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Changes in integrins αv and α5 with Nogo-A in the rat retina after optic nerve injury

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Abstract. – OBJECTIVE: The purpose of this study was to investigate whether integrin levels are associated with axon regeneration after central nervous system (CNS) injury.

MATERIALS AND METHODS: By using immunohistochemistry, we performed a detailed investigation of the changes in and colocalization of integrins αv and α5, with Nogo-A in the retina after optic nerve injury.

RESULTS: We confirmed that integrins αv and α5 were expressed in the rat retina and colocalized with Nogo-A. After optic nerve transection, we found that integrin α5 levels increased over 7 days, but integrin αv levels remained unchanged, while Nogo-A levels increased.

CONCLUSIONS: It seems that the inhibition of axonal regeneration by the Amino-Nogo-integrin signaling pathway may not occur via changes in integrin levels.

Key Words:
Integrin, Nogo-A, rat, Immunohistochemistry, Confocal microscopy, Central nervous system (CNS), Retina.

Introduction

The discovery and characterization of the myelin-associated neurite growth inhibitor Nogo-A has opened the door to a new field in central nervous system (CNS) regeneration research[1]. A GPI-linked cell surface protein, NgR, has been characterized as a receptor that binds to Nogo-A[2]. However, NgR expression is markedly in different parts of CNS. For example, NgR is expressed by neurons in the neocortex and the hippocampus in adult rodents, but not in white matter[3]. Motor neurons express NgR weakly and most other neurons in the spinal cord do not express it at all[4]. This likely limits the range of neurons that are susceptible to the growth-inhibitory effects of Nogo-A. However, even though Nogo-A does not bind to NgR, it can inhibit neurite outgrowth[5,6]. *In vitro*, the unique N-terminal domain of Nogo-A (Amino-Nogo) has been shown to inhibit both axon growth[7] and fibroblast spreading[8]. The mechanisms of Amino-Nogo inhibition are unknown. Nevertheless, antibodies directed against Amino-Nogo have been effective in promoting recovery from neurological injury in rodents[9]. This indicates that another yet-unknown receptor mechanism may mediate Nogo-specific signaling. In 2008, Hu and Strittmatter[10] reported that Amino-Nogo could inhibit dorsal root ganglion neuron axon growth by reducing the activation of certain integrins, suggesting that integrins may be additional receptors of Nogo-A. Our previous study[7] demonstrated that the Amino-Nogo domain inhibited axonal outgrowth in retinal ganglion cells (RGCs), one type of central neuron, *via* bound integrin αv. The observed integrin αv level decreased after Amino-Nogo treatment[11]. Interestingly, Tan et al[12] found that impaired integrin signaling mediated the inhibition of rat sensory neuron axon growth without affecting integrin levels. Whether integrin levels are associated with axon regeneration after CNS injury remains unknown. Therefore, we performed a detailed investigation of the change and colocalization of two integrins that are widely expressed in the adult brain, αv and α5, with Nogo-A in the retina after optic nerve injury.

Materials and Methods

Animals and Tissue Preparation

Adult Sprague-Dawley (SD) rats of either sex weighing 180-220 g were obtained from the Laboratory Animal Center (Institute of Surgery Research, Daping Hospital, Third Military Medi-

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cal University, China). The pups were taken from four groups, namely, the normal group (n=5) and three groups subjected to optic nerve transection that were euthanized at various time points after surgery (the surgical procedure was conducted as previously described[13]): the 1-day group (n=5), the 3-day group (n=5), and the 7-day group (n=5). The rats were anesthetized with anesthetic ether before transcardial perfusion was performed with 4% paraformaldehyde in 0.01M phosphate buffer. The eyeballs removed from these animals were immersed in 4 % paraformaldehyde for 24 h (4 °C).

Immunohistochemistry

Multiple immunofluorescence labeling was executed according to a previous study[14]. Eyeballs were embedded in Tissue-Tek OCT (optimal cutting temperature) compound, frozen at -20°C and sectioned on a cryostat (20 μm). The sections were mounted on poly-L-lysine-coated slides, and aird-ried for 24 h. The sections were blocked in 1% Triton-X 100 for 10 min and normal goat serum for 30 min at room temperature. The sections were incubated overnight with a monoclonal primary antibody against Nogo-A [1:100 (Millipore, Burlington, MA, USA) MAB1503] at 4°C. After washing in PBS several times, FITC-conjugated goat anti-mouse IgG (green wavelength, Sigma, 1:50) was used as a secondary antibody solution, with which the slides were incubated for 60 min at room temperature. Polyclonal primary antibodies against integrin αv [1:50 (Santa Cruz Biotechnology, Dallas, Texas, USA) sc-10719] or integrin α5 [1:50 (Santa Cruz Biotechnology, Dallas, Texas, USA) sc-10719] and a TRITC-conjugated goat anti-rabbit IgG secondary antibody [red wavelength, (Sigma, St. Louis, MO, USA) 1:50] were used for double immunofluorescence labeling. Then, a monoclonal primary antibody against βIII-Tubulin (Tuj1) [1:50 (R&D Systems, Minneapolis, MN, USA) MAB1195] and a Cy5-conjugated goat anti-mouse IgG secondary antibody [blue wavelength, (Sigma, St. Louis, MO, USA) 1:100] were used for multiple immunofluorescence labeling. The stained sections were examined and photographed with a laser confocal scanning microscope (Leica, Wetzlar, Germany). The portions of the spectrum used to collect the emission wavelengths were as follows: for blue FITC, 633 nm; for red FITC, 561 nm; and for green FITC, 488 nm.

Microscopy and Figure Preparation

Micrographs were obtained by Leica LAS AF system and immunofluorescence stacks were performed with a computer-based image analysis system (Image-Pro-Plus, Silver Spring, MD, USA). Integrated optical density (IOD) in the ganglion cell layer was analyzed within six non-overlapping areas. Mean and standard error (S.E.M.) values per field were calculated.

Statistical Analysis

Data analysis was performed with one-way ANOVA. A p-value<0.05 was considered to indicate a significant difference. The analyses were performed with the SPSS 25.0 (IBM, Armonk, NY, USA) package for Windows.

Results

The labeling of integrins, Nogo-A, and neurons, and their locations in the retina are shown in Figures 1 and 2. We found that in the ganglion cell layer, both integrin αv (Figure 1) and integrin α5 (Figure 2) immunoreactive ganglion cells were also positive for Nogo-A-immunoreactivity. Both integrin αv (Figure 1) and integrin α5 (Figure 2) were abundant in the periphery of ganglion cell bodies, suggesting the possibility of integrins on the cell membrane. Antibodies recognizing the epitope specific to Nogo-A also predominantly labeled the fragment in the periphery of ganglion cell bodies (Figures 1 and 2). Interestingly, in contrast to that of integrin αv, which remained unchanged throughout the experiment (Figure 3), the immunoreactivity of integrin α5 was increased in the ganglion cell layer 7 days after the optic nerve was transected (Figure 4). Nogo-A immunoreactivity was increased in the ganglion cell layer from days 3 through 7 (Figures 3 and 4).

Discussion

After injury, the adult mammalian CNS hardly can be repaired. Berry's study[15] showed that CNS myelin might inhibit axonal regeneration, and antibodies recognizing CNS myelin can block its ability to inhibit neurite growth in culture[16]. One of the most strongly inhibitory CNS myelin proteins, named Nogo, is a member of the reticulon family of membrane-associated molecules[17]. The Nogo gene has three splice variants, Nogo-A, Nogo-B and Nogo-C. All three Nogo isoforms share a common carboxyl terminus of 188 amino acids that contains two long hydrophobic domains and a short loop of 66 amino acids between the two hy-

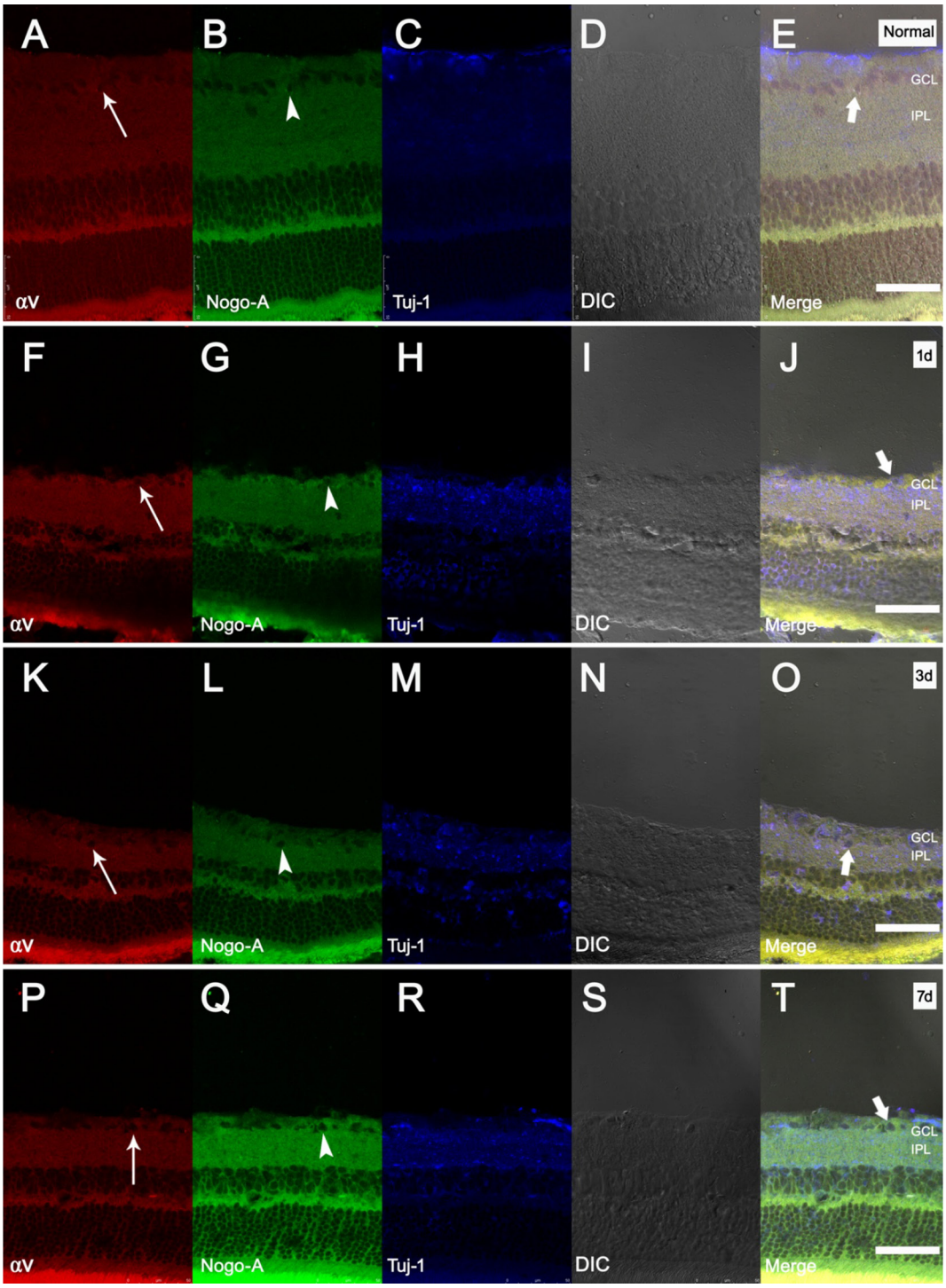

Figure 1. Confocal images illustrating the colocalization of integrin αv (*red*) with Nogo-A (*green*) in the periphery of ganglion cell bodies (*blue*) in the rat retina. Arrows point to ganglion cells positive for integrin αv, arrow heads point to ganglion cells positive for Nogo-A, *bold arrows* point to the colocalization of integrin αv with Nogo-A in the periphery of ganglion cell bodies. Scale bars: white bar, 50 μm. (DIC, differential interference contrast; GCL, ganglion cell layer; IPL, inner plexiform layer). The retina was labeled with anti-integrin αv polyclonal antibody (**A, F, K, P**). The retina was labeled with anti-Nogo-A monoclonal antibody (**B, G, L, Q**). The retina was labeled with anti-βIII-Tubulin (Tuj1) monoclonal antibody (**C, H, M, R**). The differential interference contrast image of retina (**D, I, N, S**). The merged image of labeled integrin αv, Nogo-A and ganglion cells in the retina (**E, J, O, T**).

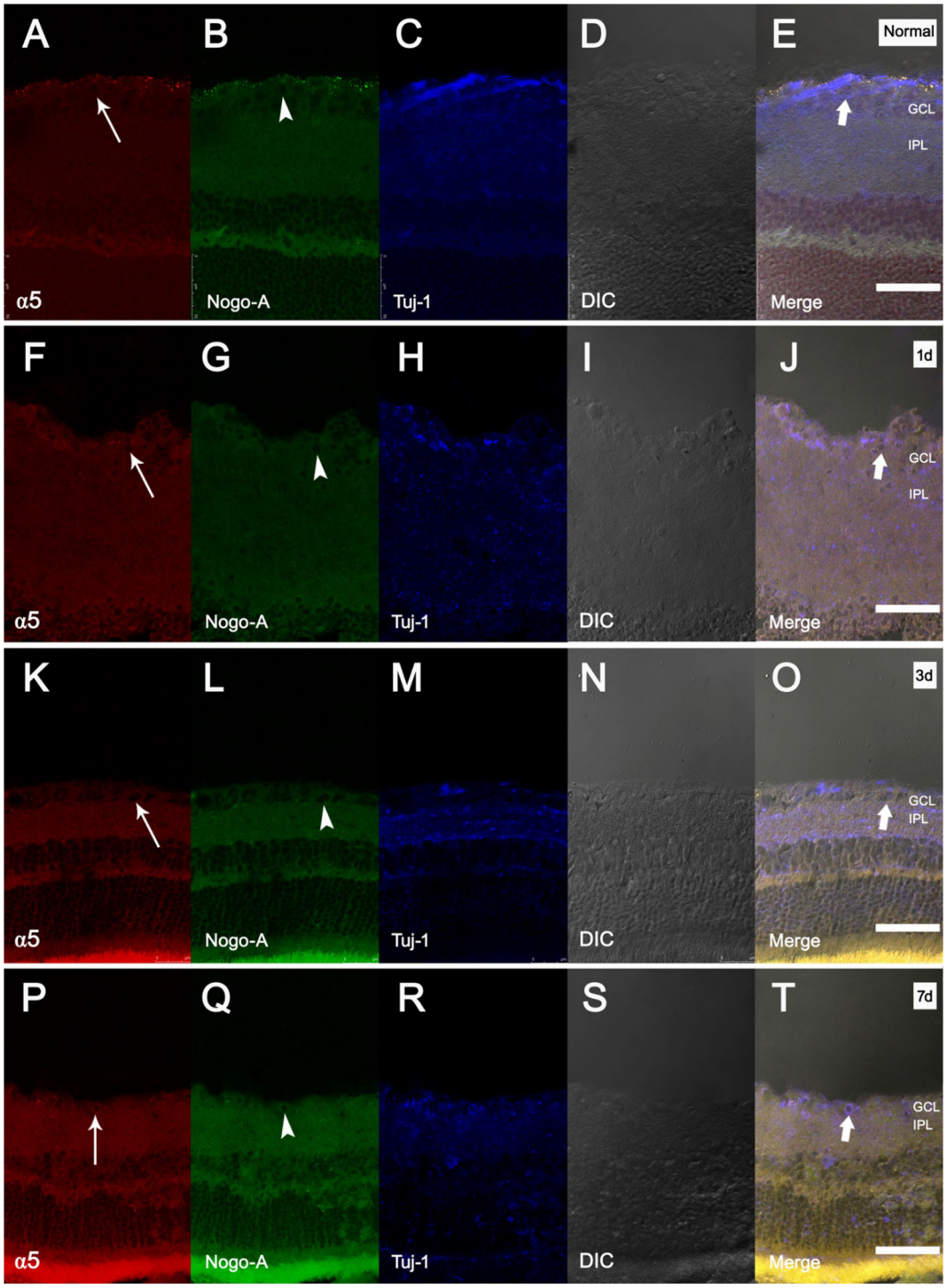

Figure 2. Confocal images illustrating colocalization of integrin α5 (*red*) with Nogo-A (*green*) in the periphery of ganglion cell bodies (*blue*) in the rat retina. Arrows point to ganglion cells positive for integrin α5, arrow heads point to ganglion cells positive for Nogo-A, *bold arrows* point to the colocalization of integrin α5 with Nogo-A in the periphery of ganglion cell bodies. Scale bars: white bar, 50 μm. (DIC, differential interference contrast; GCL, ganglion cell layer; IPL, inner plexiform layer). The retina was labeled with anti-integrin α5 polyclonal antibody (**A, F, K, P**). The retina was labeled with anti-Nogo-A monoclonal antibody (**B, G, L, Q**). The retina was labeled with anti-βIII-Tubulin (Tuj1) monoclonal antibody (**C, H, M, R**). The differential interference contrast image of retina (**D, I, N, S**). The merged image of labeled integrin α5, Nogo-A and ganglion cells in the retina (**E, J, O, T**).

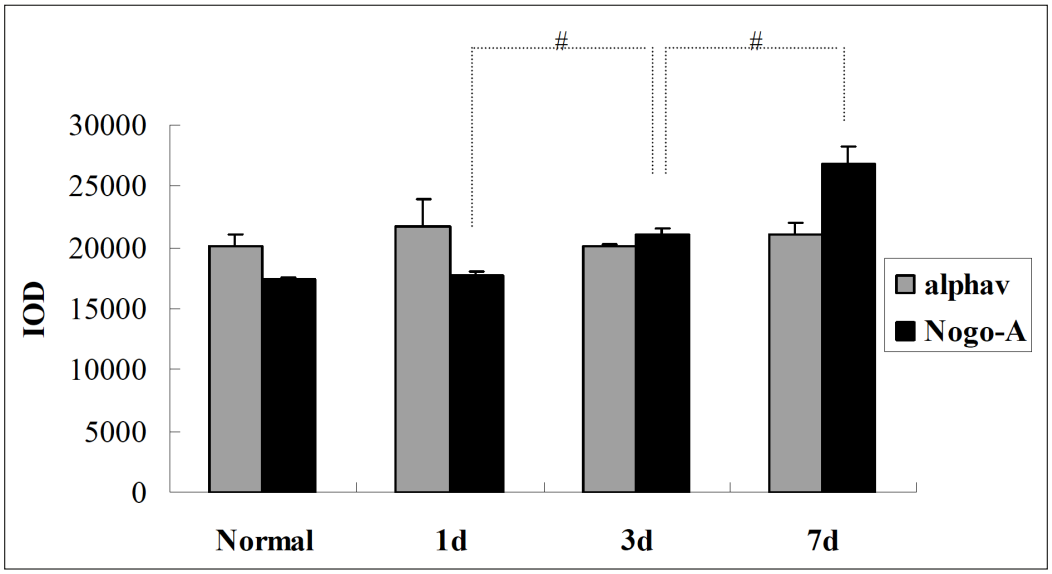

Figure 3. The quantitative analysis of integrin αv and Nogo-A in four groups (normal group, optic nerve transection 1-day group, optic nerve transection 3-day group, and optic nervetransection7-day group). $^{\#}p$ <0.05 compared with Nogo-A.

drophobic domains, called Nogo-66[1]. NgR, which is a GPI-linked cell surface protein that is widely expressed in many neurons, has been characterized as a receptor subunit that binds to the Nogo-66 site[18]. Because it lacks an intracellular domain, NgR requires either p75 or TROY and LINGO-1 as coreceptors to mediate Nogo inhibitory signaling[2]. However, if NgR is the only receptor of Nogo, why, in the absence of NgR, can Nogo also inhibit neurite outgrowth[5,6]? This indicates that another, yet unknown, receptor mechanism may mediate Nogo-specific signaling, especially Nogo-A signaling.

Aside from the Nogo-66 domain, Nogo-A still has two other active sites, the central 800-amino-acid domain and the N-terminal domain[1]. A previous study[8] showed that the unique N-termi-

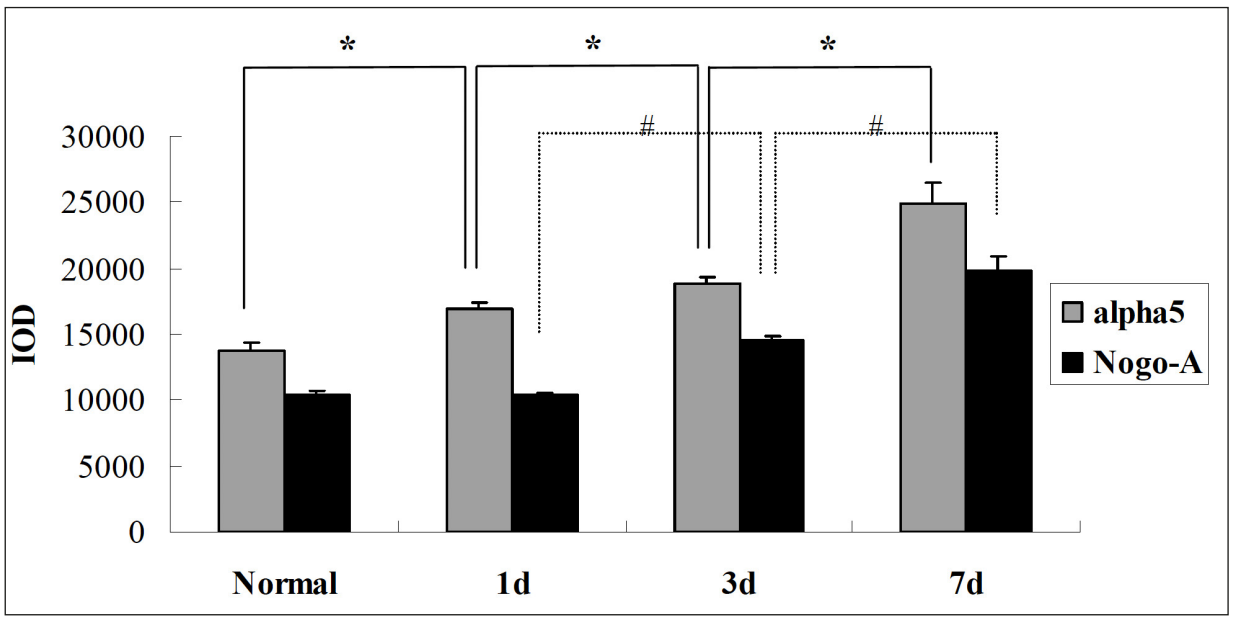

Figure 4. The quantitative analysis of integrin α5 and Nogo-A in four groups (normal group, optic nerve transection 1-day group, optic nerve transection 3-day group, and optic nervetransection7-day group). $^{*}p$ <0.05 compared with integrinα5, $^{\#}p$ <0.05 compared with Nogo-A.

nal domain of Nogo-A could inhibit axon growth. Hu and Strittmatter[10] found that the N-terminal domain of Nogo-A could inhibit dorsal root ganglion neuron axon growth by reducing the activation of certain integrins, suggesting that integrins may be additional receptors of Nogo-A.

However, to date, 18 different α and 8 different β mammalian integrin subunits have been identified to form 24 recognized αβ heterodimers[19]. Which kind of integrin is the receptor of the N-terminal domain of Nogo-A? Hu and Strittmatter[10] studied two integrins, αv and α5, which are widely expressed in the adult brain, and believed that they provided likely sites for the N-terminal domain of Nogo-A action *in vivo*. Studies[20,21] have shown that integrin αv is not detectable in the human retina and that integrin α5 is not detectable in the chicken retina. We found the expression of both integrins in the adult rat visual system, visual cortex, retina and optic nerve[11], and demonstrated that Amino-Nogo inhibited RGCs axonal outgrowth *via* the integrin αv signaling pathway[7]. In this study, we confirmed that integrins αv and α5 are expressed in the rat retina and colocalize with Nogo-A. Our previous research also revealed that the integrin αv level increases after Nogo-A knockdown but decreases after Amino-Nogo treatment[7]. However, integrin α5 expression is not significantly altered by Amino-Nogo or Nogo-A[11]. Interestingly, chondroitin sulfate proteoglycans (CSPGs), which inhibit axon regeneration after CNS lesions[22], have been reported to inhibit axon growth by impairing integrin signaling without affecting integrin levels[12].

Whether integrin levels are associated with axon regeneration after CNS injury remains unknown. We investigated the changes in integrin and Nogo-A in rats after optic nerve transection. Integrin α5 levels increased through 7 days after injury, but integrin αv levels remained unchanged, while Nogo-A levels increased as in a previous study[23]. It seems that the inhibition of axonal regeneration by the Amino-Nogo-integrin signaling pathway may not occur *via* changes in integrin levels.

Conclusions

The inhibition of axonal regeneration by the Amino-Nogo-integrin signaling pathway may not occur *via* changes in integrin levels. More investigations should be conducted to resolve the remaining uncertainties.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements
None.

Ethics Approval
This animal study was approved by the Ethics Committee of the 305th Hospital of the PLA, Beijing, China.

Informed Consent
Not applicable.

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Availability of Data and Materials
The data presented in this study are available on request from the corresponding author.

Authors' Contributions
All authors joint first authorship. Dr. Xiao-Lei Yin and Dr. Jian Ye conceived the idea and designed the study. The manuscript was prepared by Dr. Xiao-Lei Yin, Dr. Yan Huo, Dr. Xiu-Xin Li and Dr. Zhi-Peng Liu. The revision of the manuscript was mainly completed by Dr. Jian Ye, Dr. Jin-Ping Zhang and Dr. Xue-Mei Liang. All authors have read and approved the final manuscript.

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