

# Nogo-A Expression After Focal Ischemic Stroke in the Adult Rat

Joseph L. Cheatwood, PhD; April J. Emerick, PhD;  
Martin E. Schwab, PhD; Gwendolyn L. Kartje, MD, PhD

**Background and Purpose**—The Nogo-A protein is an important inhibitor of axonal remodeling after central nervous system injuries, including ischemic stroke. Interfering with the function of Nogo-A via infusion of a therapeutic anti-Nogo-A antibody after stroke increases neuronal remodeling and enhances functional recovery in rats. In this study, we describe the regional distribution of cortical neurons expressing Nogo-A in normal rats and following middle cerebral artery occlusion (MCAO).

**Methods**—Normal and post-MCAO neuronal Nogo-A expression were described via immunohistochemical analyses. All brains were processed for Nogo-A and parvalbumin expression. The level of Nogo-A expression was scored for each cortical area or white matter structure of interest. The number and fluorescent intensity of layer V neurons in contralesional sensorimotor forelimb cortex were also assessed at each time point.

**Results**—Nogo-A expression was observed in both cortical pyramidal neurons and parvalbumin-positive interneurons. Neuronal expression of Nogo-A changed over time in ipsilesional and contralesional cortical areas after MCAO, becoming globally elevated at 28 days after stroke. Nogo-A expression was not observed to fluctuate greatly in the white matter after stroke, with the exception of a transient increase in Nogo-A expression in the external capsule near the stroke lesion.

**Conclusions**—Neuronal Nogo-A expression is significantly increased at 28 days post-MCAO in all examined brain regions. Because of their robust expression of Nogo-A after stroke lesion, both excitatory and inhibitory neurons represent potential targets for anti-Nogo-A therapies in the poststroke cerebral cortex. (*Stroke*. 2008;39:000-000.)

**Key Words:** middle cerebral artery occlusion ■ interneurons ■ cerebral cortex ■ neurons

The Nogo-A protein has been shown to inhibit the outgrowth of cortico-efferent axonal processes after central nervous system injury,<sup>1,2</sup> and treatment with anti-Nogo-A antibodies after injury enhances neuroanatomical plasticity and functional recovery.<sup>3-9</sup> Although myelin-associated Nogo-A is thought to be the primary source of the Nogo-related limitation of neuronal outgrowth and sprouting after injury,<sup>1,10-12</sup> a growing body of data demonstrates that Nogo-A is expressed by many populations of neurons throughout the adult brain and spinal cord, and is not limited to oligodendrocytes.<sup>13-21</sup> Some have postulated that neuronal Nogo-A may be involved in normal cell-functioning separate from the postinjury outgrowth-inhibitory role observed for oligodendroglial Nogo-A,<sup>14,22</sup> but this remains to be determined.

Neuronal Nogo-A expression is most robust during development, but many neurons in the adult cerebral cortex do express the protein.<sup>14,20</sup> Previously, we showed that Nogo-A-

positive neurons in the rat sensorimotor forelimb cortex dramatically increase their dendritic length, dendritic branching, and spine density after stroke and anti-Nogo-A immunotherapy.<sup>8</sup> Despite the known efficacy of anti-Nogo-A immunotherapy for enhancing axonal sprouting and increasing dendritic complexity on layer V pyramidal neurons in rat sensorimotor forelimb cortex—many of which send cortico-efferent projections to adjacent cortical areas, contralateral homotopic cortical areas,<sup>23</sup> or subcortical structures important for skilled reaching movements<sup>3,5</sup>—the main site of action of anti-Nogo-A antibodies remains unknown.

One recent article demonstrated that the infusion of anti-Nogo-A antibodies into the intracerebroventricular or intrathecal spaces resulted in the binding of anti-Nogo-A antibodies to Nogo-A molecules on the cell surface of neurons and oligodendrocytes in the spinal cord and neocortex of monkeys and rats, resulting in internalization of the protein-

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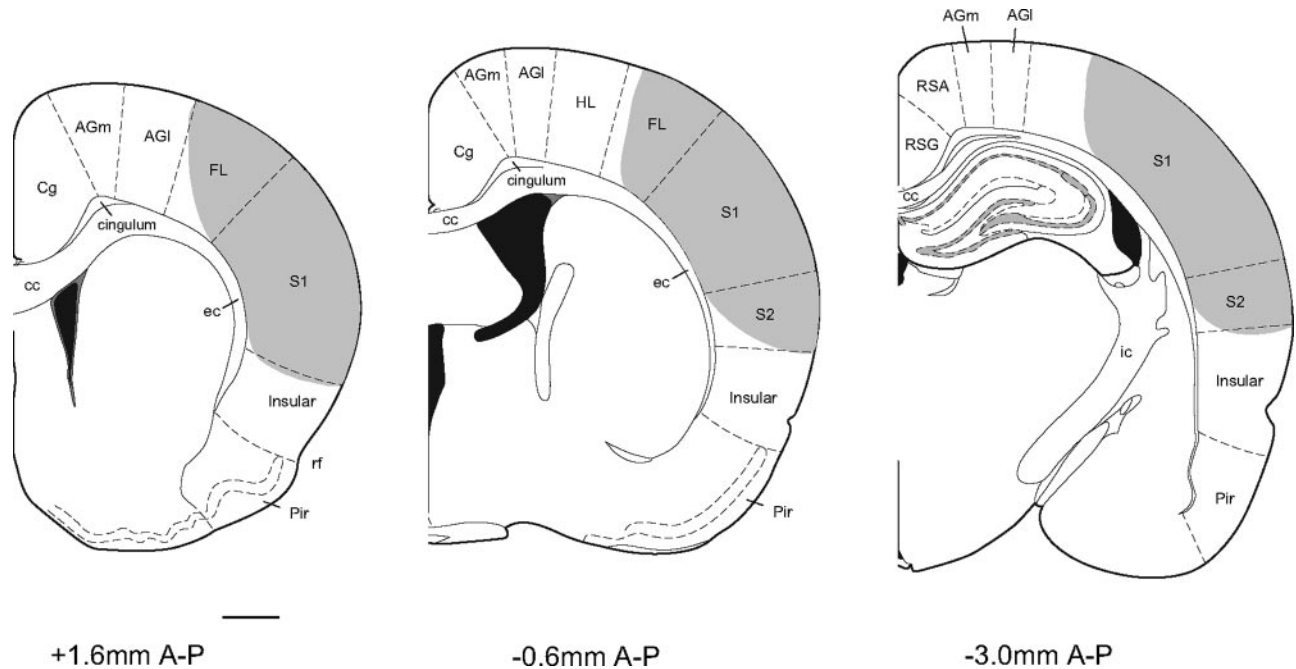
From the Research Service (J.L.C., A.J.E., G.L.K.), Hines VA Hospital, Hines, Ill, USA; the Department of Neurology (J.L.C., G.L.K.), Loyola University Medical Center, Maywood, Ill, USA; the Neuroscience Institute (J.L.C., A.J.E., G.L.K.), Loyola University Medical Center, Maywood, Ill, USA; the Brain Research Institute (M.E.S.), University of Zurich, Switzerland; the Department of Biology (M.E.S.), Swiss Federal Institute of Technology, Zurich, Switzerland; the Neurology Service (G.L.K.), Hines VA Hospital, Hines, Ill, USA; and the Department of Cell Biology, Neurobiology, and Anatomy (G.L.K.), Loyola University Medical Center, Maywood, Ill, USA.

Correspondence to Joseph L. Cheatwood, PhD, Research Service (151), Edward Hines Jr. VA Hospital, 5000 S. 5th Ave, Hines, IL 60141. E-mail jcheatwood@lumc.edu

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**Figure 1.** Scope of lesion and brain regions assessed. The typical cortical lesion observed at 28 days poststroke is depicted (gray shading). The cortical areas assessed for Nogo-A and PV staining included cingulate cortex (Cg), medial agranular cortex (AGm), lateral agranular cortex (AGI), retrosplenial cortices (RSA/RSG), sensorimotor forelimb cortex (FL), sensorimotor hindlimb cortex (HL), primary and secondary somatosensory cortices (S1 and S2), Insular cortex (Insular), and Piriform cortex (Piri). We also examined white matter structures including the corpus callosum (cc), cingulum bundle (cingulum), the external capsule (ec), and the internal capsule (ic). Figure adapted with permission from Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. 5th Ed., New York: Academic Press; 2005.

antibody complex and subsequent downregulation of Nogo-A by neurons.<sup>18</sup> This exciting finding increases the importance of defining the role of neuronal Nogo-A in limiting remodeling and recovery after stroke, beginning with the cellular and regional expression of Nogo-A in the poststroke cerebral cortex. Furthermore, a better knowledge of the neuronal cell types and populations expressing Nogo-A would provide valuable insight into the cellular processes mediating functional recovery from stroke.

We describe the pattern of Nogo-A expression by layer V pyramidal neurons and by inhibitory parvalbumin-positive GABA-ergic interneurons of the cerebral cortex in normal rats and after stroke. We discuss potential roles for both types of neurons in the modulation of recovering cortical networks after stroke and identify both excitatory and inhibitory neurons as possible targets for anti-Nogo-A immunotherapy.

## Materials and Methods

### Middle Cerebral Artery Occlusion Procedure and Immunohistochemistry

A total of 22 adult (4 to 6 months old) male Long-Evans Hooded rats (Harlan; Indianapolis, Ind) were used in this study. Nogo-A expression was examined in normal rats and at 1, 3, 7, 14, or 28 days after middle cerebral artery occlusion (MCAO). All procedures were approved by the Hines Veterans Administration Hospital Institutional Animal Care and Use Committee.

MCAO surgeries were performed as described in our other articles.<sup>5,8,24</sup> The typical size of stroke lesions resulting from this method has been described previously<sup>3,5,8</sup> and is depicted in Figure 1 (A through C; shaded area).

After the appropriate survival period, rats were euthanized via intraperitoneal injection of pentobarbital (100 mg/kg) and perfused

transcardially with heparinized 0.9% saline followed by 4% paraformaldehyde. Brains were cut on a cryostat at 50  $\mu$ m, and sections were collected and processed for immunohistochemistry as previously published.<sup>8</sup> Sections were incubated in a blocking solution of tris-buffered saline (TBS) containing 10% normal goat serum (NGS) and 0.1% Triton-X 100 (TX100) for 1 hour at room temperature. Sections were incubated overnight at 4°C in a primary antibody solution containing the mouse monoclonal anti-Nogo-A antibody 11C7 (antibody was a gift of Novartis Pharma, Basel, Switzerland)<sup>25</sup> and either a rabbit polyclonal antibody against Neurofilament-200 (a neuronal cytoskeletal marker<sup>26</sup>; N4142; Sigma-Aldrich) or a rabbit polyclonal antibody against parvalbumin (a marker for cortical GABA-ergic interneurons<sup>27</sup>; PA1-933; Affinity Bioreagents) in TBS with 5% NGS and 0.1% TX100. After incubation with the primary antibody the solution was removed and sections were washed. Sections were then incubated for 1 hour at room temperature in a secondary antibody solution containing 5% NGS and 0.1% TX100 as well as the appropriate fluorophore-labeled secondary antibodies (1:1000; Invitrogen). Sections were then washed, mounted on slides, and coverslipped with an aqueous medium (Fluoromount-G; Southern Biotech). Slides were viewed and photographed on a Leica fluorescent microscope or on a Zeiss LSM-510 laser confocal microscope. Secondary-only controls were performed for each experiment and were treated as above, with the omission of the primary antibody.

### Assessment of Nogo-A Expression

Neuronal Nogo-A expression was examined in the cerebral cortex of normal rats and in both ipsilesional and contralesional hemispheres of rats that had undergone an MCAO surgery. Only brains with negative secondary-only controls (ie, no detectable background fluorescence of neurons or neuropil) were included in the study. Cortical areas of interest included cingulate cortex (Cg), medial agranular cortex (AGm), lateral agranular cortex (AGI), sensorimotor forelimb cortex (FL), sensorimotor hindlimb cortex (HL), primary and secondary somatosensory cortices (S1 and S2), granular

and agranular retrosplenial cortices (RSA/RSG), Insular cortex (Insular), and Piriform cortex (Piri). Five to eight sections (comprising the rostrocaudal extent) were observed for each cortical region at 200x magnification, and layer V Nogo-A expression was assigned a score from 0 to 3 for each cortical area examined: 0="no Nogo-A immunopositive neurons," 1="few Nogo-A immunopositive neurons," 2="moderate number of Nogo-A immunopositive neurons," and 3="many Nogo-A positive neurons." Although Nogo-A positive neurons were present in cortical layers II through VI in some brain areas, scores were based only on staining of layer V cortical neurons because of the consistency with which these cells expressed Nogo-A, as noted elsewhere.<sup>8,19</sup> All assessments were performed twice for each brain by the same blinded observer. The resulting nominal score data were used to determine the mode for each poststroke time point. Importantly, both rating trials yielded the same results (modes), indicating a high intrarater reproducibility. To further demonstrate intrarater reliability, data obtained in each blinded rating trial were compared via Spearman Rank Order Correlations using SigmaStat 3.5 (SyStat Software, Point Richmond, Calif). The two ratings of Nogo-A expression in contralesional FL at one random time point group (1 and 3 days post-MCAO) were compared with one another for all cases (intertrial  $r=0.73$ ;  $P=0.05$ ). The same comparisons were also performed for rating data from ipsilesional AGm (intertrial  $r=0.86$ ;  $P=0.01$ ), at the same time points. The resulting correlation ( $r$ ) and probability values indicate a high degree of reproducibility between rating trials.  $P<0.05$  was considered significant.

The number and intensity of anti-Nogo-A immunolabeled neurons in layer V of contralesional sensorimotor forelimb cortex was assessed to further investigate the time course of poststroke Nogo-A expression in this key cortical region. For each brain, anti-Nogo-A immunofluorescence within sensorimotor forelimb cortex was photomicrographed on 5 evenly spaced 50- $\mu$ m-thick sections at 200x magnification using a Q-Imaging Retiga 2000R black and white 8bit CCD camera attached to a Leica LB30T fluorescent microscope. The same exposure parameters were used to digitize fluorescence across all brain sections. Using the resulting images, anti-Nogo-A immunopositive neurons were counted based on visual profiles using the NIH software ImageJ,<sup>28</sup> and a value for fluorescence intensity was simultaneously recorded for each counted neuron. Raw neuron counts were adjusted using an Abercrombie correction<sup>29</sup> in order to more accurately approximate the actual number of neurons present in each sample.<sup>30</sup> The corrected mean number of layer V neurons expressing Nogo-A was computed for each group (control, 1 and 3, 7 and 14, and 28 days poststroke) and compared via a 1-way ANOVA with Newman-Keuls Multiple Comparison post-test, using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego Calif, USA, www.graphpad.com).  $P<0.05$  was considered significant.

For analysis of fluorescence intensity, immunofluorescent neurons were divided into 3 groups based on the value of their fluorescent intensity: low intensity (0 to 85), medium intensity (86 to 170), and high intensity (170 to 255). The corrected<sup>29</sup> number of cells falling into each of these groups was compared between time points using a 2-way ANOVA with Bonferroni post test.  $P<0.05$  were considered statistically significant.

Nogo-A expression by oligodendrocytes was also examined in several white matter structures: the corpus callosum (CC), the external capsule (ec), the internal capsule (ic), and the cingulum bundle (cingulum). Oligodendrocytes were identified based on visual profiles and Nogo-A expression was scored twice by a blinded observer in all brains using a scale corresponding to that used for neurons (0=no Nogo-A expression, 1=slight Nogo-A expression, 2=moderate Nogo-A expression, and 3=high Nogo-A expression). Both evaluations resulted in the same mode score.

**Results**

**Neuronal Nogo-A Expression in the Normal Rat Cortex**

For normal rats, neuronal Nogo-A-positive neurons (Nogo-A<sup>+</sup>) were observed in all cortical areas examined (Table 1).

**Table 1. Neuronal and White Matter Nogo-A Expression in Normal Rats**

Cortical Area or White Matter Structure	Nogo-A Expression* (N=4)
Cg	++
RSA/RSG	+++
AGm	++
AGl	+++
FL/HL	+++
Insular	++
Piriform	+
S1/S2	+++
External capsule	++
Internal capsule	++
Cingulum	++
Corpus callosum	++

\*Scores are Mode of Expression Scores.  
+++ = High, ++ = Moderate, + = Low.

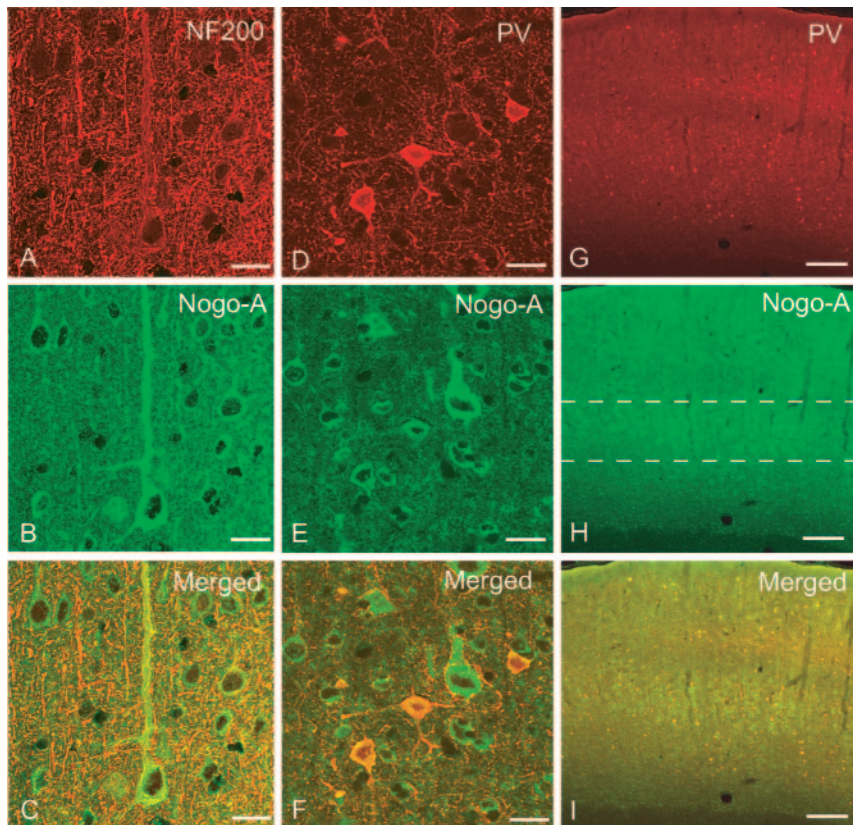
Within these areas, Nogo-A<sup>+</sup> neurons were seen in cortical layers II through VI with varying intensity between brain areas (Figure 1), with layer V neurons being labeled most robustly (Figure 2A and B). Within layer V, neuronal staining varied between cortical areas, but homotopic brain regions always exhibited consistent bilateral Nogo-A expression. The most robustly labeled layer V cortical neurons were found in FL and HL (Table 1; Figures 1 and 2). High expression of Nogo-A was also evident in layer V of AGl, S1/S2, RSA, and RSG, whereas lower immunopositivity was observed in Cg, AGm, Insular, and Piri (Table 1; Figure 1). In each of these areas, the large layer V pyramidal neurons exhibited robust Nogo-A expression which localized to the cytoplasm and dendrites, many of which were clearly labeled as they extended long distances toward the pial surface (Figure 2). Nogo-A expression was absent in the nuclei of all neurons observed.

In addition to excitatory pyramidal neurons, Nogo-A expression was also detected on parvalbumin-positive (PV<sup>+</sup>) GABA-ergic inhibitory interneurons in all layers of the cortical areas examined (Figure 2D through 2F; Figure 3). Double-labeled (PV<sup>+</sup>/Nogo-A<sup>+</sup>) interneurons were commonly observed in layers II-VI of all cortical areas inspected (Figure 2E, F, H, I; Figure 3).

**Neuronal Nogo-A Expression in Ipsilesional Cortical Areas After Stroke**

At 1 and 3 days after MCAO, neuronal Nogo-A expression in ipsilesional AGl, FL, HL, RSA/RSG, and Insular cortex appeared the same as that observed in control animals. However, Nogo-A expression was elevated above control levels in Cg, AGm, and Piri (Table 1). At these early time points, PV<sup>+</sup>/Nogo-A<sup>+</sup> neurons were observed throughout all cortical areas with the exception of the tissue immediately surrounding the lesion, where no PV<sup>+</sup> cell bodies were found.

At 7 and 14 days post-MCAO, Nogo-A expression continued to increase in Piriform cortex and remained increased above control levels in Cg. Nogo-A expression was still



**Figure 2.** Neuronal Nogo-A expression. Many neurons in the cerebral cortex express Nogo-A. Immunohistochemical staining for Neurofilament-200 (A, scale bar 20  $\mu\text{m}$ ) and Nogo-A (B) confirm the identity of Nogo-A positive cells as neurons via their colocalization (C). Parvalbumin-positive (PV<sup>+</sup>) interneurons (D, scale bar 20  $\mu\text{m}$ ) in the cerebral cortex also express Nogo-A (E). Colocalization of Nogo-A and PV occurs in many PV<sup>+</sup> cells, extending into the axons (F). PV<sup>+</sup> neurons were located in cortical layers II-VI (G, red, scale bar 600  $\mu\text{m}$ ), and neurons expressing Nogo-A were most dense in cortical layer V (area between lines; H). PV<sup>+</sup>/Nogo-A<sup>+</sup> neurons (seen in higher magnification in F) were visible in layers II-VI in normal adult rats, but were also most prominent in layer V (I).

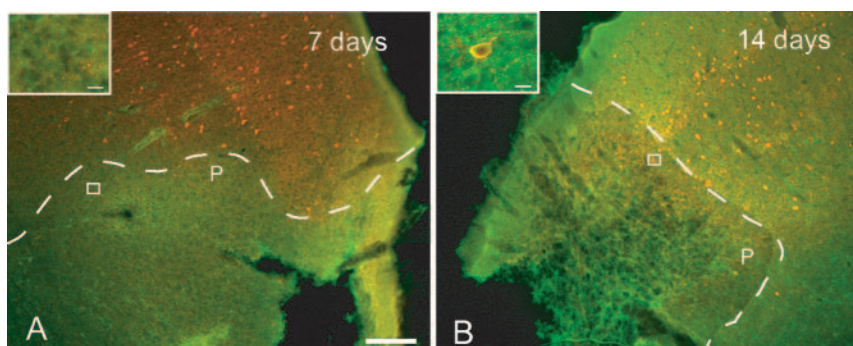
unchanged from control levels in FL, HL, and Insular cortex and returned to normal in AGm. Expression was reduced below control levels in RSA/RSG and AGI (Table 1). Parvalbumin staining appeared normal in areas farther from the lesion site and in contralesional cortex at both 7 and 14 days post-MCAO (not shown). The number of PV<sup>+</sup> neurons remained greatly reduced in and around the lesion site at day 7 (Figure 3A), but PV<sup>+</sup>/Nogo-A<sup>+</sup> neuronal cell bodies were again observed in the cortex immediately adjacent to the lesion at 14 days post-MCAO (Figure 3B).

At 28 days after stroke, layer V neuronal Nogo-A expression in the ipsilesional cortex had increased from 7 and 14 days, reaching a high level of expression in all cortical areas observed (Table 1). However, at this time point, PV had returned to control levels in all cortical areas, including the boundaries of the stroke lesion (not shown).

### Neuronal Nogo-A Expression in Contralesional Cortical Areas After Stroke

Nogo-A expression by excitatory layer V pyramidal neurons in cortical areas of the contralesional hemisphere was also altered after MCAO. At 1 and 3 days poststroke, staining was moderate in all cortical areas observed (Table 2), representing a decrease in staining intensity from control animals in RSA/RSG, AGm, AGI, FL, HL, and S1/S2. Nogo-A expression was elevated above control levels in Piri and was unchanged in Cg and Insular cortex.

At 7 and 14 days post-MCAO, Nogo-A expression remained moderate in most brain areas observed (Table 2), but increased from 1 and 3 day levels in AGI, FL, and HL. Because control Nogo-A expression in these areas was high, this increase represented a return to normal expression levels, as determined in our semiquantitative analysis (Table 2).



**Figure 3.** Neuronal Nogo-A and PV expression are diminished in perilesional cortex at early time points. At day 7 after stroke, expression of Nogo-A and PV were reduced in the perilesional cortex (P), located immediately adjacent to the stroke lesion (A, green is Nogo-A, red is PV, scale bar 600  $\mu\text{m}$ ; higher magnification inset scale bar 50  $\mu\text{m}$ ; dashed line shows edge of perilesional cortex). At day 14, Nogo-A and PV expression returned to the spared perilesional cortical tissue (B; higher magnification inset).

**Table 2. Neuronal and White Matter Nogo-A Expression After Stroke**

Cortical Area or White Matter Structure*	Poststroke Time Elapsed		
	1 and 3 Days (N=7)	7 and 14 Days (N=7)	28 Days (N=4)
<b>Cg</b>			
Ipsi	+++ (↑)	+++ (↑)	+++ (↑)
Contra	++ (→)	++ (→)	+++ (↑)
<b>RSA/RSG</b>			
Ipsi	+++ (→)	++ (↓)	++/+++ (→)
Contra	++ (↓)	++ (↓)	++/+++ (→)
<b>AGm</b>			
Ipsi	+++ (↑)	++ (→)	++/+++ (↑)
Contra	++ (→)	++ (→)	++/+++ (↑)
<b>AGI</b>			
Ipsi	+++ (→)	++ (↓)	++/+++ (→)
Contra	++ (↓)	+++ (→)	+++ (→)
<b>FL/HL</b>			
Ipsi†	+++ (→)	+++ (→)	+++ (→)
Contra	++ (↓)	+++ (→)	+++ (→)
<b>Insular</b>			
Ipsi‡	++ (→)	++ (→)	+++ (↑)
Contra	++ (→)	++ (→)	+++ (↑)
<b>Piriform</b>			
Ipsi	++ (↑)	++/+++ (↑)	+++ (↑)
Contra	++ (↑)	++ (↑)	+++ (↑)
<b>S1/S2‡</b>			
Contra	++ (↓)	++ (↓)	+++ (→)
<b>EC</b>			
Ipsi	++/+++ (↑)	++ (→)	++ (→)
Contra	++ (→)	+ (↓)	++ (→)
<b>IC</b>			
Ipsi	++ (→)	++ (→)	++ (→)
Contra	++ (→)	++ (→)	++ (→)
<b>Cingulum</b>			
Ipsi	++ (→)	++ (→)	++ (→)
Contra	++ (→)	++ (→)	++ (→)
<b>CC</b>			
Ipsi	++ (→)	++ (→)	++ (→)
Contra	++ (→)	++ (→)	++ (→)

\*Scores are Mode of Expression Scores (Relation to control score).

+++ = High, ++ = Moderate, + = Low.

↑ = Elevated above normal, → = Normal, ↓ = Decreased from normal.

†Scores for FL/HL and Insular refer to the portions not included in the lesion; ‡no score is given for Ipsilesional S1/S2 as it was wholly destroyed in stroke rats.

At 28 days poststroke, Nogo-A expression was high in all cortical areas observed. Expression increased above 7 and 14 day levels in CG, RSA, RSG, AGm, Insular, Piriform, and S1/S2. Expression scores at 28 days were high or moderate-high in all cortical areas observed.

PV<sup>+</sup>/Nogo-A<sup>+</sup> cell reactivity was present at all time points observed, and the distribution of PV<sup>+</sup> staining in cortical

areas in the contralesional hemisphere was not noticeably altered from control at any observed time point.

**Quantification of Neuronal Anti-Nogo-A Immunopositivity**

Analysis of the number of Nogo-A positive neurons in layer V contralesional forelimb motor cortex demonstrated a clear increase in Nogo-A expression in this cortical area at 28 days after stroke as compared to earlier time points (Figure 4A). The total number of neurons immunopositive for Nogo-A was increased at 28 days, at which time most of the neurons in the poststroke cortex demonstrated a moderate level of fluorescent intensity (Figure 4B), indicating that the rise in Nogo-A positivity is largely due to expression by more neurons and not because of an increase in expression by neurons already positive for Nogo-A.

**Normal and Poststroke Nogo-A Expression by White Matter Oligodendrocytes**

In normal rats, Nogo-A positive cells morphologically consistent with oligodendrocytes were regularly observed in all white matter structures examined (supplemental Figure I, available online at <http://stroke.ahajournals.org>; Table 1). After stroke, Nogo-A expression was largely unchanged in the white matter during our study period, with the exception of an early (1 and 3 days poststroke) transient increase in Nogo-A expression in the ipsilesional external capsule, which returned to baseline by 7 days poststroke (Table 2). Conversely, Nogo-A expression was transiently decreased in the contralesional external capsule at 7 and 14 days poststroke (Table 2). By 28 days poststroke, oligodendroglial Nogo-A expression returned to baseline in all white matter structures observed (Table 2).

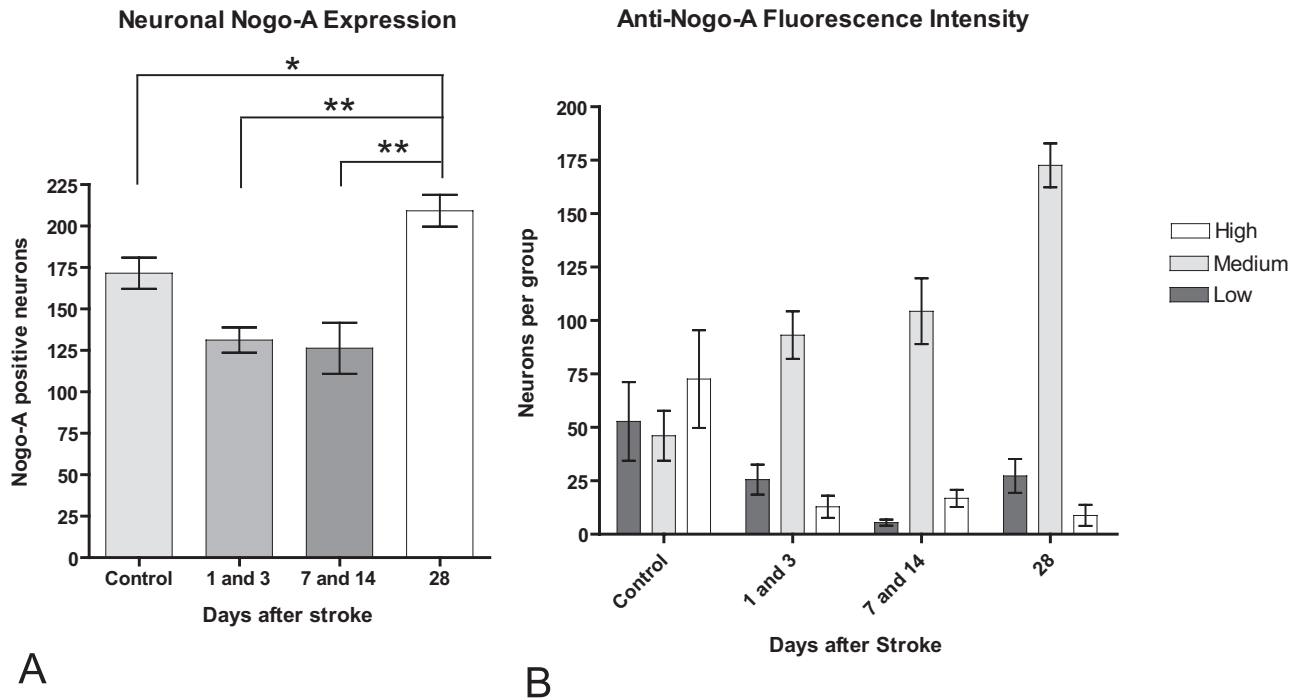
**Discussion**

**Neuronal Nogo-A Expression in Normal Rat Cortex**

In the current study, neurons in cortical layers II-VI were observed to express Nogo-A, but expression was highest in layer V pyramidal neurons in each of the cortical regions studied. This finding is consistent with our previous report regarding neuronal Nogo-A protein expression in the normal rat sensorimotor forelimb cortex,<sup>8</sup> a finding which was extended to neurons in S1 barrel cortex.<sup>19</sup> The observed cellular localization of neuronal Nogo-A to the cytoplasm and dendrites was also consistent with the pattern of localization observed by others.<sup>14-19</sup>

**Neuronal Nogo-A Expression After Stroke**

After stroke, neuronal Nogo-A expression varied across cortical areas at early time points, but expression was high at 28 days after stroke in all of the cortical areas studied. Furthermore, quantification of the number and immunofluorescent intensity of anti-Nogo-A labeled neurons in the contralateral sensorimotor forelimb cortex demonstrated that more neurons in this key cortical area expressed Nogo-A at 28 days poststroke, supporting the results of the semiquantitative analysis. Interestingly, these results demonstrate that Nogo-A<sup>+</sup> immunofluorescence does not regularly reach the



**Figure 4.** Neuronal Nogo-A expression in contralesional sensorimotor forelimb cortex. A significant increase in the number of anti-Nogo-A immunolabeled layer V neurons was detected in contralesional sensorimotor forelimb cortex at 28 days after stroke compared to earlier time points (A; \* $P < 0.05$ ; \*\* $P < 0.001$ ). The elevated number of Nogo-A immunopositive neurons at 28 days after stroke corresponded with a significant increase in the number of neurons with a midlevel fluorescence intensity over time and a reduction in the number of cells in the high and low immunofluorescence groups (B).

greatest levels of intensity after stroke, even though “high” expression was observed in one third of neurons in normal adult rats. Whether expression goes up even further at time points beyond 28 days remains to be determined.

The overall increase in neuronal Nogo-A expression observed across cortical areas might play a role—in addition to myelin-associated Nogo-A—to limit spontaneous anatomic remodeling by cortical neurons after injury, resulting in severely diminished functional recovery from poststroke deficits. This hypothesis is consistent with previous anatomic and behavioral findings which demonstrate that anti-Nogo-A immunotherapy induces axonal<sup>3–7</sup> and dendritic<sup>8</sup> changes in cortical projection neurons which coincide temporally with the impressive degree of enhanced recovery of function subsequent to anti-Nogo-A antibody treatment.

Our results are consistent with other studies of postinjury Nogo-A expression in adult rats, including the increase in neuronal Nogo-A expression described at 4 days after a cortical stab injury.<sup>14</sup> In one study of Nogo-A protein after stroke, expression of Nogo-A in the region of the cerebral cortex immediately adjacent to the stroke lesion (“perilesional cortex”) was decreased in adult rats at 1 and 2 weeks after stroke, as detected by Western blot.<sup>31</sup> Our results support this finding for neurons in the perilesional cortex (Figure 3) but further demonstrate that the transient reduction of Nogo-A expression in perilesional cortex after stroke is not indicative of a reduction in Nogo-A across the entire cerebral cortex, as evidenced by our results for neurons in more distal cortical regions (Table 2). Furthermore, our current results demonstrate that the previously described reduction

of Nogo-A expression in perilesional cortex<sup>31</sup> is transient, as levels of Nogo-A become high in all cortical areas at 28 days after stroke, a time point which was not previously examined.

Poststroke Nogo-A expression in our adult rat MCAO model differed significantly from findings described after global ischemia in neonatal rats. Although Nogo-A expression increased in pup brains after global ischemia, expression peaked within 24 hours and returned to baseline by 72 hours.<sup>32</sup> The differences in the observed results may be due to the method of inducing ischemic injury (ie, global versus focal) or the use of neonatal rats, which respond to central nervous system injury quite differently than adults.<sup>33</sup>

Anti-Nogo-A antibodies are known to enhance neuroanatomical plasticity and functional recovery after stroke when treatment begins up to 1 week after injury.<sup>3–8,24</sup> Administering anti-Nogo-A antibodies during this time period inhibits the function of Nogo-A while its expression is changing in neurons in the cerebral cortex. The effectiveness of beginning anti-Nogo-A immunotherapy during the time of rapidly changing Nogo-A expression remains to be compared with administration after neuronal Nogo-A levels become elevated in the cerebral cortex (ie, on or after 28 days poststroke).

Neurons in contralesional brain regions are known to be important for mediating anatomic plasticity after brain injuries.<sup>23,34,35</sup> Therefore, neuroanatomical plasticity after stroke and anti-Nogo-A immunotherapy has been most intensely studied in contralesional sensorimotor cortex.<sup>3–5,36–38</sup> Cortico-efferent layer V neurons in sensorimotor cortex have been shown to cross the midline and innervate the deaffer-

ented striatum, red nucleus, pontine nuclei, and the spinal cord.<sup>3,5,37</sup> Golgi-Cox analyses of dendritic morphology in this key cortical area demonstrated a specific, lasting increase in dendritic spine density, dendritic length, and dendrite branching in layer V of contralesional sensorimotor cortex after stroke and anti-Nogo-A immunotherapy.<sup>8</sup>

The poststroke increase in neuronal Nogo-A expression by layer V neurons in contralesional sensorimotor forelimb cortex observed in the current report may be directly associated with the known failure of spontaneous remodeling of cortico-efferent projections after injury.<sup>23,34,35</sup> Because the timeline of Nogo-A elevation coincides temporally with the failure of spontaneous sprouting after focal ischemic stroke,<sup>23,31</sup> Nogo-A expressed by excitatory and inhibitory cortical neurons represents a potential target of anti-Nogo-A immunotherapy. Furthermore, Nogo-A expression by cortico-efferent layer V neurons in contralesional sensorimotor forelimb cortex—which demonstrate axonal and dendritic plasticity after stroke and anti-Nogo-A immunotherapy—is of particular importance, as many of these cells project to other cortical and subcortical regions mediating forelimb movement (ie, grasping), fine touch, and proprioception. Administration of anti-Nogo-A antibodies after stroke may act to enhance recovery of function by binding neuronal Nogo-A as previously described, resulting in Nogo-A downregulation.<sup>18</sup> Such a reduction of Nogo-A expression could play a role in lengthening the period over which effective poststroke neuronal remodeling may occur in the rat cerebral cortex, resulting in an increase in functional recovery from poststroke deficits, as observed previously.<sup>3,5,24,38</sup>

### Normal and Poststroke Nogo-A Expression by PV<sup>+</sup> Interneurons

Our data demonstrate that PV<sup>+</sup> interneurons in the cerebral cortex of normal and poststroke rats express Nogo-A, as previously described in developing and uninjured adult mice.<sup>20</sup> Four subtypes of interneurons in the cerebral cortex express PV: chandelier cells, nest basket cells, small basket cells, and large basket cells.<sup>39–41</sup> In adult animals, inhibitory PV<sup>+</sup> cortical interneurons form somatic and perisomatic synapses on nearby excitatory cortical pyramidal neurons and can inhibit their firing in response to incoming excitatory postsynaptic potentials,<sup>42</sup> thereby playing a critical role in gating the activity of cortico-efferent pyramidal neurons.<sup>39</sup> In turn, these pyramidal neurons project to other brain regions or the spinal cord. Therefore, inducing plastic changes in inhibitory interneurons after stroke via anti-Nogo-A immunotherapy could result in the overall modulation of excitatory cortical output to other central nervous system structures, contributing to the anatomic remodeling of cortico-efferent projections to appropriate cortical and subcortical targets, which may subsequently result in enhanced functional recovery.

### Nogo-A Expression in White Matter Structures After Stroke

In the current study, no lasting increase was detected in any white matter structure after stroke. Although Nogo-A expression by white matter oligodendrocytes fluctuated transiently in both the ipsilesional and contralesional external capsule,

Nogo-A expression in both structures returned to baseline levels by 28 days after stroke.

In contrast to our results, oligodendroglial Nogo-A expression was demonstrated to increase over time after focal cerebral ischemia in a marmoset model of stroke.<sup>43</sup> In addition to potential species differences, this may be explained by the time points examined in the study. In contrast to the current study, which focused on the first 28 days after stroke, marmoset brains were examined at later time points (2, 3, and 4 months) after ischemia.<sup>43</sup> The possibility that Nogo-A expression in white matter becomes elevated in rodents at 2, 3, or 4 months after stroke remains to be examined.

### Summary

Herein, we demonstrate Nogo-A expression by excitatory and inhibitory layer V cortical neurons in the normal and poststroke rat brain. After stroke, Nogo-A expression by layer V pyramidal neurons changed within all of the cortical areas studied, increasing and/or decreasing over time after stroke. Importantly, neuronal Nogo-A expression was high at 28 days after stroke in all cortical areas studied. In the white matter, Nogo-A expression fluctuated transiently in the external capsule after stroke, but returned to baseline levels. Nogo-A expression remained unchanged in all other white matter structures examined.

In conclusion, our data demonstrate the importance of neuronal Nogo-A as a potential target for anti-Nogo-A immunotherapy after stroke.

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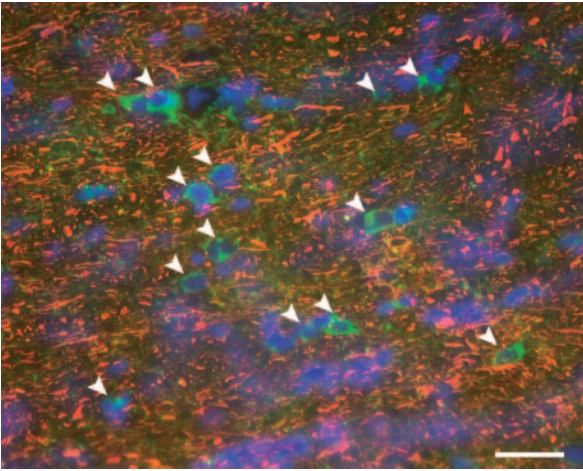
### Disclosures

None.

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**Figure 1.** Oligodendrocyte Nogo-A expression in the corpus callosum. Nogo-A positive cells (arrowheads; green; scale bar 200  $\mu\text{m}$ ) with DAPI-positive nuclei (blue) are clearly distinguished from axonal processes by their lack of NF200 positivity (red) in normal and poststroke rats.

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