NEURAL REGENERATION RESEARCH
Volume 7, Issue 1, January 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(1):13-17.

Experience-dependent expression of Nogo-A and Nogo receptor in the developing rat visual cortex

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Abstract

Nogo-A and Nogo receptor (NgR) expression in the visual cortex following a critical developmental period (postnatal days 20–60) has been previously shown. However, little is known regarding Nogo-A and NgR expression between postnatal day 0 and initiation of the critical period. The present study analyzed Nogo-A and NgR expression at four different time points: postnatal day 0 (P0), before critical period (P14), during critical period (P28), and after critical period (P60). Results showed significantly increased Nogo-A mRNA and protein expression levels in the visual cortex following birth, and expression levels remained steady between P28 and P60. NgR mRNA or protein expression was dramatically upregulated with age and peaked at P14 or P28, respectively, and maintained high expression to P60. In addition, Nogo-A and NgR expression was analyzed in each visual cortex layer in normal developing rats and rats with monocular deprivation. Monocular deprivation decreased Nogo-A and NgR mRNA and protein expression in the rat visual cortex, in particular in layers II-III and IV in the visual cortex contralateral to the deprived eye. These findings suggested that Nogo-A and NgR regulated termination of the critical period in experience-dependent visual cortical plasticity.

Key Words: monocular deprivation; neural regeneration; Nogo receptor; Nogo-A; plasticity; primary visual cortex

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Received: 2011-05-25
Accepted: 2011-07-12
(N20110225002/WLM)

Wu XY, Luo YL, Liu SZ, Li KS. Experience-dependent expression of Nogo-A and Nogo receptor in the developing rat visual cortex. Neural Regen Res. 2012;7(1):13-17.

www.crter.cn
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doi:10.3969/j.issn.1673-5374.2012.01.002

INTRODUCTION

Recovery of visual function following amblyopia in children over 7 years is exceptionally limited, leaving the affected individual with life-long poor visual acuity and a compromised quality of life. To date, there is no effective treatment for older children with amblyopia. Amblyopia treatment remains difficult due to reduced visual cortical plasticity and termination of the critical period. Although visual cortical plasticity has been widely studied since its initial discovery by Hubel and Wiesel[1-2], the description of the underlying molecular mechanisms has lagged behind. Nevertheless, reactivation of adult visual cortical plasticity is crucial for the effective treatment of adult amblyopic patients. Evidence exists that the developmental increase in intracortical inhibition contributes to reduced synaptic plasticity[3]. Nogo, a member of the reticulon family of membrane-associated molecules, has been identified in myelin of the central nervous system as a potent inhibitor of neurite outgrowth and cortical plasticity[4-6]. Nogo-A is one of the most powerful growth inhibitors among these myelin-associated inhibitors[7-8]. These inhibitory signals are mediated by a receptor complex, which includes the ligand-binding Nogo receptor (NgR)[9-10] and two signal-transducing binding partners, p75[11] and LINGO-1[12]. Activation of the neurotrophin receptor complex leads to Rho and Rho kinase pathway activation, resulting in a rearrangement of the cytoskeleton.

Previous studies have shown that the Nogo-NgR system is implicated in plasticity in the hippocampal and cortical microcircuitry, as well as in the spinal cord[13-14]. However, very little is known regarding expression and involved mechanisms in the visual cortex. As previously reported[15] in Nogo-A or NgR null mice, the adult visual cortex responds to monocular deprivation, with dramatic shifts in ocular dominance distribution of cortical neurons that favors the non-deprived eye. These results strongly suggest that Nogo-A and NgR play pivotal roles in visual cortical plasticity. Therefore, it is important to determine expression patterns of Nogo-A and NgR in the visual cortex of normal and early monocular deprivation rats.

The first goal of this study was to analyze spatiotemporal expression of Nogo-A and NgR in the rat visual cortex during normal development from postnatal day 0 to 60(P0–P60). Nogo-A and NgR mRNA and

protein expressions were measured through the use of reverse transcriptase-PCR (RT-PCR) and western blot and immunofluorescence, respectively. Because monocular deprivation affects maturation of cortical circuitry[16], the effects of monocular deprivation on Nogo-A and NgR mRNA and protein expression levels during the critical period of ocular dominance plasticity were analyzed.

RESULTS

Quantitative analysis of experimental animals

Ten Sprague-Dawley pregnant rats were included in the study. A total of 84 rat pups were randomly assigned to six groups according to postnatal days and visual manipulation: NorP0 (neonatal period; *n* = 10), NorP14 (before critical period; *n* = 10), NorP28 (during critical period; *n* = 10), NorP60 (after critical period; *n* = 20), MDP28 (*n* = 10), and MDP60 (*n* = 20). For the monocular deprivation (MD) model, a previously described method was utilized at P21[17]. Four rat pups were excluded, because model establishment was not successful. In total, 80 rat pups were included in the study.

Nogo-A and NgR mRNA expression in the rat visual cortex (Figure 1)

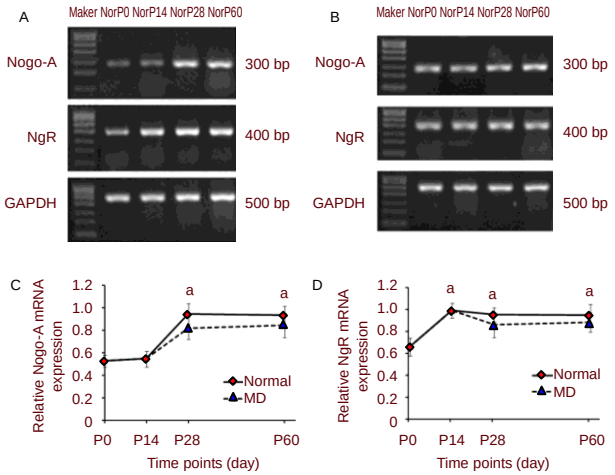

Figure 1 Reverse transcriptase-PCR analysis and quantification of Nogo-A and NgR mRNA expression in the visual cortex of normal development rats and rats with monocular deprivation (MD). GAPDH: Glyceraldehyde phosphate dehydrogenase; NgR: Nogo receptor.

(A, B) Total RNA was isolated from the visual cortex of P0 to P60 normal rats and age-matched rats with MD. Three bands, corresponding to Nogo-A (300 bp), NgR (400 bp), and GAPDH (500 bp), were amplified.

(C, D) Relative Nogo-A and NgR mRNA quantification was normalized to GAPDH mRNA. Values are expressed in relative absorbance value and represented as mean ± SD (*n* = 5 in each group). Nogo-A and NgR mRNA expression is significantly increased at P28 and P14, respectively (^aP < 0.01, *vs.* P0 group; one-way analysis of variance followed by least significant difference-*t*-test), and reached a plateau at P60. In response to MD, Nogo-A and NgR mRNA expression is slightly reduced at P28 and P60, but is not significant (unpaired Student's *t*-test, *P* > 0.05).

Nogo-A and NgR mRNA expression, as well as the internal control glyceraldehyde phosphate dehydrogenase (GAPDH), were amplified by RT-PCR. Bands corresponding to Nogo-A (300 bp), NgR (400 bp), and GAPDH (500 bp) were detected (Figure 1). In general, Nogo-A mRNA expression was significantly increased at P28 (*P* < 0.01) and maintained at a steady level between P28 to P60 (Figures 1A and 1C). Similarly, NgR mRNA was significantly increased at P14 (*P* < 0.01) and was maintained at a high level to P60 (Figures 1A and 1D). In response to MD, both Nogo-A and NgR mRNA expression were slightly reduced at P28 and P60, although neither of these changes were significant (*P* > 0.05; Figures 1B–D).

Nogo-A and NgR protein expression in the rat visual cortex

Western blot was used to quantify Nogo-A and NgR protein expression during development and in response to MD (Figure 2).

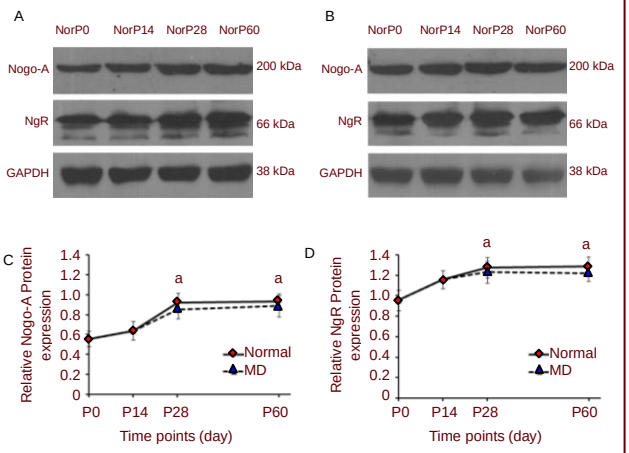

Figure 2 Western blot analysis and quantification of Nogo-A and NgR protein expression in the visual cortex of normal development rats and rats with monocular deprivation (MD). GAPDH: Glyceraldehyde phosphate dehydrogenase; NgR: Nogo receptor.

(A, B) Equal amounts of protein samples were analyzed with rabbit anti-Nogo-A and anti-NgR antibodies. GAPDH immunoblots were used to document equal protein loading.

(C, D) Quantification of Nogo-A and NgR protein expression. Data were obtained by densitometry and were normalized using GAPDH as the loading control. Values are expressed as a relative absorbance value and represented as mean ± SD (*n* = 5 in each group). Nogo-A and NgR protein expressions are significantly increased at P28 and remained at high levels up to P60 (^aP < 0.01, *vs.* P0 group; one-way analysis of variance followed by the least significant difference-*t*-test). MD minimally reduces Nogo-A and NgR expression in the primary visual cortex compared with age-matched normal controls (unpaired Student's *t*-test, *P* > 0.05).

Expression of 200 kDa and 66 kDa bands, corresponding to Nogo-A and NgR protein, respectively, were significantly elevated at the peak critical period (P28) and expression remained high up to P60 (Figure 2A). Following normalization with GAPDH expression, there was a 1.6-fold Nogo-A protein increase and 1.3-fold NgR

protein increase at P28 compared with protein expression at P0 ($P < 0.01$; Figures 2C, D). Following MD induction, Nogo-A and NgR protein expression decreased minimally in the primary visual cortex compared with age-matched normal controls ($P > 0.05$; Figures 2B–D).

Nogo-A and NgR expression in all visual cortex layers

In the NorP60 and MDP60 groups, Nogo-A and NgR protein expression in the rat primary visual cortex was observed using immunofluorescence. Several Nogo-A and NgR immunoreactive cells were detected in all visual cortex layers. Notably, Nogo-A and NgR expression significantly decreased in layers II-III and IV in the visual cortex contralateral to the deprived eye following monocular deprivation (Figure 3). These results were not consistent with results from RT-PCR and western blot analysis.

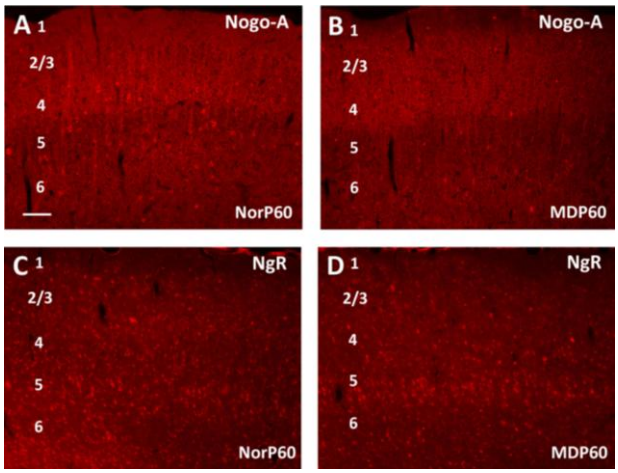

Figure 3 Immunofluorescence demonstrates Nogo-A (A and B) and Nogo receptor (NgR) (C and D) expression in the primary visual cortex of NorP60 (after critical period) and MDP60 (monocular deprivation) rats (red = Cy3 labeling, scale bar = 200 μm).

For rat models of monocular deprivation, the right eyelids were sutured together, and the left primary visual cortex was observed. Several Nogo-A and NgR immunoreactive cells are visible in all visual cortex layers (A–D). Notably, monocular deprivation significantly decreases Nogo-A and NgR protein expression in layers II–III and IV in the visual cortex contralateral to the deprived eye (B and D).

DISCUSSION

In comparison to the adult brain, the adolescent brain is thought to exhibit more robust plasticity due to an immature neuronal circuitry. During early postnatal periods, changes in visual input can lead to specific neuronal connectivity and functional changes. In rats, the so-called “critical period” begins at the time of eye opening (P14), reaches a peak at P28, and ends at P32[16]. Decreased or absent synaptic plasticity in the adult visual cortex contributes to difficulties in amblyopia treatment and other cortical-based visual disorders. It is wildly accepted that the Nogo-NgR system takes part in axonal outgrowth inhibition, as well as neuronal plasticity[13-14]. Therefore, analysis of Nogo-A and NgR expression patterns in the visual cortex in normal developmental and monocular deprivation rats is important to determine the mechanisms of action.

Results from the present study demonstrated Nogo-A/NgR mRNA and protein expression in the visual cortex of neonatal rats, which suggested that expression did not inhibit axonal outgrowth in the visual cortex during early development, but might participate in axonal guidance. These results are consistent with previous results[18]. Nogo-A and NgR mRNA and protein levels increased after birth and reached a plateau by adulthood. It was hypothesized that high neuronal levels of Nogo-A or NgR resulted in a locked visual cortex state in the adult rat; in addition, one or both of these proteins must be effectively downregulated to reactivate cortical plasticity. During normal development, mRNA and protein expression is consistent with decreased visual cortical plasticity with age. However, when compared with NgR mRNA expression during normal development, the upregulation of protein expression lagged behind, which could be due to a longer translation process from mRNA to protein.

Monocular deprivation also led to slightly decreased Nogo-A and NgR expression in the visual cortex, as determine by RT-PCR and western blot analysis, which was consistent with previous results[19]. It is possible that the mixture of all cortical layers in RT-PCR and western blot analysis results in obscured selective expression changes in specific cortical layers most critical for plasticity. Therefore, immunofluorescence analysis allowed for layer-specific assessment of expression. Nogo-A and NgR protein expression was significantly decreased in layers II–III and IV in the visual cortex after monocular deprivation. In the thalamus, the lateral geniculate nucleus receives inputs from both eyes and relays information to layer IV in the primary visual cortex, which subsequently transmits to layer II–III *via* synapses[20]. It was hypothesized that significantly decreased inputs to the visual cortex following monocular deprivation could lead to decreased expression of Nogo-A and NgR. The layer-specific downregulation of Nogo-A and NgR protein levels following monocular deprivation suggested a role for the Nogo-NgR system in visual cortical plasticity. Recent identification of this pathway, from myelin Nogo-A to its receptor NgR to intracellular Rho/Rho kinase pathway, provides an opportunity to develop interventions for reactivation of visual cortical plasticity in adult rats.

In conclusion, targeting of the Nogo-NgR system could promote plastic mechanisms underlying recovery from amblyopia in the visual system, as well as from various forms of damage in other regions of the central nervous system. Results from the present study provided a basis

for future studies to more precisely explore possible roles for the Nogo-NgR system.

MATERIALS AND METHODS

Design

Comparative, observational, developmental, neurobiological study.

Time and setting

The experiments were performed at the Laboratory of Neurobiology, Xiangya School of Medicine, Central South University, Changsha, China, from March 2009 to July 2010.

Materials

Ten female, Sprague-Dawley rats with litters of mixed gender were obtained from the Laboratory Animal Center of Central South University in China (License No. SCXK (Xiang) 2006-0002). All animal research followed the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Every effort was made to minimize animal suffering and to reduce the number of animals. All experiments were performed under double-blinded conditions to minimize errors. The mother was provided food and clean water *ad libitum* and was housed in a temperature- and light-controlled facility.

Methods

Establishment of monocular deprivation (MD) models

All surgical procedures were performed under 10% chloral hydrate (0.2 mL/100g, intraperitoneally). The lid margins of the right eye were removed, and the eyelids were sutured together using 6-0 silk. A small bead of antibiotic ointment was placed between the lids prior to closing. The suture was checked daily until the rat was sacrificed at P28 or P60. Minimal eye opening was excluded.

RT-PCR

A subset of pups at P0, P14, P28, and P60, as well as rats from the monocular deprivation model at P28 and P60, were sacrificed and the binocular zone of the left primary visual cortex was removed and frozen for RT-PCR analysis. Frozen samples were homogenized and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed with an RT-PCR kit (MBI Fermentas, Vilnius, Lithuania) according to manufacturer instructions. Rat GAPDH was amplified as a control for the PCR reaction. Primer sequences (synthesized by Invitrogen) were as follows:

Primer	Sequence	Product size (bp)
Nogo-A	Sense: 5'-TCA AAG GTG ACT GAG GCA GC-3' Antisense: 5'-ACT GGG CTG CAC TAC AGA AG-3'	300
NgR	Sense: 5'- CCA GAG AAT CTT CCT GCA CG-3' Antisense: 5'-ACT GGG GAT ACG GTT GCC AT-3'	400
GAPDH	Sense: 5'-GGG CCA AAA GGG TCA TCA TC-3' Antisense: 5'- AAC CTG GTC CTC AGT GTA GC-3'	500

Nogo-A amplification was performed for 30 cycles of 1 minute at 94°C for denaturing, 45 seconds at 55°C for annealing and 3 minutes at 72°C for extension. For NgR and GAPDH, the annealing temperature was 54°C and 56°C, respectively. Amplified products were subjected to electrophoresis on a 1% agarose gel and were stained with ethidium bromide. Gels were photographed under ultraviolet transillumination. The ratio of Nogo-A or NgR to GAPDH product was obtained by analyzing the absorbance value of the corresponding bands using Quantity One (Bio-Rad, Hercules, CA, USA).

Western blot analysis

The binocular zone of the left primary visual cortex was immediately resected according to coordinates from *The Rat Brain in Stereotaxic Coordinates*[21]. The samples were then homogenized in ice-cold lysis buffer. Following sonication and centrifugation, supernatants were collected and protein concentrations were determined using the Bradford method (Bio-Rad), with bovine serum albumin serving as the standard[22]. Protein samples were denatured in sample buffer at 100°C for 8 minutes. Equal amounts of protein were loaded into each lane of an 8% sodium dodecyl sulfate polyacrylamide gel and were separated by electrophoresis. Protein bands were then transferred to nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, England). Transfer efficiency was analyzed by Ponceau-S red staining. The membrane was then incubated in blocking buffer at room temperature for 2 hours while shaking, following by rabbit anti-Nogo-A polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1: 800) or rabbit anti-NgR polyclonal antibody (Santa Cruz Biotechnology; 1: 300) overnight at 4°C. Mouse anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology; 1: 200) was used as the loading control for protein quantification. Subsequently, the membrane was washed three times with Tris-buffered saline/Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; 1: 7 500) or rabbit anti-mouse IgG (Santa Cruz Biotechnology; 1: 7 500) in blocking solution for 1 hour. After three washes with TBST, the membranes were incubated with ECL Plus reagent for 3 minutes. Band densities were captured on X-ray films and were quantified using NIH Image (Bethesda, Maryland, USA).

Immunofluorescence

Immunofluorescence was performed according to standard protocols[23]. Animals were euthanized and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Brain specimens were removed, post-fixed overnight, and then placed in 20% and 30% sucrose until the samples sank. A series of frozen, coronal, brain sections, which included the visual cortex, were cut into 20-μm thick sections using a freezing microtome. The sections were then blocked with 5% bovine serum albumin plus 0.1% Triton-X 100 for 2 hours at 37°C. The sections were incubated in rabbit anti-Nogo-A polyclonal antibody

(Santa Cruz Biotechnology; 1: 100) or rabbit anti-NgR polyclonal antibody (Santa Cruz Biotechnology; 1: 100) for 2 hours at 4°C, followed by Cy3-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; 1: 100) for 30 minutes at 37°C. The sections were then extensively washed with phosphate-buffered saline, mounted on slides, air-dried, and coverslipped. Images were obtained using the Leica LAS AF system (Leica Microsystems, Heidelberg, Germany) using appropriate filters for Cy3.

Statistical analysis

SPSS 14.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Relative absorbance data of corresponding bands by RT-PCR and western blot were expressed as mean ± SD. Statistical differences between normal groups were analyzed using one-way analysis of variance followed by least significant difference-*t*-test. The unpaired Student's *t*-test was used to evaluate significant differences between normal and MD groups of the same age. $P < 0.05$ was considered statistically significant.

Author contributions: Xiaoying Wu, Yulin Luo, and Shuangzhen Liu designed the study; Yulin Luo and Kuanshu Li performed the experiments; Yulin Luo and Xiaoying Wu analyzed the data; Yulin Luo and Xiaoying Wu wrote the manuscript.

Conflicts of interest: None declared.

Funding: This study was supported by the Graduate Degree Thesis Innovation Foundation of Central South University, No. 2009BSXT050.

Ethical approval: This study was approved by the Central South University Committee on Animal Research.

Acknowledgments: We thank Xuegang Luo and Kun Xiong from the Laboratory of Neurobiology, Xiangya School of Medicine, Central South University in China, for technical support. The authors also wish to thank Zhenghai Liu, Lei Shang, and Jie Zeng at the same laboratory for their help.

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(Edited by Wang JF, Li JT/Qiu Y/Song LP)