Stroke is a prevalent and devastating disorder, and no treatment is currently available to restore lost neuronal function after stroke occurs. One unique therapy that may improve functional recovery after stroke is blockade of the neurite inhibitory protein Nogo-A with the monoclonal antibody IN-1, through enhancement of neuroanatomical plasticity from uninjured areas of the central nervous system. In the present study, we combined IN-1 treatment with an ischemic lesion (permanent middle cerebral artery occlusion) to determine the effect of Nogo-A neutralization on cortical plasticity and functional recovery. We report here that, following ischemic stroke and treatment with IN-1, adult rats demonstrated functional recovery on a forelimb-reaching task and new cortico-efferent projections from the opposite, unlesioned hemisphere. These results support the efficacy of Nogo-A blockade as a treatment for ischemic stroke and implicate plasticity from the unlesioned hemisphere as a mechanism for recovery.

Cerebrovascular disease, or stroke, is one of the leading causes of death and the most common cause of adult disability. Neuronal loss due to stroke may result in permanent deficits in visual, sensory, language, and motor capabilities. There is no specific treatment for improving functional recovery after stroke except for rehabilitative strategies, which have had limited success.

Injury to the adult mammalian central nervous system (CNS) shows virtually no capacity for cut axons to regenerate and very limited capacity for neuroanatomical plasticity, the new growth of uninjured pathways. In contrast, neuroanatomical plasticity is commonly seen after CNS injury in the young. Importantly, because CNS injury in the young has been associated with better functional recovery compared with similar injury at maturity in both clinical and animal studies, neuroanatomical plasticity is thought to be the underlying mechanism for functional recovery. That the developing human brain is capable of significant plasticity has been shown in several studies. Therefore, strategies to improve neuroanatomical plasticity following adult brain injury should lead to improved rehabilitative potential.

The inability to achieve large-scale “rewiring” after adult CNS lesions may be attributable to several factors, including glial scars, lack of neurotrophins, and growth-inhibitory molecules, such as proteoglycans and myelin-associated proteins. Of particular interest is the recently cloned myelin-associated neurite inhibitory factor Nogo-A (formerly known as neurite growth inhibitor NI-250). This protein is primarily expressed on the surface of oligodendrocytes and their product myelin. Neutralization of Nogo-A/NI-250 by treatment with the monoclonal antibody (mAb) IN-1 allowed neurite outgrowth on CNS myelin or cultured oligodendrocytes in vitro. In vivo treatment with the mAb IN-1 led to regeneration of corticospinal tract (CST) fibers after adult CNS lesions and functional recovery after spinal cord injury in adult rats. Neutralization of Nogo-A with the mAb IN-1
resulted in compensatory neuroanatomical plasticity and functional recovery after pyramidotomy of a lesion.\textsuperscript{17,24,25} Furthermore, pertinent to this study of cortical stroke is our earlier work describing cortico-efferent plasticity following cortical aspiration of a lesion and treatment with the mAb IN-1.\textsuperscript{26,27}

To test directly the effect of Nogo-A neutralization on cortico-efferent plasticity and functional recovery following focal ischemic stroke, we administered the mAb IN-1 to adult rats following middle cerebral artery occlusion (MCAO). Rats were tested with a skilled forelimb reaching task that requires an intact CST and the integration of the rubrospinal tract (RST).\textsuperscript{28–30} We show in the present study that treatment with the mAb IN-1 following stroke leads to functional recovery demonstrated by the forelimb reaching task. Furthermore, we demonstrate neuroanatomical plasticity originating from the opposite unabluted cerebral cortex, indicating a possible important role of the intact hemisphere in recovery after stroke.

### Materials and Methods

#### Animals

Adult male Long Evans black-hooded rats (200–300g, ages 6–8 weeks) were divided into three experimental groups: (1) MCAO only (n = 9), (2) MCAO plus the mAb IN-1 (n = 11), and (3) MCAO plus control antibody treatment (n = 6). Animals were number-coded and investigators were blinded to the treatment groups. Experiments were approved by the Joint Institutional Animal Care and Use Committee of Loyola University and Hines Veterans Affairs Hospital.

#### Stroke Surgery

MCAO was performed as described by Chen et al.\textsuperscript{31} Animals were anesthetized with sodium pentobarbital (50mg/kg, administered intraperitoneally). Bilateral common carotid arteries (CCAs) were isolated, a vertical 2cm incision was made between the eye and the ear, and the temporalis muscle was retracted. A burr hole was made to expose the MCA, which was permanently occluded by cauterization and then transected with microscissors. The CCA ipsilateral to the MCAO was permanently occluded with a 4-0 suture and the contralateral CCA was temporally occluded for 45 minutes. Body temperature was maintained with a heating pad and monitored with a rectal probe. The wound was then closed, and the animals were warmed under a heating lamp until they awoke.

#### TTC (2,3,5-Triphenyl-2H-Tetrazolium Chloride) Reaction

For demonstration of stroke size, some animals that were not used for behavior studies were examined using TTC. Three days after MCAO surgery, brains were removed, cut into 3mm slices, and incubated in 2% TTC (Sigma, St. Louis, MO) at 37°C for 15 minutes, then fixed in 4% paraformaldehyde (PFA) for examining stroke location.

#### Antibody Application

Immediately after MCAO, a cell suspension (6µl) containing a total of $1 \times 10^5$ of either the mAb IN-1\textsuperscript{20} or the control antibody (anti-HRP)\textsuperscript{21} secreting hybridoma cells was injected posterior to the lesion (4mm caudal, 5mm lateral, and 5mm ventral to the bregma) on the side of the lesion. All animals, including those in the MCAO-only group, received cyclosporin A (10 mg/kg, administered intraperitoneally) daily, starting from the day before surgery to 14 days after surgery, to prevent rejection of the hybridoma xenograft.

#### Detection of Distribution of the Monoclonal Antibody IN-1

Polyclonal rabbit antimouse immunoglobulin M (1:200; Dako, Carpinteria, CA) was applied to tissues at 4°C overnight. Tissues were further incubated with biotin-conjugated goat-antirabbit antibody (1:200, Dako) at room temperature for 1 hour. Cy3-conjugated streptavidin (1:1000; Jackson ImmunoResearch, West Grove, PA) was applied to sections at room temperature for 1 hour. Sections were rinsed, mounted, and viewed with a Leica DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

#### Behavioral Test

Animals were reduced to $<95\%$ of their original body weight before testing and maintained on a restricted diet for the duration of the testing period. All animals were trained for up to 3 weeks to establish limb preference and baseline measurements (defined as the average of the last three testing sessions of preoperative testing). The forelimb reaching task was performed as described,\textsuperscript{32} using a transparent Plexiglas chamber ($30 \times 36 \times 30$cm) with a rectangular opening ($1.5 \times 3$cm) in the front wall on either the left or the right side. A Plexiglas shelf was attached outside, underneath the opening. Sucrose pellets (45mg; Bilaney Consultants, Frenchtown, NJ) were placed one after the other on the shelf at a distance of 1.5cm from the opening. We determined limb preference and transferred animals to a testing chamber, where the placement of the opening biased the animal to use the preferred limb. In the 20-pellet paradigm, performed daily (Monday through Friday), animals had to reach through the opening and obtain 20 pellets that were placed on the shelf one after the other. The parameters that were measured included (1) the success score, defined as the number of pellets grasped with the appropriate limb and placed into the mouth; (2) the rate of recovery; and (3) the amount of time needed to obtain 20 pellets. A maximum time limit of 10 minutes per testing session was given. Each animal was tested 2 days after surgery and then daily for up to 8 weeks. Baseline and weekly sessions were videorecorded.

#### Biotinylated Dextran Amine Tracing

After 8 weeks of behavioral testing, animals were anesthetized with sodium pentobarbital (50mg/kg, administered intraperitoneally). The sensorimotor cortex opposite to the stroke lesion site was exposed, and 2 injections of 1µl each of a 10% biotinylated dextran amine (BDA) solution (Molecular Probes, Eugene, OR) were placed stereotaxically into the forelimb sensorimotor cortex as described by Neafsey et al.\textsuperscript{33} Two weeks after BDA injection, animals were overdosed
with sodium pentobarbital and perfused with 4% PFA. Brains were cut into 50µm coronal sections and reacted for BDA by the semi-free-floating method. Alternate sections were processed for Nissl stain and analyzed for lesion size.

**Neuroanatomical Analysis**

All anatomical structures were identified using the atlas of Paxinos and Watson. The cortico-efferent projections to the red nucleus were quantitatively analyzed ipsi- and contralaterally to the injection site, as described by Z’Graggen et al. All the slides were coded and investigators were blind to the treatment group. Sections were analyzed by computer-aided image analysis using NIH Image software (National Institutes of Health, Bethesda, MD). The hardware was comprised of a Macintosh Centris 650 computer interfaced through a digitizing board (Data Translation, Marlboro, MA) to a digital camera (model MOS; Hitachi, Brisbane, CA) attached to a Leitz DMR microscope.

**QUANTIFICATION OF CST LABELING.** The number of labeled CST fibers in the cerebral peduncle ipsilateral to the injection site was determined and used to correct for vari- ances among animals in BDA tracing. Two consecutive sections per animal, from the same midpoint level, were analyzed. Briefly, the area of the cerebral peduncle was measured first using a ×10 objective. Then four squares, each measuring 3,015µm², were placed systematically over the cerebral peduncle, and the BDA-positive fibers within these squares were counted using a ×40 objective. The four values were averaged and the total number of labeled CST fibers was estimated by extrapolating for each section. Values from the two consecutive sections were averaged.

**QUANTIFICATION OF CORTICORUBRAL PROJECTION.** The corticorubral projection to the contralateral red nucleus was analyzed by counting all BDA-positive fibers crossing the midline on each section through the rostral to caudal extent of the red nucleus. To correct for tracing differences, the number of midline-crossing BDA-positive fibers was divided by the total number of CST fibers (calculated as described in the previous paragraph).

**Stroke Size Analysis**

The stroke volume of each animal (excluding 1 animal from the MCAO–control antibody group that could not be analyzed) was quantitatively analyzed on Nissl stained sections (+4.7 to −5.2 mm from the bregma, according to Paxinos and Watson), using the method described by Kawamata et al. The total area of the intact contralateral hemisphere minus the total area of the intact ipsilateral hemisphere, multiplied by the total distance between sections. Stroke size was expressed as a percentage of the intact contralateral hemispheric volume.

**Statistics**

Analysis of all data was performed with SAS software (SAS Institute, Cary, NC). For behavioral data, a one-way analysis of variance (ANOVA) was used for comparison of the mean values for one session (success score), and a repeated-measures ANOVA was used for comparison of the rate of recovery (the slope of the line). For nonparametrical behavior data, the likelihood ratio test statistic was used for comparison within the same experimental group, i.e., between baseline and postoperative testing (time measurements).

Analysis of fiber-count data (midline-crossing fibers) was performed using a Poisson regression analysis for comparison of the mean values between the experimental groups. Stroke size analysis was performed using a one-way ANOVA for comparison of the mean values between the experimental groups. A p value less than or equal to 0.05 was considered significant. All data are presented as mean values ± standard error of mean (SEM).

**Results**

**Localization of Stroke Lesions in the Sensorimotor Cortex**

All stroke lesions included the sensorimotor cortex (Fig 1; shaded area; Fig 2). Analysis showed no difference in stroke volume between groups (MCAO only, n = 9, 12.22% ± 2.83 SEM; MCAO plus control antibody, n = 5, 7.76% ± 6.07 SEM; MCAO plus the mAb IN-1, n = 11, 8.08% ± 1.41 SEM) (p = 0.55, one-way ANOVA).

**Distribution of the Monoclonal Antibody IN-1**

The mAb IN-1 penetrated into the CNS parenchyma, including the gray and white matter (Fig 3). The antibody-producing hybridoma cells originally implanted in the hippocampus were found in the cerebrospinal fluid circulation (see Fig 3).

**Functional Recovery in Animals Treated with IN-1**

To determine whether neutralization of the myelin-associated inhibitory protein Nogo-A after MCAO resulted in functional recovery, the rats were tested with the forelimb reaching task (Fig 4a). Rats were trained daily for up to 3 weeks to establish limb preference and
baseline measurements prior to stroke surgery. The MCAO resulted in the ischemic lesion affecting the preferred limb. Animals were then tested daily for the next 8 weeks. We first determined the success score, defined by the number of pellets grasped with the preferred limb in the 20-pellet paradigm. The reaching behavior over the 8-week testing period revealed two important findings (Fig 4b). First, all three animal groups showed marked deficits in successfully obtaining pellets with the stroke-affected limb 1 to 3 days after MCAO, with no significant difference between groups \((p > 0.05)\). Second, animals treated with the mAb IN-1 continued to improve from 2 weeks postlesion to exceed the other two control groups, so that by 6 weeks they were significantly different \((p < 0.05)\). Furthermore, at 8 weeks postlesion, animals treated with the mAb IN-1 demonstrated a recovery of 80% of the prelesion behavioral performance, which was significantly different from the observations of control animals \((p < 0.01)\). In contrast, rats that had no treatment or control antibody treatment showed a slight improvement in performance beginning at 2 weeks postlesion, which plateaued at 4 weeks with no further improvement. In addition, there was no significant difference between the two control groups at all time points \((p > 0.05)\). In the second analysis, we measured the rate of recovery demonstrated by the forelimb-reaching task over the postoperative time period (Fig 4c). Compared with both control lesion groups, which showed no difference between their rates of recovery \((p > 0.05)\), for animals treated with the mAb IN-1 the rate of recovery was significantly different \((p < 0.0001)\). In the third analysis, we measured the time required for animals to obtain 20 pellets with their affected limb (Fig 4d). In baseline preoperative testing, there was no difference between groups, and all animals obtained 20 pellets within about 75 seconds. At 1 to 3 days following stroke, animals in all groups showed a marked increase in the time needed to obtain pellets, significantly different from their baseline values. Over the next 8 weeks of postoperative testing, animals in the MCAO-only group slightly improved, requiring less time to obtain 20 pellets (229 seconds), but never reached baseline values \((p < 0.01)\). In contrast, by 8 weeks following stroke, animals treated with the mAb IN-1 had fully recovered in terms of the time needed to obtain 20 pellets (99 seconds), and these results

Fig 2. TTC (2,3,5-triphenyl-2H-tetrazolium chloride)–reacted coronal brain sections 3 days after permanent middle cerebral artery occlusion (MCAO) demonstrated a representative stroke size and location in the ipsilateral sensorimotor cortex. Viable tissue is red (appears gray in this figure) as a result of the reduction of the dye by functional mitochondrial enzymes; the area of the ischemic infarction appears white. Scale bar = 0.5cm.

Fig 3. Photomicrograph of the monoclonal antibody (mAb) IN-1 immunoreactivity, demonstrating the distribution of the mAb throughout the brain parenchyma. Intense staining of the corpus callosum (Cc) crossing the midline was seen. Note mAb IN-1–producing hybridoma cells (insert, arrows) in the cerebral spinal fluid circulation in the lateral ventricle (V) distant from their implantation site. The asterisk (*) signifies the cortical lesion site. The negative control, the adjacent section, showed no staining. Scale bar = 50μm.
were not significantly different from baseline values ($p < 0.05$). Animals in the MCAO–control antibody group still required more time (189 seconds) to retrieve pellets at 8 weeks, but the difference between this result and baseline values did not reach statistical significance ($p > 0.05$).

**Neuroanatomical Plasticity Parallels Functional Recovery**

The significant functional recovery observed in the rats treated with the mAb IN-1 led us to investigate whether anatomical changes paralleled this recovery. At the level of the brainstem, we examined the red nucleus that receives direct input from the primary motor cortex, i.e., the corticorubral projection.\textsuperscript{37,38} This unilateral projection to the red nucleus ends primarily in the ipsilateral parvocellular part, with only a small number of fibers crossing the midline to project to the contralateral nucleus. Figure 5a shows a representative BDA injection site centered in the forelimb motor cortex. In animals in the MCAO-only (Fig 5b) and MCAO–control antibody groups (Fig 5c), the corti-
The corubral projection was only ipsilateral, with little evidence of fibers crossing to the contralateral red nucleus. In contrast, the animals that underwent stroke surgery and treatment with the mAb IN-1 showed significantly more BDA-positive fibers crossing the midline and terminating in the contralateral red nucleus in appropriate target areas mirroring the nondeafferented red nucleus (Fig 5d, e). Quantitative analysis confirmed these results and showed a significant increase in the fibers crossing in animals treated with the mAb IN-1 (469 ± 81 SEM) compared with animals in the MCAO-only (232 ± 37 SEM) and MCAO–control antibody groups (166 ± 50 SEM) (Fig 6).

Discussion
This study demonstrates that intracerebral application of the mAb IN-1 results in a high degree of functional recovery in skilled forelimb movements after MCAO in adult rats. Furthermore, mAb IN-1 treatment after MCAO increases neuroanatomical plasticity in the fibers originating from the forelimb area of the opposite, unablated cortex, resulting in a bilateral innervation to the red nucleus.

The execution and coordination of skilled forelimb movements in the normal rat is dependent on several cortico-efferent pathways that primarily include the corticospinal and rubrospinal tracts. Because a rat’s skilled movements constitute an action pattern and are highly conserved, they are useful to study as a model for motor recovery following cortical lesions. Such lesions, when they include the sensorimotor cortex in the adult rat, significantly affect the performance of these skilled movements in the forelimb contralateral to the lesion, resulting in permanent impairments with limited spontaneous recovery. Our results of studying control rats with MCAO lesions alone (given no treatment or control antibody treatment) support these data in that animals were shown to be permanently impaired when the forelimb reaching test was given. Although these animals showed some improvement over time, they plateaued at approximately 50% of baseline values. In contrast, our results demonstrated a recovery of 80% in skilled forelimb movements after MCAO and mAb IN-1 treatment. In addition, these results agree with previous work from our group, which showed hindlimb functional recovery after treatment of thoracic spinal cord lesions with mAb IN-1 and recovery of skilled forelimb movements after mAb IN-1 treatment for higher CST lesions at the level of the medullary pyramid. These earlier studies attributed functional recovery after mAb IN-1 treatment to either regeneration of cut CST axons or, as in the

Corubral axons established a bilateral projection in the red nucleus after middle cerebral artery occlusion (MCAO) and treatment with the monoclonal antibody (mAb) IN-1. (a) Representative biotinylated dextran amine (BDA) injection site in the forelimb motor cortex. (b) Corubral projection after stroke only, with scarce BDA-positive labeled fibers crossing the midline (vertical line) to the denervated side (left). (c) Similarly, animals with stroke that received the control antibody showed very few BDA-positive fibers crossing the midline. (d) In contrast, with stroke and mAb IN-1 treatment there were many midline BDA-positive crossing fibers and an increased innervation pattern to the side denervated by the lesion (left), seen in the boxed area and shown at a higher power in panel (e). (e) Higher magnification of the boxed area in (d) shows BDA-positive crossing fibers (arrows) and a dense termination pattern to the denervated red nucleus. Therefore, the corubral projection appears to form new connections to the denervated red nucleus after stroke and mAb IN-1 therapy. Dashed vertical lines indicate midline. Cc = corpus callosum, RN = red nucleus. Scale bar in (a) = 500μm; all others = 50μm.
The present study, neuroanatomical plasticity of uninjured pathways.17,24,25 The high degree of functional recovery observed here in rats treated with the mAb IN-1 has also been seen after the occurrence of similar lesions in neonatal animals that had no treatment, ie, spontaneous recovery.6,7,28,29 Interestingly, such functional recovery following lesions in the perinatal period coincides with a high degree of neuroanatomical plasticity. Unilateral cortical lesions in the neonatal rat result in the development of bilateral projections to subcortical motor areas from the opposite, unablated hemisphere, including the striatum,9 thalamus,42 red nucleus,38,43 basilar pontine nuclei,4,45 and spinal cord.2,46,47 This reorganization of neuroanatomical pathways following CNS injury in the young is thought to be the underlying substrate for functional recovery.3,10,48 Therefore, mechanisms to increase similar cortico-efferent plasticity after the occurrence of stroke lesions in adults, such as mAb IN-1 therapy, could significantly improve the rehabilitative potential of stroke victims. In our animal study, we used the antibody-secreting hybridoma cells to deliver Nogo-A–blocking antibodies intraventricularly. Further clinical applications could utilize the intraventricular delivery of humanized anti-Nogo-A antibodies currently under development.

The corticorubral pathway in the adult rat is primarily an ipsilateral projection with a very minor contralateral component.25,49 We show that after MCAO and treatment with the mAb IN-1, sprouted corticorubral fibers originating from the unlesioned hemisphere cross the midline to arborize in the parvocellular portion of the red nucleus, which was denervated from the stroke lesion, mirroring the nondeafferented red nucleus. Furthermore, our results support previous work showing increased plasticity of corticostratial,26 corticopontine, and corticorubral27 fibers after unilateral cortical aspiration lesions and mAb IN-1 treatment. Thus, the neuroanatomical plasticity observed in the present study resulted in the opposite, unablated cortex influencing the deafferented red nucleus and thereby possibly indirectly affecting spinal targets to attain functional recovery. Whether similar structural plasticity and functional recovery occurs in more clinically relevant older animals after stroke and delayed treatment with mAb IN-1 is currently under investigation.

The functional recovery observed in the present study most likely involves multiple underlying mechanisms. First, the mAb IN-1 recognizes and neutralizes the potent neurite inhibitory protein Nogo-A.18,20,50 Properties of its inhibitory role include growth cone collapse and inhibition of neurite outgrowth.18,51,52 Consequently, neutralization of Nogo-A creates a permissive environment that allows for new neurite growth, thus resulting in the development of new neuronal pathways, as shown in our study.

Next, the stroke lesion alone may result in several molecular and cellular responses that could stimulate growth and lead to partial functional recovery, ie, the limited spontaneous recovery seen after adult cortical lesions.53 In this regard, stroke lesions induce growth-promoting factors, such as brain-derived neurotrophic factor (BDNF)54 and basic fibroblast growth factor (bFGF).36,55 and growth-associated molecules, such as GAP-43,56,57 Moreover, blockade of bFGF expression after cortical lesions in the rat resulted in decreased spontaneous recovery in the affected forelimb as measured by the forelimb reaching task.58 In addition to molecular responses, widespread cellular changes, such as increased dendritic branching59,60 and increased number of synapses,57,61 have been observed in both hemispheres after unilateral stroke lesions. Therefore,
following ischemic lesions the adult CNS can reexpress important growth-related molecules and induce cellular responses needed for new neurite growth and synaptic formation. \(^{62}\) Taken collectively, strategies that take advantage of the growth potential of the injured CNS as well as the permissive environment created by blockade of neurite growth–inhibitory factors may lead to new neuronal pathways and functional recovery in adult patients following ischemic stroke.

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References