

Research report

Nogo-A inhibition induces recovery from neglect in rats

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Abstract

Neglect is a complex human cognitive spatial disorder typically induced by damage to prefrontal or posterior parietal association cortices. Behavioral treatments for neglect rarely generalize outside of the therapeutic context or across tasks within the same therapeutic context. Recovery, when it occurs, is spontaneous over the course of weeks to months, but often it is incomplete. A number of studies have indicated that anti-Nogo-A antibodies can be used to enhance plasticity and behavioral recovery following damage to motor cortex, and spinal cord. In the present studies the anti-Nogo-A antibodies IN-1, 7B12, or 11C7 were applied intraventricularly to adult rats demonstrating severe neglect produced by unilateral medial agranular cortex lesions in rats. The three separate anti-Nogo-A antibody groups were treated immediately following the medial agranular cortex lesions. Each of the three antibodies induced dramatic significant behavioral recovery from neglect relative to controls. Severing the corpus callosum to destroy inputs from the contralesional hemisphere resulted in reinstatement of severe neglect, pointing to a possible role of interhemispheric mechanisms in behavioral recovery from neglect.

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One of the major cognitive disorders induced by brain damage and stroke is hemineglect, or neglect. Neglect is a neuropsychological disorder characterized by the failure to report or respond to stimuli presented to the side of the body opposite a brain lesion. Neglect is a complex spatial–attentional disorder involving changes in affect, as well as auditory, visual, and tactile functioning. Neglect occurs in approximately 40% of all brain injuries, most frequently after right hemisphere damage and cannot be explained by more elementary sensory-motor deficits [26,38]. The presence of neglect at 53 days post-stroke is the single best predictor of a poor recovery [16], and recovery, when it occurs, is spontaneous and often incomplete [26].

A rodent cortical model of neglect has been developed which has focused on the rodent analog of the frontal eye fields, the medial agranular cortex (AGm), and the posterior parietal cortex (PPC) [4]. As in primates, the AGm and PPC are multimodal convergence zones which are reciprocally interconnected [12]. Complete unilateral lesions of the AGm result in neglect that does not recover spontaneously, but recovery can be induced pharmacologically by systemic injections of apomorphine or by an environmental manipulation, 48 h of light deprivation [5,11,15].

Previous studies of recovery from neglect produced by unilateral AGm lesions have found that recovery from AGm-induced neglect is correlated with alterations in immediate early gene expression in the dorsolateral striatum [55]. In that study, increases in NMDA and kainate receptors in the ipsilesional dorsolateral striatum were correlated with behavioral recovery from

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neglect. More recent studies have suggested that the critical area for recovery in the striatum is the convergence zone of the striatal afferents from AGm and PPC, the dorsal central striatum (DCS) [8,43,50,52]. Unilateral excitotoxic DCS lesions result in severe multimodal neglect that does not recover spontaneously and light deprivation and apomorphine treatment are ineffective [53]. These results have led to the conclusion that the integrity of the DCS is vital for recovery following unilateral lesions of AGm. Further, these findings suggest that producing plastic changes within the DCS may be a viable option for treating neglect.

While neurite outgrowth in the central nervous system (CNS) occurs readily during the early stages of development, as higher vertebrates reach maturity the plasticity of the brain decreases dramatically [22,59]. The inability of the brain to fully recover following damage is often attributed to the inhibition of neurite growth [46]. Several factors have been identified as being inhibitory to neural regeneration. One of the major contributors to the inhibition of neural outgrowth in vivo is Nogo-A, a membrane bound molecule associated with CNS myelin sheaths [17,22,34,59].

Nogo-A can be inhibited in several ways including, neutralization by the monoclonal antibody IN-1 [9,17], which binds to the amino specific part of Nogo-A [17], blockade of Nogo by a soluble receptor fragment [18], or by receptor blockade of the Nogo-66 receptor NgR [19,21]. Anti-Nogo-A antibodies work by binding to the surfaces of oligodendrocytes and significantly reducing their non-permissiveness to neurite outgrowth [9,46]. IN-1 has been shown to neutralize the inhibition of axon growth and regeneration and results in enhanced recovery of function after lesions of spinal cord or sensorimotor cortex. Very similar results were recently obtained with two other anti-Nogo-A antibodies, 11C7 and 7B12, in spinal cord injury as well as stroke models [1,25,40,41,46].

Perhaps the most striking findings indicate that enhanced recovery in anti-Nogo-A-antibody treated animals may be a result of plasticity originating from the contralesional cortex [2,25,40,59,60]. Previous studies have found that treatment with IN-1 can produce plasticity in the corticostriatal projections from the contralesional homotopic cortex following sensorimotor cortex lesions [25]. Given the plasticity observed in striatal anatomy and physiology that is correlated with behavioral recovery from neglect [55], substantial enhancement of plasticity might result in recovery from neglect. Further, if plasticity from the contralateral homotopic cortex does indeed underlie recovery, then recovery from neglect should be systematically related to the transection of the inputs from contralesional AGm. Support for the functional role of contralateral inputs in recovery has been found for spontaneous recovery from neglect. Crowne et al. [13] found that neglect could be reinstated by callosal sections following spontaneous recovery. These findings support the potential role of inputs from the contralesional side in recovery from neglect.

In part 1 of the present study, anti-Nogo-A antibodies IN-1, 7B12, or 11C7 were administered following complete unilateral aspiration lesions of AGm to determine if neural plasticity and behavioral recovery from neglect can be induced by these antibodies. In order to determine if contralesional projections

underlie recovery, a second series of experiments involved transections of the corpus callosum that either disrupted or spared the connections from contralesional AGm to ipsilesional DCS.

1. Experiment 1

1.1. Materials and methods

1.1.1. Surgical procedures

Rats were anesthetized using ketamine/xylazine i.p. (1.5 ml of 100 mg/ml xylazine and 10 ml of 100 mg/ml Ketamine, at 0.1 ml/100 g). Upon exhibiting no eyeblink response or response to ear bar placement, the rats were fixed in a stereotaxic apparatus using blunt-tipped ear bars. Surgical procedures for AGm removal were identical to those described in prior studies from our laboratory [28,29,51–53]. The underlying rostral and caudal AGm were aspirated through a fine gauge pipette. Following the aspiration of AGm, the subjects were assigned to one of three groups. For the IN-1 group ($n=9$), hybridoma cells containing IN-1 in a cell suspension (6 μ l) containing a total of 1×10^5 cells were injected via a Hamilton syringe intracerebrally into the region of the ventral hippocampus on the same side as the lesion (4 mm caudal, 5 mm lateral to bregma, and 5 mm ventral to the cortical surface; as in Papadopoulos et al. [40]). For the HRP group ($n=9$) the same injection procedures with hybridoma cells were used, but they contained antibodies to HRP. The HRP group served as an antibody-hybridoma control. The cerebral injection site was chosen due to its remoteness to AGm. A third AGm lesion group served as a control for the effects of cyclosporine A ($n=5$).

Postoperatively, the IN-1 animals and controls were maintained on cyclosporin A for a minimum of 9 days to a maximum of 14 days (10 mg/kg, i.p. daily for week 1, 5 mg/kg daily for 1 week 2) to prevent loss of the introduced hybridoma cells by immune response. The range of cyclosporine treatment durations was distributed among all of the groups. The initial injection of cyclosporin A was given the day prior to AGm surgery.

1.1.2. Hybridoma cells

Antibody-secreting hybridoma cells were originally raised by immunization of mice with NI-250 [6]. These mouse hybridoma cells produce an IgM antibody against the rat neurite growth inhibitory protein NI 250, or Nogo-A [9]. Monoclonal control antibodies against horseradish peroxidase (anti-HRP, antibody without inhibitory effect on HRP enzymatic activity) were generated from the same parent myeloma line. Hybridoma cells are grown in Iscove's modified Dulbecco's medium using standard mammalian tissue culture techniques. Before implantation into the brain, hybridoma cells are tested for inhibitory neutralizing activity in a bioassay using neurons or fibroblasts on a myelin-protein substrate [44] and are also tested for IgM production by FITC-coupled anti-mouse antibodies. The production of antibodies in the brain have been detected in previous studies by staining brain sections with anti-mouse FITC-conjugated immunoglobulin, and by the presence of IN-1 antibodies in the serum [47]. This allows a continuous supply of antibodies from the CSF. Previous studies from our group have found a high level of mouse antibodies in the tumors, the ventricles, and the brain parenchyma [40,45]. Weak staining has also been found in the parenchyma of the brainstem [60]. IN-1 secreting hybridoma cells have been used in early studies of Nogo-A inhibition, before the availability of the purified forms, 7B12 and 11C7. The advantage of using hybridoma cells is the higher amount of antibody produced; however, the resulting tumor growth is difficult to control [47]. Also, it is not likely that IN-1 could be infused by minipump due to the inability of the cells to remain in suspension for the duration of time needed for infusion; therefore cells are implanted by syringe. The clinical implications of using tumor-creating, hybridoma cells to deliver anti-Nogo-A antibodies do not make it a viable option for therapeutic use in humans.

1.1.3. Surgical procedures for 7B12 and 11C7 infusion groups

Immediately after the AGm lesion, rats were randomly assigned to receive continuous infusion of either experimental antibody (7B12 ($n=7$) or 11C7 ($n=9$), all at 5 mg/1.68 ml in PBS) or control IgG antibody ($n=8$) into the

lateral ventricle on the ipsilesional side using osmotic minipumps over a 14 day period (5 μ l/h, Alzet model 2002, Palo Alto, CA) [59]. Two weeks of antibody delivery was based on previous research from our group showing that hybridoma cells survive and continue to secrete antibody for approximately 2 weeks [47].

For both groups, immediately following the medial agranular cortex lesion, a midscapular incision was made approx 25 mm in length, and a hemostat was used to make a short, subcutaneous tunnel from the scalp incision to the midscapular region under the dorsal fascia of the neck. Opening and closing the hemostat (blunt dissection) created a large subcutaneous pocket to house the osmotic pump and to create an opening connecting the scalp and midscapular incisions. A hole was drilled at the desired a–p (–2.28), m–l (+3.20), and d–v (–4.00 from the dura) coordinates using bregma as a landmark and designed to place the cannula into the cerebral ventricle on the same side as the AGm lesion. The tubing from the cannula was drawn back through the mid scapular incision, the minipump filled, and the connection was made between the cannula and the pump. Once the connection between the cannula and pump was found to be functional, the cannula was placed through the hole in the skull and fixed in place with a drop of superglue. This was followed quickly by using dental acrylic to surround the cannula and two small stainless steel screws placed around the skull window to anchor the cannula in place. Once the cement hardened, both the scalp and midscapular incisions were closed using individual stitches and sterile suture. All of the above procedures were carried out during the same surgical session.

Postoperatively the animal was placed in a clean, warm environment and continuously monitored until ambulatory, typically 1–2 h, to ensure that postoperative pain was minimized.

After a period of 21 days the rats were anesthetized with ketamine/xylazine and placed in a stereotaxic as previously described. During this second surgery, the minipump was removed. The subject then received the remainder of the behavioral testing beginning 48 h after minipump removal. All of the subjects were capable of being tested behaviorally the following day with no evidence of pain or discomfort.

1.1.4. 7B12 and 11C7 antibodies

Previous studies have shown through enzyme-linked immunosorbent assay that antirat monoclonal antibody 7B12 binds to rat NiG (the protein domain specific to Nogo-A) and rat NiG delta-6 fragment [39]. NiG and delta-6 were coated on enzyme-linked immunosorbent assay plates at a concentration of 2 μ g/ml and incubated with 7B12 monoclonal antibody. The binding of 7B12 to the two proteins was quantified by using a goat antimouse horseradish peroxidase-coupled antibody and peroxidase substrate (Roche Diagnostics, Rotkreuz, Switzerland) and reading the optical density of the color reaction in an enzyme-linked immunosorbent assay plate reader at 450 nm (Spectracount; Packard, Mississauga, Ontario, Canada).

1.1.5. Behavioral testing

1.1.5.1. Circling. As in our prior studies, circling behavior was assessed because a tendency to circle consistently either ipsi- or contralesionally might confound the interpretation of the behavioral measures. While our previous studies indicate that significant circling is not observed using the proposed procedures [51–53] it was essential that circling be assessed in the subjects injected with hybridoma cells because of the potential for nonspecific behavioral effects produced by the tumor cells or cannula implantation.

The subject was placed in its home cage on the testing platform and the number of ipsi- and contralesional turns were counted to the nearest 1/2 turn for a 2 min period prior to behavioral testing for neglect [51–53]. All testing was conducted during the light phase of the light/dark cycle in a room with standard overhead fluorescent lighting, with the experimenter blind to the experimental treatment. Orientation testing was a modified version of that developed by Crowne et al. [13] and was designed to reflect simple bedside testing for neglect in humans. These procedures have been used in a number of previous studies (e.g., [51–53]).

1.1.5.2. Orientation testing. After the assessment of circling behavior, the animal was taken out of its cage, and placed directly on the test platform marked to delineate 0°, 30°, 45°, and 60° angles in either direction from a central line running the length of the testing board. The subject was gently restrained by

hand from behind without restricting head movement and aligned with the center line. Stimuli were presented only when there was no evidence of struggling, no asymmetry of body posture, and when the head was oriented in direct line with the body. Typically, the animal's body had to be realigned several times during testing. The early extensive handling minimizes struggling and “freezing”. Visual, tactile, and auditory stimuli were presented in turn. The visual stimulus consisted of the presentation of a silver metallic rod 10.0 cm in length (8 mm in width), which was waved in a small circle (approximately 5.0 cm in diameter) five times within the animal's visual field at a distance of 7.5–10.0 cm from the animal. Care was taken not to contact the vibrissa with the metallic rod. The auditory stimulus was a single 114-dB (SPL) click generated by a clicking device held at midbody approximately 5 cm from the subject. The tactile stimulus was a single caudal-to-rostral stroke through the vibrissa with a 15 cm Puritan applicator (Harkwood Products Co., No. 807). Although such stimulation has a visual component, testing under red light conditions yielded identical results.

Three cycles of testing comprised one test session. One cycle consists of a single presentation of each of the three stimuli to each body side in turn. Stimuli are presented in the order: visual, tactile, and then auditory. We have found that order of presentation does not influence performance [54]. The experimenter rated the degree of head turning toward or away from (allegesia/allokinesia) the stimuli as measured by the position of the tip of the snout over the test platform markings. Head turns of less than 30° received a zero score, between 30° and 45° a 1.0, between 45° and 60° a 1.5, and greater than 60° a 2.0. Orientations later than 2 s after stimulus presentation received a zero score. Orientations to the visual stimulus after the third revolution (3 s) received a maximum score of 1.5. The maximum score for each body side is 6.0 for each of the three modalities, 18.0 in total. In prior studies this rating scale produced an interrater reliability of 1.0 for the direction of orientation, and above 0.9 for the magnitude of orientation [11]. Subjects were tested 2 times per week for 10 weeks. A total neglect ratio was derived from the formula (contralateral total)/(ipsilateral total) [5,11]. Neglect ratios were also calculated to compare the non-neglected to the neglected body side for the visual, tactile, and auditory modalities. Because neglect ratios give no indication of whether an asymmetry in orientation results from lower contralesional (non-neglect side), or higher ipsilesional (neglect side) scores, separate analyses of raw scores for ipsi and contralesional responding were conducted. Significant increases in responding on the neglected side have always accompanied recovery [51–53].

Responses to the inappropriate side, away from the side of stimulation (allegestic responses), are rated identically. Allegestic responses were analyzed separately from responses which occur to the side appropriate to stimulus presentation. All behavioral testing occurred between 07:00 and 19:00 of the light portion of the 12-day/12-night cycle.

1.1.5.3. Behavioral recovery of function. Recovery on the orientation task was considered to have occurred if a subject attained a total neglect ratio ≥ 0.60 for two consecutive tests. If a subject did not reach the criterion for recovery they were tested for a maximum of 10 weeks [11,57].

1.1.5.4. Histological procedures. After behavioral testing was completed the subjects were given an overdose of sodium pentobarbital (65 mg) and when totally unresponsive (absence of a corneal reflex, unresponsiveness to tail pinch, and cessation of respiration), intracardially perfused with normal saline, followed by 4% paraformaldehyde. The brain was removed from the skull, placed in 4% paraformaldehyde for 1–3 h, then into 30% sucrose-paraformaldehyde until the brain sank. The brain was frozen and sectioned in the coronal plane at 40 μ m.

For the AGm operates every sixth section through the extent of the lesion and through the thalamus was saved, mounted, and stained with cresyl violet. All lesions were examined to determine the extent of damage to adjacent areas and the AGm. The thalamus was examined for any signs of calcification, gliosis, or shrinkage of nuclei.

Lesion size was measured by tracing the extent of the lesion and areas of gliosis through an image analysis program (Optimus, BioScan). Lesion extents were traced onto standard brain diagrams [42]. To ensure a blind analysis, all brains were identified by an arbitrary number, and not associated with a particular group. Further, all histological processing and analyses were performed prior to the behavioral analyses.

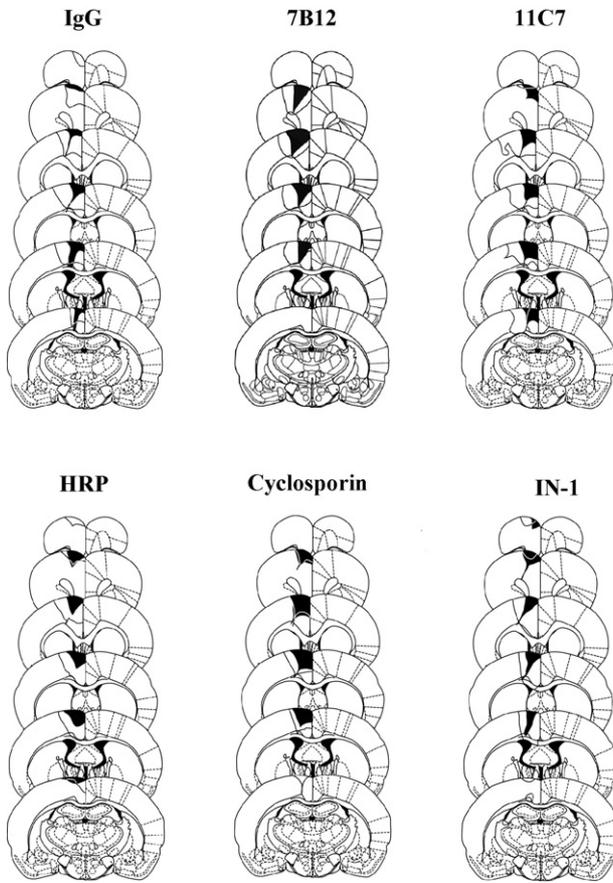


Fig. 1. Maximum and minimum (black) lesion extents of individual subjects in each group. There were no differences in lesion sizes among the six groups.

1.2. Results

1.2.1. Histology

Extensive damage to the AGm was seen in all subjects. As found in prior studies, the typical lesion tended to encroach medially on cingulate cortex with no damage to the contralesional hemisphere. There was minor, variable amount of damage laterally to lateral agranular cortex and hindlimb cortex [3,5,11,14,28,54,57]. A few of the lesions extended ventrally through the white matter across several groups, but this was confined to the caudal portion of the lesion and there was some evidence of white matter damage across all groups. Enlargement of the ipsilesional lateral ventricle or both ventricles occurred across all groups, and was not systematically related to the behavioral findings.

Fig. 1 illustrates the maximum and minimum lesion sizes of individual subjects in each group. A simple one-way between-groups analysis of variance (ANOVA) indicated that there were no differences in lesion size among the groups ($F(5, 45) = 0.51, p = 0.770$).

1.2.2. Behavioral analyses

1.2.2.1. Circling. Circling behavior was analyzed using non-parametric statistics because of the high number of zero scores. Within subjects Wilcoxon Signed-Ranks tests revealed no significant differences between ipsi- and contralesional circling

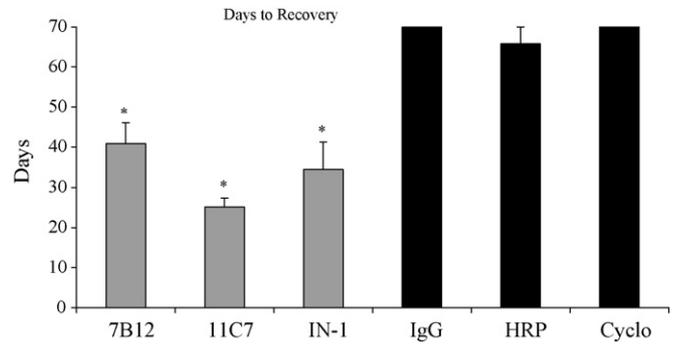


Fig. 2. Mean number of days to recovery from neglect in the 7B12, 11C7, IN-1 experimental groups and the IgG, HRP and Cyclosporin control groups. *Significant difference between-groups in the number of days it takes to reach recovery ($p < 0.01$). Recovery was defined as a Mean Total Neglect Ratio of 0.6 or higher for two consecutive test days. Error bars represent standard error of the mean.

within the IN-1, 7B12 and IgG groups (all p 's > 0.05). Further, Kruskal–Wallis tests revealed no significant differences in ipsi- and contralesional circling between-groups (all p 's > 0.05). Therefore, any behavioral differences among the groups cannot be explained by a consistent circling bias.

1.2.2.2. Recovery. To determine whether treatment with IN-1, 7B12, or 11C7 resulted in behavioral recovery from hemispatial neglect, the groups were compared for differences in the number of days to reach behavioral recovery using a simple one-way ANOVA. As illustrated in Fig. 2, the analysis revealed that there was a significant difference among the groups ($F(5, 42) = 29.30, p = 0.001$). Post hoc analysis showed that the IN-1, 7B12, and 11C7 groups recovered significantly faster than the HRP, cyclosporine A, and IgG control groups (all p 's = 0.01). There were no significant differences among experimental groups (all p 's > 0.05) or among control groups (all p 's > 0.05). Only one of the control subjects, in the HRP control group, recovered within the 10-week (70 day) testing period (Fig. 3). In contrast, only one experimental subject, in the 7B12 group, did not demonstrate recovery within 70 days.

1.2.2.3. Total neglect ratios. A 6×2 group ($6 \times$ weeks (2)) mixed ANOVA was done to compare the total neglect ratios among the three treatment groups (IN-1, 7B12, 11C7) and the

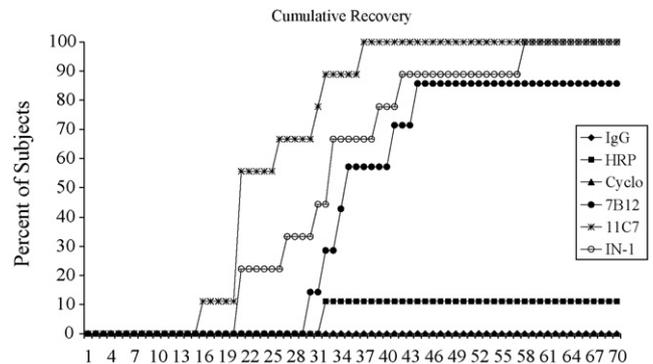


Fig. 3. Cumulative percentage of subjects demonstrating recovery over the 10 week (70 day) testing period. Subjects in the 7B12, 11C7, and IN-1 groups recovered faster than subjects in the control groups.

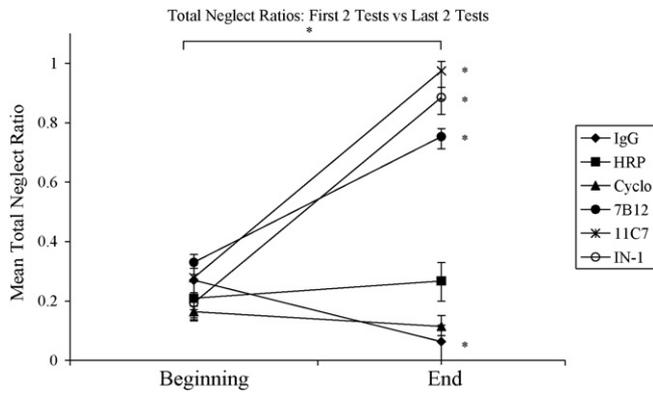


Fig. 4. Comparison of total neglect ratios among the three treatment groups (IN-1, 7B12, 11C7) and the control groups (HRP, Cyclosporine A, IgG) for the first two neglect tests and last two neglect tests. Results indicated no differences among groups for the first two tests ($p > 0.05$), but a significant difference between all treatment groups (IN-1, 7B12, 11C7) and all control groups (HRP, Cyclosporine A, IgG) on the last two tests ($p = 0.000$). There was a significant decrease in severity of neglect in the three experimental groups (all p 's < 0.01) and a significant increase in severity of neglect in the IgG group ($p < 0.01$).

control groups (HRP, cyclosporine A, IgG) for the first two neglect tests (week 1) and the last two neglect tests (final week of testing). The results indicated significant main effects for group ($F(5, 41) = 28.57, p < 0.001$), test ($F(1, 41) = 53.69, p < 0.001$), and a significant group \times week interaction ($F(5, 41) = 20.19, p < 0.001$).

In order to further examine the interaction, individual ANOVAs were done to compare the total neglect ratios among the groups for the first and last two neglect tests (Fig. 4). The results of the analyses indicated that the groups did not differ for the first week ($F(5, 41) = 1.75, p > 0.14$), however, there was a significant difference among the groups for the last week ($F(5, 41) = 32.51, p < 0.001$). A Tukey's post hoc analysis revealed that the IN-1, 7B12, and 11C7 groups differed significantly from the cyclosporine A, IgG, and HRP groups (all p 's < 0.001). None of the anti-Nogo-A antibody groups differed from one another.

The within-groups comparisons were done using t -tests for dependent means to compare the total neglect ratios for the first week and last week of neglect testing within each of the groups. The results indicated that there was a significant decrease in the severity of neglect in the IN-1, 7B12, and 11C7 groups (all p 's < 0.01). The only difference within the control groups was found in the IgG group which demonstrated a significant increase in the severity of neglect across testing ($p < 0.01$).

1.2.2.4. Raw scores. To ensure that recovery was the result of an increase in responding on the contralesional (neglected) side and not purely a decrease in responding on the ipsilesional side, separate group \times test mixed ANOVAs were done to compare the ipsilesional and contralesional raw scores for the first two behavioral tests and the last two tests. Responding in the ipsilesional direction showed a significant main effect of test indicating that overall responding decreased between the beginning and the end of testing ($F(1, 41) = 127.63, p = 0.001$, Fig. 5A). This finding has been reported before in results from our laboratory [51]. Ipsilesional scores tend to decrease during prolonged test-

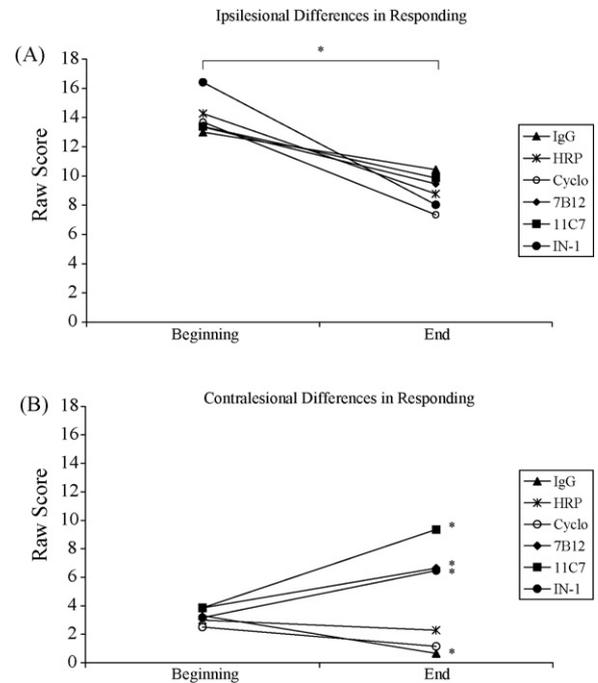


Fig. 5. (A) Responding in the ipsilesional direction decreased at the end of testing compared to the beginning ($p = 0.00$). (B) Responding in the contralesional direction showed no difference between-groups at the beginning of testing ($p = 0.66$), but a significant increase in responding for the 7B12, 11C7 and IN-1 groups at the end of testing ($p = 0.00$). The IgG group showed a significant difference in responding at the end of testing in the opposite direction.

ing across all groups. Responding on the contralesional side (neglected side), however, showed a significant group \times test interaction ($F(5, 41) = 11.36, p = 0.001$). As expected, there were no differences between-groups at the beginning of testing ($F(5, 46) = 0.65, p = 0.662$), but the 7B12, 11C7 and IN-1 groups showed a dramatic significant increase in responsiveness on the contralesional side at the end of testing ($F(5, 46) = 23.80, p = 0.000$, Fig. 5B). The IgG group also showed a significant difference in contralesional responding at the end of testing, however responding in this group was in the opposite direction and showed a decrease in responding on the contralesional (neglected) side. Thus, the raw score data corroborate those for the total neglect ratios and indicate that anti-Nogo-A antibodies increase responsiveness on the neglected side.

1.2.2.5. Modalities. To further explore the effects of anti-Nogo-A therapy on recovery of function from neglect statistical analysis were done on the three modalities. Individual group \times week repeated measures ANOVA were run for the visual, tactile and auditory modalities. In the visual, tactile and auditory modalities there were no significant group \times week interactions and no main effect of weeks or group for the IN-1, 7B12 or 11C7 treatments (all p 's > 0.01).

A comparison of responding at the beginning and end of testing was also performed on the visual, tactile and auditory modalities to see if overall responding increased over time. These results showed significant group \times test interactions for the visual ($F(5, 41) = 6.00, p = 0.001$, Fig. 6A) and tactile modalities ($F(5, 41) = 4.03, p = 0.005$, Fig. 6B). There was no group \times test

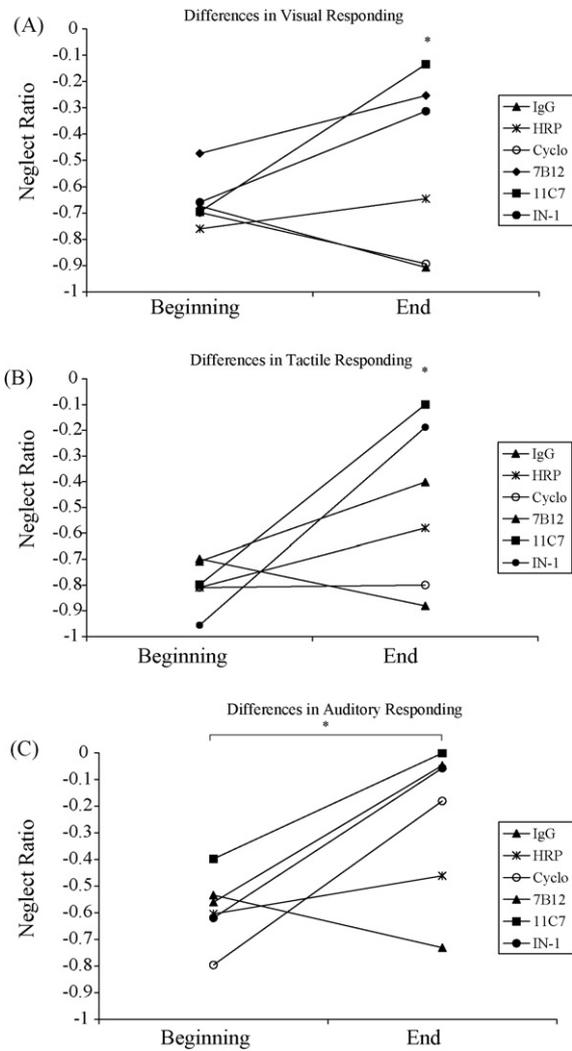


Fig. 6. (A) In the visual modality all experimental groups differed significantly from control groups (all p 's < 0.05), except the IN-1 group, which did not differ from the HRP group ($p = 0.177$), but differed from the IgG and cyclosporin control groups (p 's < 0.05). (B) The only difference observed in the tactile modality was between the 11C7 and IgG groups ($p = 0.025$). (C) In the auditory modality there was a main effect of test ($p = 0.00$) showing a general increase in responding at the end of testing.

interaction in the auditory modality ($F(5, 41) = 2.10, p = 0.085$), but there was a main effect of test ($F(1, 41) = 14.61, p = 0.001$, Fig. 6C) showing a general increase in responding at the end of testing. In the visual modality all experimental groups differed significantly from control groups (all p 's < 0.05), except the IN-1 group, which did not differ from the HRP group ($p = 0.177$), but differed from the IgG and Cyclosporin control groups (p 's < 0.05). The only difference observed in the tactile modality was between the 11C7 and IgG groups ($p = 0.025$).

1.2.2.6. *Allesthesia/allokinesia*. There were no significant changes in allesthesia/allokinesia.

1.3. Discussion

The results of experiment 1 indicate that anti-Nogo-A treatment can produce recovery of function from severe neglect

induced by unilateral AGm lesions. The IN-1 group, and the specific anti-Nogo-A antibodies 7B12 and 11C7 groups, demonstrated recovery of function compared to the control groups when analyzing both total neglect and the individual modalities. Recovery in the treated groups typically began at approximately 4 weeks post-surgery. Recovery was due mainly to increased responsiveness on the neglected side. Perhaps most impressive is that only one of the anti-Nogo-A treated rats, in the 7B12 group, failed to reach the criterion for behavioral recovery.

2. Experiment 2

Previous research has indicated that recovery from lesion-induced deficits may be the result of plasticity originating from the contralesional hemisphere [2,25,40,59,60]. In experiment 2 we examined whether projections from the contralesional hemisphere underlie behavioral recovery by transecting a component of the corpus callosum in animals that had previously recovered from neglect.

2.1. Materials and methods

2.1.1. Subjects

Twelve subjects that demonstrated behavioral recovery from experiment 1 were used in experiment 2.

2.1.2. Behavioral testing

Testing procedures were identical to those used in experiment 1.

2.1.3. Knife-cut surgery

Immediately after demonstrating behavioral recovery following IN-1 treatment, rats were anesthetized using ketamine/xylazine i.p. (1.5 ml of 100 mg/ml xylazine and 10 ml of 100 mg/ml Ketamine, at 0.1 ml/100 g). Upon exhibiting no eyeblink response or response to ear bar placement, the rats were fixed in a stereotaxic apparatus using blunt-tipped ear bars. A scalp incision was made and the dura carefully reflected. Care was taken not to produce any additional damage to the original AGm lesion, and any remaining gel-foam was left undisturbed. The subjects then received either a medial knife-cut (KC) designed to sever the corpus callosum medial to the DCS, or a lateral callosal KC just lateral to the DCS on the ipsilesional side. Corticostriatal axons from contralesional AGm travel in the dorsal half of the corpus callosum and enter the ipsilesional striatum about 1.5 mm lateral to the midline. Therefore, a sagittally oriented KC made at the appropriate depth 0.5 mm lateral to the midline and extending from +1.8 mm to -1.5 mm AP on the ipsilesional side will sever these axons (and other corticocortical axons). Our prior experience suggested that the depth of the blade needed to be varied from 3.7 to 2.7 mm as the cut is made in the rostrocaudal direction. Control lesions were made on the ipsilesional side 2.5 mm lateral to the midline, using the same AP extent and appropriate depth (2.5–2.3 mm rostral to caudal) to lesion the white matter. Knife-cuts were made as we have done in a previous study [4].

A microknife fabricated from a no. 11 surgical blade [4] was lowered at 0.5 mm lateral to the center of the sagittal sinus. The blade was lowered 0.4 mm ventrally into the substance of the callosum at +1.8 mm AP, then moved caudally to -1.5 mm AP, gradually raising it to a depth of 0.2 mm to compensate for the decreasing thickness of the corpus callosum caudally. The lateral KC should not disrupt fibers traveling through the corpus callosum from the intact contralesional AGm.

2.1.4. Post-knife-cut behavioral testing

Beginning 48 h post-surgery all subjects received standard neglect testing. Testing was continued until meeting the criterion for recovery in experiment 1, which was two consecutive tests of ≥ 0.60 , or up to a maximum of 4 tests over the course of 2 weeks.

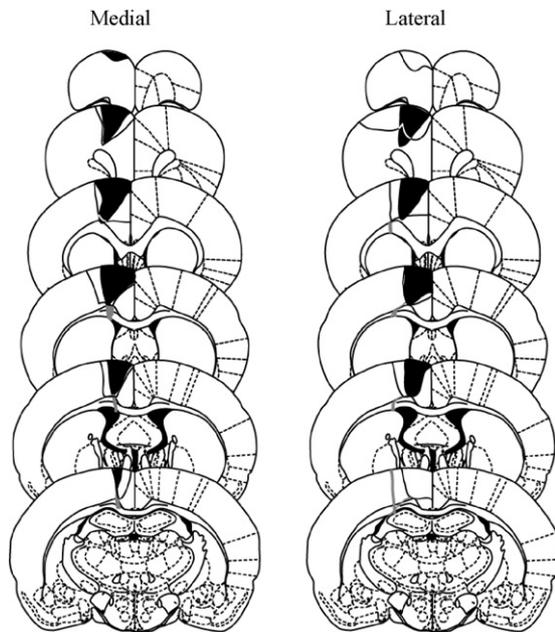


Fig. 7. Maximum and minimum lesion extents of individual subjects in each group. There were no differences in lesion sizes between the two knife-cut groups and the cuts were in the intended target area.

2.1.5. Histological procedures

The histological procedures were identical to those used in experiment 1.

2.2. Results

2.2.1. Histology

As illustrated in Fig. 7 there were no differences in lesion size between the two KC groups, and the cuts were in the intended target area specified in the methods. The medial knife-cuts severed the corpus callosum medial to the cingulum bundle. The lateral knife-cuts were all lateral to the cingulum. The KCs in both groups typically extended from +1.6 to 1.8 mm and –1.5 to 1.7 mm relative to bregma.

2.2.2. Behavioral analysis

2.2.2.1. Circling. Circling behavior was analyzed as in the previous experiment and revealed no significant differences between ipsi- and contralesional circling within the medial and lateral knife-cut groups (all p 's > 0.05). Likewise, there were no differences in circling found between-groups (all p 's > 0.05). Therefore, any behavioral differences among the groups cannot be explained by a consistent circling bias.

2.2.2.2. Neglect testing. The rats that were chosen from experiment 1 all reached recovery within 39 days. Following recovery animals were quasi-randomly assigned to receive either a medial or lateral knife-cut of the corpus callosum. Analysis of the original group assignment revealed that prior to the KC procedures there was no significant difference in the number of days to reach recovery between the medial and lateral knife-cut groups ($p > 0.05$).

A mixed design group \times test ANOVA was done to compare the total neglect ratios for the two tests during the last week

Table 1
Mean total neglect ratios and contralesional raw scores

Group	Neglect ratios		Raw scores	
	Pre-KC	Post-KC	Pre-KC	Post-KC
Lateral	1.13	1.17	8.39	8.07
Medial	0.93	0.06 ^{*,‡}	6.35	0.90 ^{*,‡}

* Significant between pre- and post-KC groups at $p < 0.01$.

‡ Significant between medial and lateral post-KC groups at $p < 0.01$.

of pre-knife-cut testing to the week of testing immediately following the KCs for both the medial and lateral KC groups. The analysis revealed a significant group \times test interaction ($F(1, 10) = 37.17, p < 0.001$), and significant main effects for group ($F(1, 10) = 107.26, p < 0.01$) and test ($F(1, 10) = 30.55, p < 0.001$).

To further examine the interaction, a series of t -tests were done to compare the groups just prior to, and after the KCs. As reported in Table 1, just prior to the knife-cuts the two groups did not differ in the severity of neglect, however after the KCs the medial KC group was significantly impaired relative to the lateral group ($t = 16.15, p < 0.001$). In fact, all of the subjects in the lateral group demonstrated recovery within two tests, the minimum number of tests to demonstrate recovery. In contrast, following the medial cuts severe neglect was reinstated and lasted for the duration of behavioral testing (2 weeks).

Individual within-subjects t -tests were done to examine within-group changes resulting from the KCs. The level of total neglect did not change as a result of the lateral KC. However, as reported in Table 1, the medial KC group demonstrated a significant and dramatic increase in the severity of neglect ($t = 7.80, p < 0.001$).

Changes in the neglect ratio may reflect changes in responsiveness on the ipsilesional, contralesional or both sides so a group \times weeks mixed ANOVA was done to compare the total orientation raw scores on the neglected side between the KC groups. It was expected that there would be a significant decrease in orientation scores on the neglected side in the medial KC group. The analysis revealed a significant group \times week interaction ($F(1, 10) = 21.08, p < 0.001$), and significant main effect for group ($F(1, 10) = 32.56, p < 0.001$) and week ($F(1, 10) = 26.70, p < 0.001$). Individual analyses were done to further examine the interaction. As found in experiment 1, the two groups did not differ in responsiveness on the neglected side prior to the KCs, but following the KCs there was a significant decrease in responsiveness on the neglected side in the medial group relative to the lateral group (Table 1; $t = 7.41, p < 0.001$). Within-group comparisons revealed that there was no change in the orientation scores of the lateral group, however the medial group demonstrated a significant decline in orientation scores as a result of the KC ($t = 5.31, p < 0.006$).

2.2.2.3. Individual modalities. A series of group \times test ANOVAs were run on the neglect ratios for each of the modalities to compare the last 2 days of pre-KC testing to the first 2 days of post-KC testing. The KCs produced significant changes within each of the modalities.

Table 2
Mean modality neglect ratios

Group	Pre-KC	Post-KC
Visual		
Lateral	−0.30	−0.37
Medial	−0.15	−0.90 ^{*,‡}
Tactile		
Lateral	0.22	0.36
Medial	0.06	−0.87 ^{*,‡}
Auditory		
Lateral	0.36	0.17
Medial	−0.04	−0.89 ^{*,‡}

* Significant between pre- and post-KC groups at $p < 0.05$.

‡ Significant between medial and lateral post-KC groups at $p < 0.05$.

In the visual modality there was a significant main effect for test ($F(1, 10) = 13.41, p < 0.005$) and a significant group \times test interaction ($F(1, 10) = 9.396, p < 0.02$). In order to examine the interaction, separate t -tests were done to compare pre-KC visual responsiveness and post-KC visual responsiveness between the groups. The results indicated that there was no difference in responsiveness between the recovered groups prior to the KC, however the medial KCs resulted in significant visual neglect (Table 2; $t = 2.36, p < 0.05$). Within-groups comparisons revealed that there was a significant pre-KC vs. post-KC difference in the medial group ($t = 4.47, p < 0.02$).

The group \times test ANOVA for tactile responsiveness revealed significant main effects for group ($F(1, 10) = 31.48, p < 0.001$), test ($F(1, 10) = 5.71, p < 0.04$), and a group \times test interaction ($F(1, 10) = 10.38, p < 0.01$). Separate comparisons of the pre- and post-KC comparisons between the groups revealed that there was no pre-KC difference between the groups, but there was a significant post-KC difference with the medial group demonstrating tactile neglect (Table 2; $t = 6.29, p < 0.001$). Within-groups comparisons revealed that only the medial group showed a significant return of tactile neglect ($t = 3.24, p < 0.05$).

The group \times test ANOVA for the auditory modality found significant main effects for group ($F(1, 10) = 44.27, p < 0.001$), test ($F(1, 10) = 13.20, p < 0.01$), and a significant group \times test interaction ($F(1, 10) = 5.43, p < 0.05$). To further explore the interaction independent t -tests were done to compare pre- and post-KC responsiveness between the groups. There was no pre-KC difference between the groups, but post-KC the medial group demonstrated significant auditory neglect relative to the lateral group (Table 2; $t = 7.49, p < 0.001$). Within-group t -tests revealed that there was no change in auditory responsiveness in the lateral group, but a significant decline in auditory responsiveness produced by the medial KC ($t = 4.03, p < 0.02$).

The results of the modality data corroborate the findings of the total neglect ratios and raw score changes. The medial KCs produced significant neglect across all modalities, whereas the lateral KCs did not.

2.2.2.4. *Allesthesia/allokinesia*. There were no significant changes in allesthesia/allokinesia as a result of the KCs.

2.3. Discussion

The results of experiment 2 suggest that axons crossing from the intact hemisphere are responsible for recovery from neglect. The medial knife-cut group showed a reinstatement of the neglect deficit following transection of the corpus callosum. Recovery was unaffected in the lateral knife-cut group. These results are consistent with previous findings which indicate that recovery from sensorimotor deficits may be the result of plasticity originating from the contralesional cortex [2,25,40,59,60].

While these results strongly support the role of contralesional projections for behavioral recovery, they do not answer the question of whether contralesional projections are necessary for recovery. McNeill and colleagues have suggested that plasticity in corticostriatal projections follow a hierarchy such that when one avenue of plasticity is blocked (e.g., via fiber transection) the next level in the hierarchy (converging projections containing the same neurotransmitter) may demonstrate plasticity. Following cortical lesions there is clear evidence for plasticity in the inputs to the denervated striatum [10]. In subsequent studies [35–37], it was discovered that following cortical lesions, projections from the homotopic cortex are most likely to sprout in the denervated striatum, followed by glutamatergic thalamic inputs, and last by dopaminergic inputs. Three subjects from experiment 1, one each from the 11C7, IN-1, and 7B12 groups demonstrated complete transection of the white matter at the time of the initial aspiration lesion. In spite of the transection induced by aspiration, all three rats demonstrated behavioral recovery from neglect. Thus, while contralesional inputs are sufficient, they may not be necessary for behavioral recovery from neglect. Ipsilesional projections from the posterior parietal cortex also converge on many of the same regions as the AGm, including the DCS. It may be that plasticity in these projections occurs when contralesional inputs are blocked via transection. Finally, subjects in the medial KC group were tested for 2 weeks post-surgery. It is possible that with longer survivals the subjects may have recovered spontaneously, however, based on our findings, which indicate a virtual absence of recovery in the untreated controls, this would appear unlikely.

3. General discussion

The results of these studies indicate that anti-Nogo-A treatment can induce recovery from neglect and suggest that fibers crossing from the intact contralesional side are responsible for recovery. In experiment 1 anti-Nogo-A administration resulted in fewer days to reach recovery from neglect in the IN-1, 7B12, and 11C7 groups compared with their corresponding control groups (HRP; Cyclo; IgG). All of the subjects in the experimental groups recovered during the testing period except for one in the 7B12 group. Conversely, only one of the control subjects from the HRP group demonstrated recovery within the 70 days of testing. Consistent with other studies, the behavioral differences among and between the groups cannot be explained by differences in lesion size or as a result of a bias in circling behavior [51–53]. Although this study did not stain for the presence of antibodies, previous studies from our group have found

high levels of antibodies in the tumors produced by IN-1, the ventricles, and the brain parenchyma [40,45].

The present findings support those of previous studies which have shown that behavioral recovery can occur following damage to other parts of the brain by neutralizing Nogo-A with IN-1. Papadopoulos et al. [40] have shown that application of IN-1 following a stroke affecting forelimb motor cortex results in functional recovery accompanied by new corticorubral projections from the opposite, unlesioned hemisphere. These projections crossed the midline to innervate the deafferented red nucleus. Similar results of plasticity have been shown in the spinal cord of the adult rat following a unilateral lesion of the cerebral spinal tract [49]. Treatment with IN-1 showed topographically appropriate sprouting in both intact and lesioned tracts as well as full recovery in both motor and sensory tests. Corticostriatal plasticity has also been reported in subjects treated with IN-1 following unilateral lesions of the sensorimotor cortex [25]. This effect has been shown to work on aged [31,33] and hypertensive animals as well [59]. The results of the present studies support and extend the use of anti-Nogo-A antibodies to include recovery from severe neglect following unilateral AGm aspiration lesions. The therapeutic effectiveness of 11C7, which acts at a domain common to rats as well as human and non-human primates, is particularly significant and further points to the relevance of the rodent model for the potential treatment of neglect in humans.

Bregman et al. [1] has shown that recovery can be eliminated in animals that have recovered locomotor function due to treatment with IN-1. Spinal cord lesioned animals that had previously recovered locomotor function with IN-1 treatment showed a reinstatement of locomotor deficits following a lesion of the contralateral sensorimotor cortex. Recovered animals showed regeneration and plasticity of corticospinal pathways, which suggests that the recovery is dependent on regrowth of damaged pathways. The results of experiment 2 provide further support for the role of plasticity originating from the contralesional hemisphere. Comparing the effects of the medial and lateral cuts in experiment 2 indicates that projections from contralesional inputs to areas medial to the cingulum bundle may underlie recovery. The reinstatement of neglect following medial cuts suggests that plasticity in these fibers may be responsible for functional recovery. Based on a series of behavioral [50,51,53] and anatomical studies [56] which support the role of the DCS in recovery, the most likely candidate would appear to be those from the contralesional hemisphere to the ipsilesional DCS. However, as indicated from the results of three subjects receiving anti-Nogo-A antibody treatment, if those connections are severed concurrent with the initial AGm surgery, recovery can still occur. These results implicate the potential role of intrahemispheric connections in recovery or compensation if interhemispheric connections are disrupted. While the specific mechanisms which would underlie recovery are unknown, previous studies have supported the role of the perilesion cortex [7].

These findings suggest that anti-Nogo-A antibodies may be effective in a wide variety of contexts including lesions involving white matter disruption. In addition to being effective in a variety

of brain areas, Nogo-A inhibition has also been shown to work following a delay in administration. Wiessner et al. [59] have shown that 7B12 infused 24 h after stroke improved behavioral recovery in rats. Similarly, Seymour et al. [47] reported recovery of skilled forelimb function following a 1-week delay in administration of IN-1 after focal cerebral ischemia to the forelimb sensorimotor cortex. We have preliminary data which indicates that Nogo-A inhibitors are effective in producing recovery from severe AGm-induced neglect when administered 2 days post-surgery. These studies help demonstrate the efficacy of Nogo-A neutralization even if administration is delayed 1 week after stroke.

There are numerous clinical implications for the use of anti-Nogo-A therapies for the treatment of CNS damage. It is known that IN-1 and 7B12 treatment result in recovery of function following damage to various parts of the brain in rats, and that this recovery is associated with plasticity. While evidence suggests that recovery may be a result of plasticity originating from the contralesional cortex [2,24,40,47,60], it was not established, until recently, whether plasticity also occurs in areas of the brain not associated with the impaired function. Recent evidence indicates that transient increases in dendritic arbors can occur in normal animals treated with IN-1 at 2 weeks post-treatment, but there is a return to normal by 6 weeks [41].

Another approach to inhibiting Nogo-A is through the Nogo-66 receptor, NgR, which is not specific to Nogo-A. Myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) are inhibitory molecules that also recognize and bind NgR [32,58]. Even though NgR binds three types of inhibitory molecules, studies have indicated that Nogo knockout mice show axonal regeneration following spinal cord lesions [27,48]. This suggests that Nogo-A may be primarily responsible for neural inhibition. However, axonal regeneration in knockout mice is somewhat controversial, as not all studies have been able to duplicate this effect [61]. Regardless, the binding ability of NgR makes it a prime target for blocking inhibition. Enzymatic cleavage of NgR results in neurons that are no longer responsive to Nogo-66 inhibition [23]. Likewise, forced expression of NgR results in sensitivity to Nogo-66 inhibition in normally unresponsive neurons. Receptor antagonists, such as the peptide NEP1-40, have been used to block Nogo-66 and prevent inhibition of axonal outgrowth, which results in significant axon elongation [21]. Additionally, Li and Strittmatter [30] have shown that subcutaneous or intraperitoneal injections of NEP1-40 in mice result in extensive axonal sprouting and enhanced recovery of locomotor activity following thoracic spinal cord injury. It is likely that NEP1-40 enters the spinal cord through a focally disrupted blood–brain barrier (BBB) at the site of injury. This treatment has also shown to be effective following a delay of at least 1 week. This is the first study to report a systemic therapy for axonal regeneration following CNS injury.

Following brain damage, both motor and cognitive deficits may result. Thus, far, most of the research on the effects of Nogo-A inhibition has concentrated on motor deficits. Neglect is a severe and prevalent cognitive disorder and treatment for patients with neglect following stroke has been met with limited success. The presence of neglect and the associated lack

of awareness and affective changes also complicate recovery from associated disorders, such as hemiplegia [16,20]. Given that there are currently no generally accepted therapies for the treatment of neglect, it is essential to examine any promising therapeutic intervention. The present findings suggest that anti-Nogo treatment can induce dramatic recovery from severe neglect and that this recovery may be due to plasticity from the contralesional hemisphere. A significant finding in the present studies is that the specific anti-Nogo-A antibody 11C7, which acts on a domain common to rats, as well as human and non-human primates, is effective in the treatment of neglect in the rodent [53].

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