGCs and changes in the expression of integrins and their downstream components FAK (focal adhesion kinase) and RhoA [20, 24–28]. Primary RGCs were treated with Nogo-A small interfering RNA (siRNA), a Nogo-66 antagonist peptide (Nep1–40) or a recombinant Amino-Nogo Fc chimeric protein (△20). Our results demonstrated that the Amino-Nogo domain primarily bound integrin αv and inhibited RGC axonal outgrowth via an inhibitory effect on the downstream signaling pathway.

Materials and Methods

Materials and Animals

Dulbecco's modified Eagle's medium (DMEM)/F12, 2.5 mM L-glutamine, 15 mM HEPES, fetal bovine serum (FBS) and trypsin were purchased from Gibco (MD, USA). The mouse monoclonal Thy-1 antibody was obtained from Becton–Dickinson (CA, USA), and antibodies targeting Nogo-A, integrin αv, integrin α5, FAK (sc-932), and phospho-FAK (p-FAK, sc-81493) were purchased from Santa Cruz (CA, USA). The Nogo-66 (1–40) antagonist peptide (Nep1–40), poly-L-lysine and 5-bromo-2′-deoxyuridine were purchased from Sigma Aldrich (MO, USA). The recombinant rat Nogo-A Fc chimeric protein (△20) was purchased from R&D Systems (MN, USA), and the RhoA activation assay kit was purchased from Cytoskeleton (CO, USA). pAKD.CMV.bGlobin.eGFP.H1.shRNA was purchased from Neuron Biotech (SH, China). The enhanced chemiluminescence (ECL) substrate was purchased from Pierce (CO, USA).

Neonatal Sprague-Dawley (SD) rats of either sex at postnatal days 1–3 and adult SD rats of either sex weighing 180–220 g were provided by the Animal Experimental Center (Institute of Surgery Research, Daping Hospital, Third Military Medical University, China). The Animal Research Committee of the Third Military Medical University approved the study protocol.

Integrin αv, Integrin α5 and FAK Immunohistochemistry

Adult SD rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) and transcardially perfused with 4 % paraformaldehyde. The brain, eyeball and optic nerve were removed and further fixed by immersion in 4 % paraformaldehyde overnight. Serial 3-μm sections of the brain, eyeball and optic nerve were cut from paraffin blocks, and the sections were incubated with primary antibodies (anti-integrin αv, anti-integrin α5 and anti-FAK at 1:200) at 4 °C overnight. The sections were visualized using the ABC system with 3, 3′-diaminobenzidine tetrahydrochloride after three washes with phosphate-buffered saline (PBS) and counterstained with hematoxylin. Sections were incubated with PBS instead of primary antibodies for negative controls.

RGC Culture and Identification

Neonatal SD rats at postnatal days 1–3 were disinfected in 75 % alcohol. Eyeballs were excised, and the retinas were dissected under a microscope. A 0.25 % trypsinase solution was added for digestion, which was terminated by the DMEM/F12 medium containing 10 % FBS. Cell suspensions were prepared and seeded onto poly-L-lysine-pretreated 12-well plates at a density of 5–6 $\times$ 10^5 cells/ml in DMEM/F12 medium containing 100 kU/L penicillin, 100 mg/L streptomycin and 10 % FBS. The cells were cultured at 37 °C in 95 % air and 5 % CO_2. Then, 5-bromo-2′-deoxyuridine (20 μg/L) was added, and half of the medium was removed after 24 h to inhibit the growth of non-neuronal cells. Immunofluorescence labeling was performed using

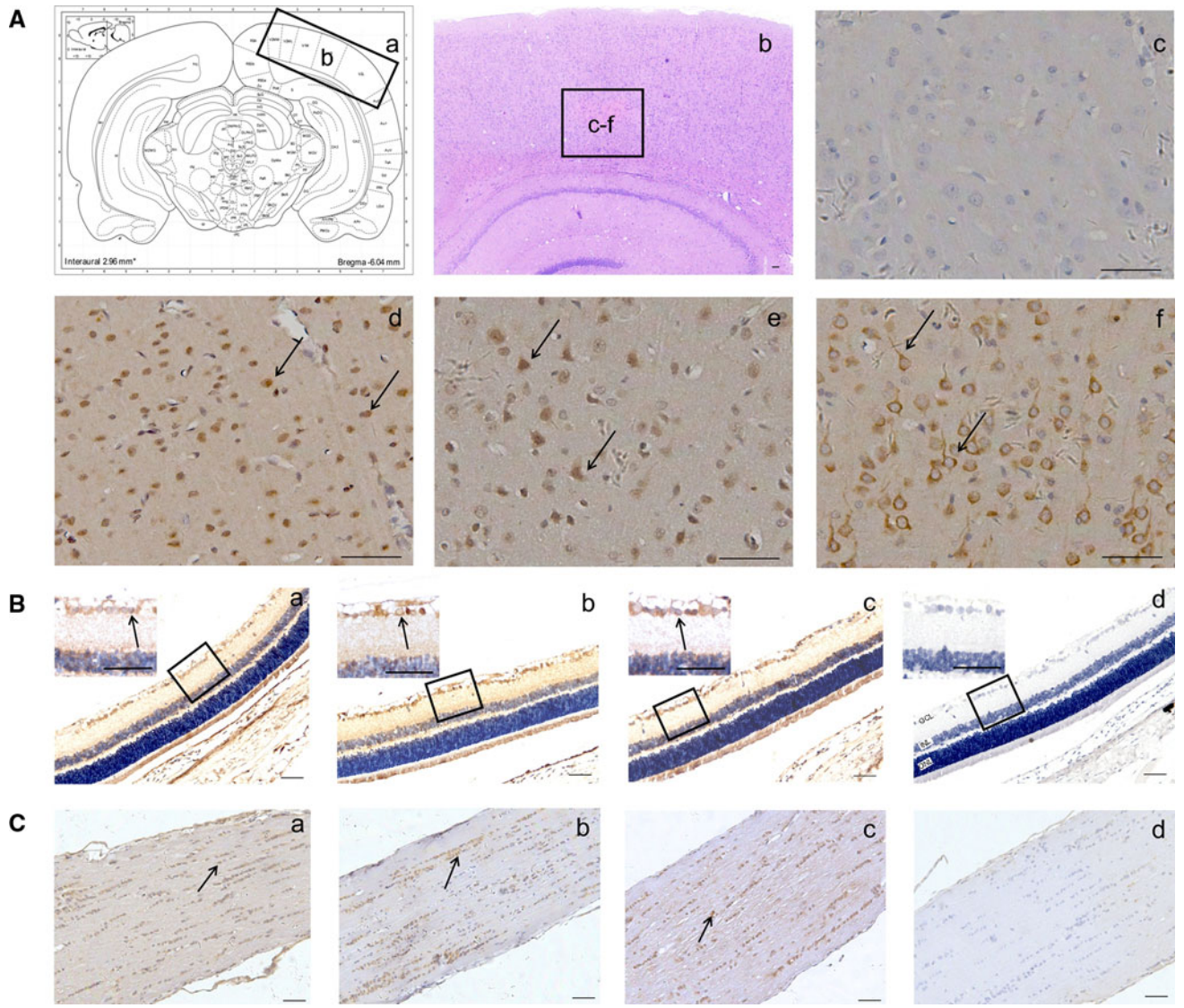

Fig. 1 Integrin αv, integrin α5 and FAK expression in the visual system. Serial 3–-μm-thick paraffin sections of the brain, eyeball and optic nerve were analyzed using immunohistochemistry. Anti–integrin αv, anti–integrin α5 and anti–FAK primary antibodies were used at a 1:200 dilution, and PBS was applied as a negative control. **A** A coronal slice of the visual cortex determined using a rat brain atlas (*a*). Hematoxylin and eosin staining on sections from normal visual cortex is presented (*b*). PBS was applied as a negative control (*c*). Integrin αv (*d*), integrin α5 (*e*) and FAK (f) are widely expressed in the normal visual cortex. **B** Representative sections of a normal retina stained for integrin αv (*a*), integrin α5 (*b*), FAK (*c*) and the PBS control (*d*). Integrin αv-, integrin α5- and FAK-positive cells were distributed primarily in the RGCs of the ganglion cell layer (GCL), inner nuclear layer (INL). **C** Representative sections of a normal optic nerve stained for integrin αv (*a*), integrin α5 (*b*), FAK (*c*) and the PBS control (*d*). Positive signals for the integrin αv, integrin α5 and FAK proteins were located in a regular beaded arrangement along the long axis of the optic nerve. The *arrows* indicate positive expression (*brown*). $N = 6$. *Scale bar*: 50 μm (Color figure online)

anti-Thy-1, a specific marker of RGCs, to verify that the cultured cells were RGCs. RGCs were obtained by incubation with DMEM/F12 medium for 7 days.

Virus Production and Purification

Three small interfering RNA (siRNA) duplexes targeting Nogo-A were used: siRNA1 5′-GAGGCAGATTATGTTACAA-3′; siRNA2 5′-GGTGCAGATAGATCATTAA-3′; and siRNA3 5′-GATCCA GGCTATCCAGAAA-3′. BLAST analysis revealed that these sequences exhibited no homology to any rat genes other than Nogo-A. The negative control (NC) sequence, 5′-TTCTCCGAACGTGTCACGT-3′, exhibited no homology to any rat genes. An H1 RNA polymerase III promoter drove the expression of Nogo-A. The Nogo-A siRNA was packaged with pAKD. CMV .bGlobin.eGFP.H1.shRNA. Large-scale recombinant

adeno-associated virus2/8 (rAAV) production and purification were performed as described previously [17].

RGC Outgrowth Assay

Retinal ganglion cells were seeded on poly-L-lysine-pretreated 12-well plates. Nep1–40 (50 nM) [29], and △20 (40 nM) [22] were added to the DMEM/F12 medium. NC siRNA and Nogo-A siRNAs were transfected into RGCs 24 h after plating, and half of the culture medium was replaced every 24 h. The RGCs were stained with anti-Thy-1 and axonal outgrowth of RGCs was detected by staining with anti-GAP-43 after 7 days. Axonal lengths were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA), and at least 60 neurons in each experimental group were quantified in duplicate wells from six independent experiments.

Western Blotting

RIPA buffer was used to lyse unstimulated RGCs (control) or RGCs treated with NC siRNA, Nogo-A siRNA, Nep1–40 (50 nM) and △20 (40 nM). The protein samples were electrophoretically resolved using 10 % SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked for 2 h at room temperature in 5 % skim milk powder diluted with Tris-buffered saline Tween-20. The membranes were incubated overnight at 4 °C with diluted anti-integrin αv, anti-integrin α5, anti-p-FAK, anti-FAK (1:500) and anti-β-actin (1:2000) antibodies in 5 % milk. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature (rabbit anti-mouse HRP and goat anti-rabbit HRP, 1:5000). Signals were detected using ECL-Plus and membranes were exposed to film. The signal intensity of each band on the western blot was quantified using Labwork 4.6 and normalized to that for β-actin.

RhoA Activity Assay

RhoA GTPase activity was measured in cell lysates prepared from control and NC siRNA-, Nogo-A siRNA-, Nep1–40- (50 nM), and △20- (40 nM)-stimulated RGCs using a commercially available G-LISA RhoA activation assay kit. Lysate protein concentrations were measured, and lysis buffer was added to the cellular extracts to yield identical protein concentrations. The samples, a blank control and a RhoA-positive control were placed on an orbital microplate shaker (400 rpm) at 4 °C for 30 min. Antigen-presenting buffer was added. The samples were then incubated at room temperature for 2 min. The samples were incubated with a diluted anti-RhoA primary antibody (1:250) for 45 min. They were then incubated with diluted secondary antibody for 45 min, followed by incubation with the HRP detection reagent at 37 °C for 15 min. HRP stop buffer (50 μl) was added, and the signal absorbance was assessed at 490 nm using a microplate spectrophotometer.

Statistical Analysis

All of the results are reported as the mean ± SD of at least six independent experiments. Comparisons of two groups were made using the unpaired *t* test. Multiple-group statistical analyses were performed using one-way ANOVA, followed by least significant difference post hoc tests. All statistical analyses were performed using SPSS software (version 10.0 for Windows; SPSS Inc., Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

Results

Expression of Integrin αv, Integrin α5 and FAK in the Visual Cortex, Retina and Optic Nerve

Tissues from adult SD rats were subjected to immunohistochemistry to detect the expression of integrin αv, integrin α5 and FAK proteins in the visual system. Integrin αv, integrin α5 and FAK expression were observed in the visual cortex, retina and optic nerve (Fig. 1). The location of the visual cortex was determined using a rat brain atlas (Fig. 1A a) [30]. Integrin αv-, integrin α5- and FAK-positive cells were commonly located in neuronal soma membranes and neurites in visual cortex slices (Fig. 1A d–f). Integrin αv-, integrin α5- and FAK-positive cells were distributed primarily in the RGCs of the ganglion cell layer (GCL) and inner nuclear layer (INL) in the retina (Fig. 1B a–c). Many cells in the optic nerve were notably stained, and positive signals for integrin αv, integrin α5 and FAK proteins were regularly observed in a beaded arrangement along the optic nerve long axis (Fig. 1C a–c).

Amino-Nogo Inhibits RGC Axonal Outgrowth

siRNAs against Nogo-A were used to examine the functions of Amino-Nogo. We investigated the efficacy of the rAAV2/8-Nogo-A-siRNAs by detecting Nogo-A protein level using western blots 7 days after transfection. The rAAV2/8-Nogo-A-siRNA3 group exhibited the strongest knockdown of Nogo-A compared with the level in the rAAV2/8-NC-siRNA group ($P < 0.01$). A weaker Nogo-A knockdown effect was observed in the rAAV2/8-Nogo-A-siRNA1 and rAAV2/8-Nogo-A-siRNA2 groups compared with the level in the rAAV2/8-NC-siRNA group ($P < 0.01$). These results confirmed that rAAV2/8-Nogo-

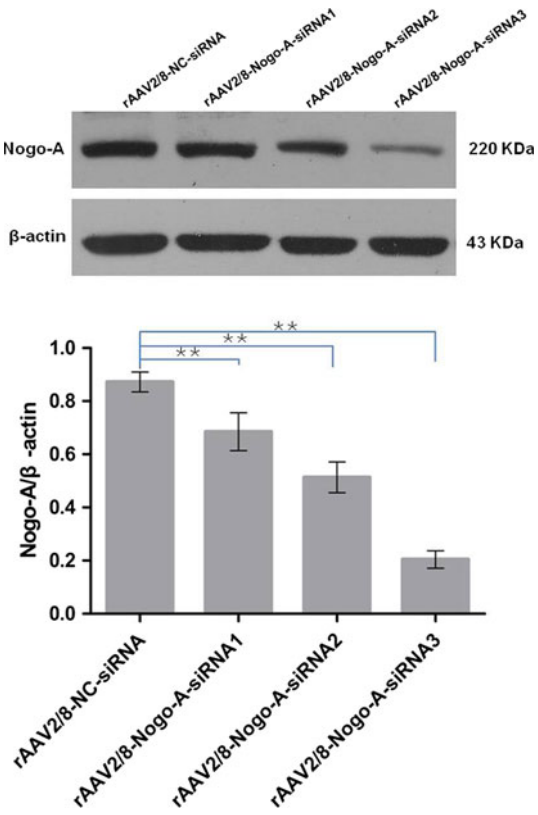

Fig. 2 Expression of Nogo-A in different rAAV2/8-Nogo-A-siRNA-transfected RGCs. The RGCs were transfected with rAAV2/8-Nogo-A-siRNA1, rAAV2/8-Nogo-A-siRNA2, rAAV2/8-Nogo-A-siRNA3 and rAAV2/8-NC-siRNA for 7 days. Nogo-A protein expression was determined using western blotting. Nogo-A protein expression was significantly reduced in all siRNA groups compared to the rAAV2/8-NC-siRNA group. *Error bar* = SD, $n = 6$. $^{**}P < 0.01$

A-siRNA3 efficiently knocked down Nogo-A, so this siRNA was used in further experiments (Fig. 2).

We next examined the axonal length of RGCs to observe the effect of Amino-Nogo on RGCs axonal outgrowth. The axonal length of Nogo-A siRNA-treated RGCs (Fig. 3c) was the greatest, but Nep1–40 treatment (Fig. 3d) also yielded longer axons than those in the control group (Fig. 3a). The axonal length of △20-treated RGCs (Fig. 3e) was the shortest among the treated groups. The axonal length of NC siRNA-treated RGCs (Fig. 3b) was not significantly different from that of the control group (Fig. 3a). These results demonstrated that Amino-Nogo inhibited RGC axonal outgrowth.

Nogo-A knockdown Increases the Level of Integrin αv and p-FAK and Inhibits the Activation of RhoA

Nogo-A siRNA treatment significantly increased integrin αv expression ($P < 0.01$), but no significant change in integrin α5 expression was observed (Fig. 4). The phosphorylation of FAK at Tyr397 was also evaluated. Nogo-A siRNA treatment increased the p-FAK level ($P < 0.05$), but no changes in total FAK level were observed (Fig. 5). RhoA activation was measured using the G-LISA method, and Nogo-A siRNA treatment significantly suppressed RhoA activation ($P < 0.01$) (Fig. 6). These results revealed that the integrin signaling pathway was regulated by Nogo-A and that the integrin subunit involved might be integrin αv.

Nep1–40 Inhibits FAK Phosphorylation and RhoA Activation but does not Alter Integrins αv and Integrin α5 Expression

Western blotting revealed that Nepl–40, a specific antagonist of Nogo-66, did not alter the expression of integrin αv or integrin α5 (Fig. 4). However, Nep1–40 treatment decreased p-FAK ($P < 0.05$) (Fig. 5). RhoA activation was suppressed in the Nep1–40 treatment group ($P < 0.05$) (Fig. 6). Our data suggested that the Nogo-66 domain did not regulate the integrin signaling pathway. Furthermore, these results suggested that the Amino-Nogo domain was the specific Nogo-A domain influencing the integrin signaling pathway.

△20 Inhibits Integrin αv and p-FAK Level and Activates RhoA

We used △20 to determine whether the Amino-Nogo domain was the specific Nogo-A domain that influenced the integrin signaling pathway. Western blotting revealed that Amino-Nogo treatment attenuated integrin αv expression ($P < 0.01$) (Fig. 4) and p-FAK level ($P < 0.01$) (Fig. 5), but no significant change in integrin α5 expression was observed (Fig. 4). RhoA activation was increased significantly ($P < 0.05$) after △20 treatment (Fig. 6). Together, these results demonstrated that Amino-Nogo inhibited axonal outgrowth via the integrin αv signaling pathway.

Discussion

Regeneration in the CNS after injury has been a research hotspot in recent years, and much research has focused on the optic nerve as a representative of the CNS. The axons of RGCs form the optic nerve. Axonal regeneration in RGCs is limited after optic nerve injury. Understanding the mechanism underlying the inhibition of axonal outgrowth in RGCs would provide a better understanding of the capacity for regeneration in the CNS after injury. The present study revealed that integrin αv, integrin α5 and FAK, which are integrin signaling pathway-related proteins, were all expressed in the visual cortex, retina and optic nerve of the visual system (Fig. 1). Our results

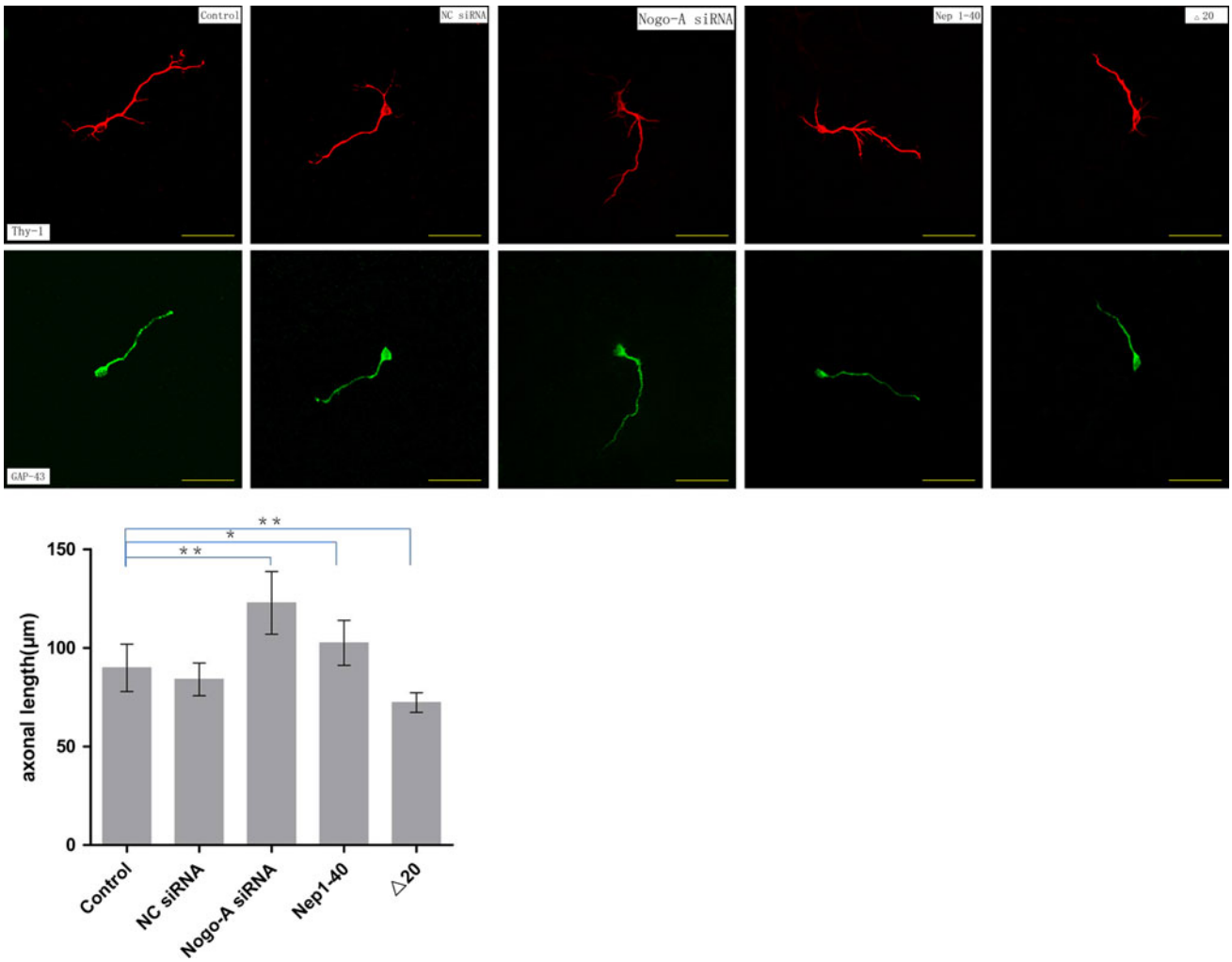

Fig. 3 Representative images of RGCs with Thy-1 (*red*) and GAP-43 (*green*) staining in the indicated groups. The RGCs were treated with NC siRNA, Nogo-A siRNA, Nep1–40 (50 nM) and △20 (40 nM). The RGCs were stained with anti-Thy-1 and the axonal lengths were analyzed by immunofluorescence staining with anti-GAP-43 after 7 days in culture. The RGC axonal length following Nogo-A siRNA treatment was the longest among the indicated groups; RGC axonal length following Nep1–40 treatment was longer than that of the control. The axon length following △20 treatment was the shortest among the treated groups. There was no significant difference between the control group and NC siRNA group. *Error bar* = SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$. *Scale bar*: 50 μm (Color figure online)

suggest that an Amino-Nogo–integrin signaling pathway exists in the optic nerve (Fig. 7). Further research into the components of the Amino-Nogo-integrin signaling pathway in optic nerve is required.

Nogo-A is expressed primarily by oligodendocytes and myelin in the adult CNS [31–33]. It is mainly known for its inhibitory effects on axon regeneration and compensatory sprouting after CNS injury [34, 35]. In addition to its glial expression, Nogo-A is also found on central neurons, which express it at high levels during development [12, 32, 33, 36, 37]. Focusing on the outgrowth of RGCs, one type of central neurons, we detected that Nogo-A was highly expressed in RGCs (Fig. 2). It is likely that Nogo-A plays a role in RGC outgrowth and that knocking down Nogo-A could promote RGC outgrowth, as demonstrated by our finding that Nogo-A siRNA significantly promoted RGCs axonal length increased. The presence of Nogo-A on the cell surface was verified, and cell-surface Nogo-A acted on neighboring neurites and cells [38]. As elevated levels of Nogo can inhibit the activation of integrins [22], it is likely that a Nogo-A siRNA that decreases Nogo-A expression should relieve the inhibition of the integrin signaling pathway; however, results from our study showed that our Nogo-A siRNA also increased integrin levels, and similar results have been reported in previous articles [39–42]. Moreover, the change in integrin expression can lead to activation of downstream molecules [39, 40]. This finding suggests that Nogo-A inhibits RGC axonal outgrowth through the integrin signaling pathway.

The inhibition of axonal regeneration by Nogo-A is attributable to its two functional domains, the amino terminal domain and Nogo-66 [5–8], which interact with

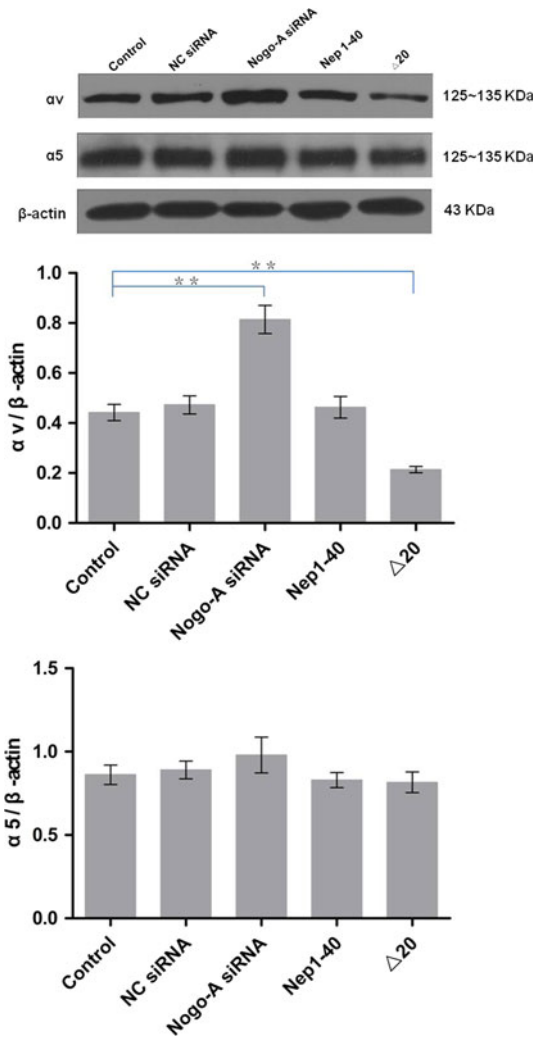

Fig. 4 Expression of integrin αv and integrin α5 proteins in the indicated groups. The RGCs were treated with NC siRNA, Nogo-A siRNA, Nep1–40 (50 nM) and △20 (40 nM) for 7 days; protein expression was detected using western blotting. The expression of integrin αv was increased by Nogo-A siRNA and suppressed by △20, but no change was observed in the Nep1–40 treatment group. None of the treatment groups exhibited a change in integrin α5 expression. There was no significant different between the control group and NC siRNA group in terms of the expression of integrin αv and integrin α5 proteins. *Error bar* = SD, $n = 6$. $^{**}P < 0.01$

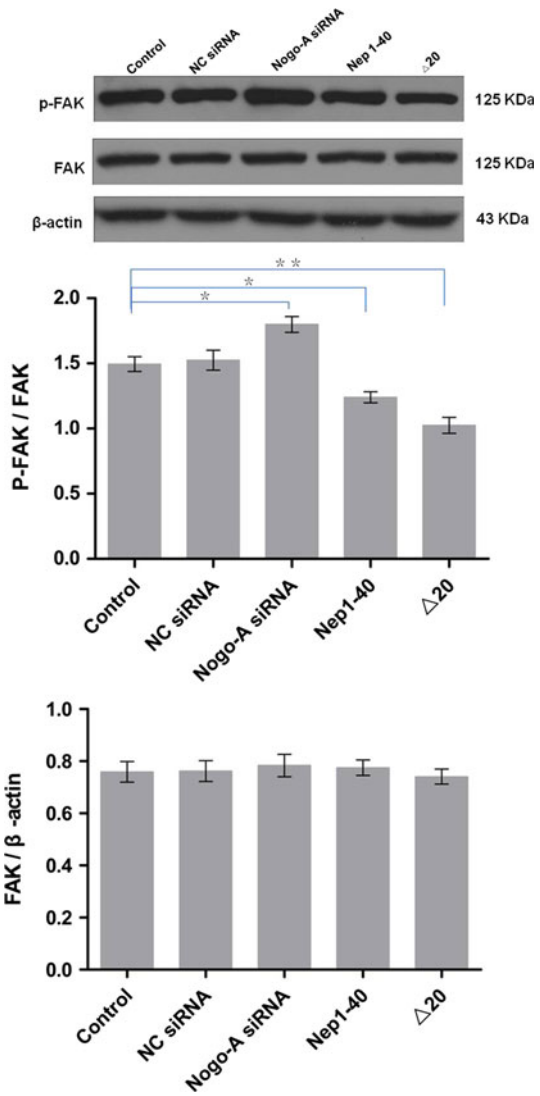

Fig. 5 Expression of p-FAK and total FAK protein in the indicated groups. The RGCs were treated with NC siRNA, Nogo-A siRNA, Nep1–40 (50 nM) and △20 (40 nM) for 7 days; protein expression was detected using western blotting. Nogo-A siRNA treatment increased the level of p-FAK, but △20 and Nep1–40 treatment significantly suppressed the p-FAK level. The level of total FAK was not significantly changed in the treated groups. There was no significant difference between the control group and NC siRNA group in terms of expression of p-FAK and total FAK protein. *Error bar* = SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$

different receptors and exert different biological effects. To deepen our understanding of the roles of the two functional domains, we used △20 to mimic the effect of Amino-Nogo and Nep1-40 to block Nogo-66. RGCs treated with △20 showed significantly inhibited integrin levels and reduced axonal length (Fig. 3). However, this was not the case in RGCs treated with Nep1-40 [29, 43]. There were no changes in integrin level when RGCs were treated with Nep1-40. Collectively, these results suggest that the Amino-Nogo domain, but not the Nogo-66 domain, acts through the integrin signaling pathway in RGCs, similar to previous findings from research on cell adhesion [22].

Integrins contain several subunits, and it is possible that the Amino-Nogo domain exerts an inhibitory function via a specific integrin subunit. Both integrin αv and integrin α5 were found to be expressed in the visual system in our study (Fig. 1). Treatment of RGCs with Amino-Nogo decreased integrin αv level, but Nep1–40 treatment did not alter integrin αv expression. Furthermore, the integrin αv level increased after Nogo-A knockdown, which decreased after Amino-Nogo treatment. However, integrin α5 expression was not significantly altered by Amino-Nogo or Nogo-A

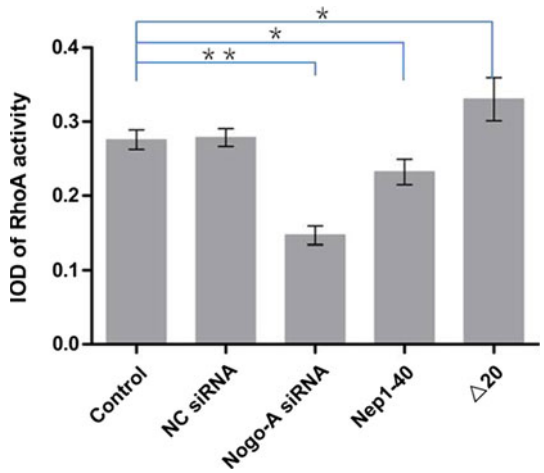

Fig. 6 RhoA activation was measured using the G-LISA™ kit BK124. RGCs were treated with NC siRNA, Nogo-A siRNA, Nep1–40 (50 nM) and △20 (40 nM); unstimulated cells (*control*) were included. The absorbance at 490 nm was measured. Nogo-A siRNA treatment and Nep1–40 treatment significantly suppressed RhoA activation. Amino-Nogo (△20) treatment increased RhoA activation in the RGCs. There was no significant difference between the control group and NC siRNA group. *Error bar* = SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$

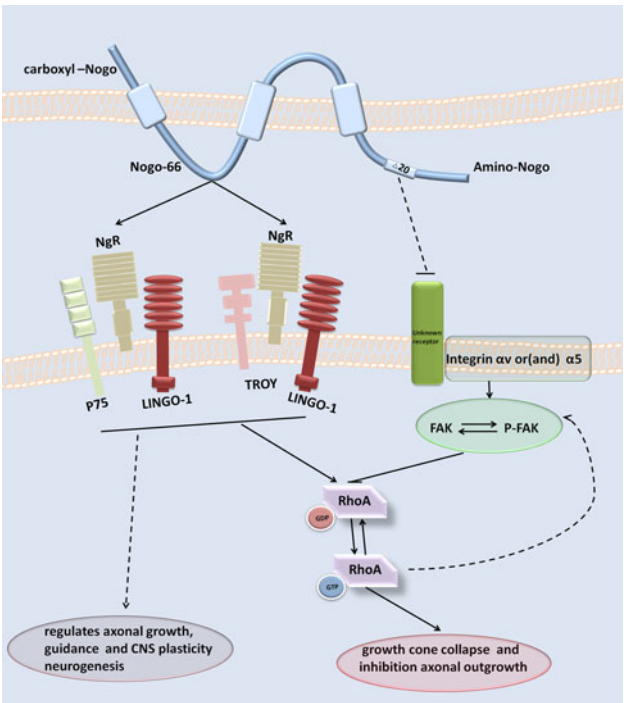

Fig. 7 Schematic depiction of the Amino-Nogo–integrin αv–FAK–RhoA signaling pathway. Amino-Nogo inhibits the expression of integrin αv and phosphorylation of FAK, and further results in the activation of RhoA

siRNAs (Fig. 4), which suggested that the Amino-Nogo domain of Nogo-A primarily inhibits the integrin αv signaling pathway. In addition, the lack of significant change in integrin α5 level in our study was possibly due to the fact that integrin α5 is not a major participator in the Amino-Nogo–integrin signaling pathway, unlike integrin αv. Additional investigations are required to resolve these issues.

Focal adhesion kinase is a key tyrosine kinase in the integrin transduction pathway [24]. FAK is phosphorylated at tyrosine 397 after integrin activation [26], and it inhibits growth cone dynamics and axonal path-finding via RhoA [25, 27, 28]. Amino-Nogo treatment significantly suppressed FAK phosphorylation. Nogo-A knock down by siRNA activated FAK, and the level of phosphorylated FAK was increased (Fig. 5). We also found that axonal outgrowth in RGCs was inhibited when RhoA was activated following △20 treatment. However, knockdown of Nogo-A inhibited RhoA and promoted RGC axonal outgrowth (Fig. 6). These observations are consistent with those of Niederost et al. [44], who suggested that RhoA was activated by Amino-Nogo, which was triggered through the inhibition of integrins. We elucidated that the Amino-Nogo–integrin αv–FAK–RhoA signaling pathway was involved in the inhibition of axonal outgrowth.

Interestingly, the phosphorylated FAK level was attenuated by Nep1–40 treatment (Fig. 5). A previous study showed that the Nogo-66–NgR co-receptor could activate RhoA [45]. The decrease in RhoA activation results from Nep1–40 preventing Nogo-66 from binding to the NgR (Fig. 6) [29, 43, 45–48]. Theoretically, there is no change of FAK level because there is no alteration in integrin αv level following in the Nep1–40 treatment (Fig. 4). A possible explanation for this may be that RhoA regulates the activation of FAK in turn, as previous studies have suggested [49–51]. However, this phenomenon requires further investigation.

In conclusion, our data suggests that integrin αv is the primary component that links Amino-Nogo and FAK. Integrin αv may play an important role in the inhibitory effects of Nogo-A on RGC axonal outgrowth, and RhoA activation is related to the inhibition of integrin signaling by Amino-Nogo. These data have uncovered a new molecular mechanism for the promotion of axonal regeneration in the CNS, and may provide new and more specific targets to treat and prevent injury of the CNS. However, this signaling pathway requires further verification in vivo.

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Conflict of interest The authors declare that they have no conflict of interest.

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