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Extended Treatment Window Exists for Improving Sensorimotor Recovery Using Anti-Nogo a Immunotherapy in Adult Rats with Chronic Stroke Deficits

Katherine Marie Podraza
Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

EXTENDED TREATMENT WINDOW EXISTS FOR IMPROVING SENSORIMOTOR RECOVERY USING ANTI-NOGO-A IMMUNOTHERAPY IN ADULT RATS WITH CHRONIC STROKE DEFICITS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDANCY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

KATHERINE MARIE PODRAZA

CHICAGO, IL

MAY 2015
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Dla moich rodziców i dla mojego kochanego Nitin
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
</tr>
<tr>
<td>CFA</td>
<td>Caudal Forelimb Motor Cortex</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>EEG</td>
<td>Electroencephalography</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<td>GAP-43</td>
<td>Growth-associated-protein-43</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidyl Inositol</td>
</tr>
<tr>
<td>ITT</td>
<td>Intention to treat</td>
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<td>kD</td>
<td>KiloDalton</td>
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<td>LIMK1</td>
<td>Lin-11, Isl-1, Mec-3 Kinase I</td>
</tr>
<tr>
<td>LTD</td>
<td>Long Term Depresssion</td>
</tr>
<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-Associated Glycoprotein</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>NEP1-40</td>
<td>Nogo-66 Antagonist Peptide</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>OMgp</td>
<td>Oligodendrocyte and Myelin Glycoprotein</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<tr>
<td>PP</td>
<td>Per-Protocol</td>
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<td>RFA</td>
<td>Rostral Forelimb Area</td>
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ABSTRACT

Stroke related death ranks as the fourth most common cause of mortality in the United States. However, even with those who survive the initial ischemic insult, profound neurological disability may remain. Current therapeutic options following stroke are limited and therefore the development of a treatment that can improve functional outcome after the acute phase of the stroke has passed would have a great impact on the level of disability and quality of life of stroke sufferers. One potential therapy involves the use of anti-Nogo-A immunotherapy post-stroke. Our laboratory has shown that the administration of this novel treatment results in sensorimotor recovery in adult and aged rats. This therapy has been shown to be efficacious when applied immediately, 24 hours, and 1 week post-stroke in rats with mild to moderate sensorimotor deficits. In addition, recent results suggest that administration of anti-Nogo-A immunotherapy up to 9 weeks after stroke still induces functional recovery and neuroanatomical plasticity in adult rats with mild to moderate sensorimotor deficits. However, whether and how long anti-Nogo-A immunotherapy is efficacious when administered in the chronic stroke phase in rats with moderate to very severe sensorimotor deficits is unknown. In the present project we sought to investigate the effectiveness of anti-Nogo-A immunotherapy as a therapeutic intervention to improve sensorimotor recovery when applied to chronic stroke-impaired adult rats with moderate to very severe sensorimotor deficits. Prior to testing the efficacy of anti-Nogo-A immunotherapy we set out to evaluate the spontaneous motor recovery
profiles in adult rats after stroke. We found that animals displayed different recovery profiles based on initial deficit severity after stroke and overall, all spontaneous recovery occurred within the first 4 weeks post-stroke. Therefore, the neurological deficit in the stroke animals had plateaued by the time anti-Nogo-A immunotherapy was given at 9 and 25 weeks post-stroke. We found that animals with moderate to very severe deficits treated with anti-Nogo-A immunotherapy at 9 or 25 weeks post-stroke showed improved sensorimotor recovery at both time points. However, the improved sensorimotor function was not associated with dendritic changes in either the perilesional or contralesional cortex. Our finding of improved sensorimotor recovery even when treatment was started 25 weeks post-stroke demonstrates the promising therapeutic potential for anti-Nogo-A immunotherapy to treat stroke sufferers long after the initial ischemic damage has taken place. Further neuroanatomical correlates of the improved sensorimotor function are being investigated.
CHAPTER ONE
OVERVIEW AND HYPOTHESIS

Stroke is a leading cause of long-term disability worldwide and it is estimated nearly 795,000 people experience a new or recurrent stroke every year (AHA, 2013). The only clinical treatment for acute stroke is thrombolytic therapy and it must be given in the first hours after stroke onset, thereby limiting its usefulness. Therefore, it is important to study possible therapeutic interventions that can help repair the brain after the initial damage has already occurred. One potential therapy involves the use of anti-Nogo-A immunotherapy following stroke. Our laboratory has shown that the administration of this novel treatment results in sensorimotor recovery after stroke in adult and aged rats and is mediated by dendritic and axonal plasticity (Markus et al., 2005; Papadopoulos et al., 2006). Administration of anti-Nogo-A immunotherapy has been shown to be efficacious when applied immediately, 24 hours, and 1 week after ischemic stroke in the rat (Papadopoulos et al., 2002; Wiessner et al., 2003; Seymour et al., 2005; Markus et al., 2005; Tsai et al., 2007). However, given the vast number of people living with chronic stroke deficits, the question of whether anti-Nogo-A immunotherapy can be efficacious when administered long after the ischemic damage has occurred is of extreme importance. Recent data from our laboratory suggests that administration of anti-Nogo-A immunotherapy up to 9 weeks following ischemic stroke in adult rats that have mild to moderate sensorimotor deficits induces functional recovery and neuroanatomical
plasticity (Tsai et al., 2010). We aimed to extend these exciting results in this dissertation by investigating the important preclinical question of whether there exists a critical treatment window for the application of anti-Nogo-A immunotherapy in the chronic stage following ischemic injury in adult rats with moderate to very severe sensorimotor deficits.

**HYPOTHESIS:**

Inhibiting the Nogo-A protein using anti-Nogo-A antibodies at 9 or 25 weeks post-stroke in adult rats with moderate to very severe sensorimotor deficits will result in improved functional recovery and enhanced dendritic plasticity.

**Specific Aim 1:** Determine the magnitude and time course of spontaneous sensorimotor recovery in adult rats during the first 8 and 24 weeks post-stroke. Male adult rats will be tested using the skilled forelimb-reaching task post-stroke for up to 8 or 24 weeks post-stroke to determine sensorimotor recovery.

**Specific Aim 2:** Evaluate the critical treatment window for administration of anti-Nogo-A immunotherapy to induce sensorimotor recovery in the chronic stage after ischemic stroke in the adult rat. The same animals in Aim 1 will be used in Aims 2 and 3. At either 9 or 25 weeks post-stroke, adult rats with severe sensorimotor deficits will be treated with intracerebroventricular infusion of anti-Nogo-A immunotherapy, a control antibody, or will receive no treatment. Sensorimotor recovery will be tested using the
skilled reaching task, the skilled horizontal ladder walking task, and the bilateral adhesive removal task over the course of 12 additional weeks.

Specific Aim 3: Determine whether treatment with anti-Nogo-A immunotherapy in the chronic phase after stroke in adult rats will result in enhanced dendritic plasticity in the perilesional and contralesional cortex. The brains from Aim 2 will be processed for Golgi-Cox staining and neurons in the perilesional and contralesional caudal forelimb motor cortex will be evaluated for dendritic complexity. Specifically, layer V pyramidal neurons will be evaluated in the perilesional cortex and layer II/III and V pyramidal neurons will be evaluated in the contralesional cortex.
CHAPTER TWO
LITERATURE REVIEW

CHAPTER OVERVIEW

This chapter outlines the relevant concepts and literature background that is pertinent to the experiments in this dissertation. Firstly, a general discussion of neuronal plasticity and the capacity for neuronal regeneration after injury is presented in the context of the developing, adult, and aged central nervous systems. Additionally, previous work on factors found within the central nervous system that restrict neuronal plasticity, especially the protein Nogo-A, is presented. Finally, the therapeutic potential of blocking Nogo-A after various central nervous system injuries is discussed. Specifically, the potential of blocking Nogo-A in chronic ischemic stroke, which forms the basis of this dissertation, is addressed in detail.

NEURONAL PLASTICITY IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM AFTER INJURY

Definition of Neuronal Plasticity

Neuronal plasticity, as used in this dissertation, refers to any change in the structure or function of neurons in the central nervous system (CNS) in response to a
specific internal or external stimulus. Such stimuli can include day-to-day environmental experiences of an animal, pharmacological interventions, or even brain injury. Previously, the medical and neuroscience community regarded the mature brain as immutable and unable to undergo such repair or neural circuit reorganization. It is now known that varying degrees of neuronal plasticity take place not only in the developing and young CNS but also, given the appropriate circumstances, in the adult and aged CNS as well.

One important type of neural plasticity seen in response to brain and spinal cord injury takes the form of neuronal structural reorganization. This reorganization is characterized by the growth of new axons and dendrites and is ultimately thought to lead to the recovery of function after injury. There are two main types of axonal structural rearrangements that take place after injury, namely regeneration and compensatory plasticity (Kartje et al., 2006). During regeneration, a severed axon is able to reconnect with its deafferented targets by re-growing from its damaged end. In the case of compensatory plasticity, deafferented targets are reinnervated by new axonal growth from undamaged spared neurons that can be located quite far from the original injury site. Additionally, changes in dendritic complexity may also serve to enhance the functioning of damaged circuits. It is thought that these mechanisms play a large role in the spontaneous albeit limited improvements seen after brain and spinal cord injury. However, the magnitude of neuronal plasticity that can take place in response to injury is dependent on the age of the animal as well as whether the injury took place in the CNS or the peripheral nervous system (PNS). By studying the differences in reorganizational potential between the developing, adult, and aged CNS as well as in the PNS, important factors
governing neuronal plasticity have been discovered and are the basis of new therapeutic investigation.

Reorganizational Potential of the Mammalian Developing CNS

Seminal studies on the recovery of motor function in primates after early brain injury were performed by Margaret Kennard in the 1900s and led to the general premise that functional recovery after brain injury is faster and more complete in young animals than adolescent or adult animals (Kennard et al., 1936, 1938, 1942). This concept became known as the “Kennard Principle” and postulates that there is a negative linear correlation between recovery potential and age at brain or spinal cord injury, although exceptions have been reported (Schneider, 1979). The neuroanatomical corollary of improved motor function in young animals after brain injury was evaluated in numerous studies and evidence accumulated indicating that the young central nervous system remodels after injury by compensatory plasticity. The undamaged cortex opposite to the injury extends newly formed axons across the midline toward the injured side and reinnervates denervated targets. Specifically, the opposite undamaged cortex sends projections to the denervated striatum (Kolb et al., 1992), thalamus (Yu et al., 1995), red nucleus (Nah & Leong et al., 1976; Naus et al., 1985), basilar pontine nuclei (Castro and Mihailoff, 1983; Kartje-Tillotson et al., 1986), and spinal cord (Castro, 1975; Kartje-Tillotson et al., 1985; Rouiller et al., 1991). Since it is thought that neuroanatomical plasticity is the underlying mechanism of functional recovery after CNS injury in young animals, strategies that could induce the adult and aged CNS to display similar levels of
plasticity as seen in the young CNS could make a large difference in the rehabilitation of humans after brain and spinal cord injury.

Reorganizational Potential of the Mammalian Adult CNS

The reorganizational potential of the mammalian adult CNS is much more limited than during development or in the PNS. Classic experiments performed by Aguayo et al. investigated whether cut adult CNS axons were capable of long distance regenerative growth when exposed to a peripheral sciatic nerve graft (David et al., 1981). An autologous 35 mm sciatic nerve segment was grafted into the lower portion of the medulla oblongata and lower cervical or upper thoracic spinal cord of adult rats. After 22-30 weeks post grafting, it was found that axons from spinal and medullary neurons had elongated more than 30 mm into the sciatic nerve graft. Another experiment by Aguayo et al. showed that when optic nerves were implanted into sciatic nerves, the regenerating peripheral axons almost completely avoid the optic nerves (Aguayo et al., 1978; Weinberg and Spencer, 1979). Results from these experiments suggest that although CNS neurons are not capable of long distance regeneration within the environment of the CNS, the neurons were capable of long distance growth within the environment of the peripheral nerve segment. Seemingly, it was the CNS environment exerting a non-permissive effect that rendered the CNS neurons incapable of meaningful re-growth.

Since the molecular basis of the differences within the CNS and PNS environment were unknown at that time, Schwab and colleagues decided to investigate whether the CNS
environment lacked a potential trophic factor or whether the CNS environment contained a non-permissive substrate for neurite growth in the CNS. Neonatal rat sympathetic or sensory ganglia were grown in the presence of nerve growth factor (NGF) in a chamber culture system where the neurons could grow into explants of either sciatic nerves or optic nerves. Schwab et al. found that the neurons very frequently grew into the sciatic nerve explants, but never into the optic nerve explants (Schwab et al., 1985). Additionally, the same findings were found with previously frozen optic and sciatic nerves that did not contain living glial cells. This experiment suggested the existence of a non-permissive substrate in the optic nerves that could not be overcome by the strong fiber outgrowth promoting effects of NGF.

The Schwab laboratory continued to investigate the possible source of the non-permissive substrate in the CNS and therefore studied the interaction of CNS neurons and CNS glia. Rat sympathetic, sensory ganglion, or fetal retinal cells were plated onto cultures with dissociated optic nerve glial cells of young rats. They found that while immature oligodendrocytes and astrocytes were often touched by neurons and neurites, mature oligodendrocytes acted as a non-permissive substance for neuronal adhesion, neurite growth, and fibroblast spreading (Schwab et al., 1988). They also showed that adult rat spinal cord myelin acted to inhibit cell attachment, neurite outgrowth, and fibroblast spreading (Schwab et al., 1988). Rat CNS myelin was analyzed and size fractionation of myelin protein by SDS-Page resulted in the identification of two membrane bound proteins (a 35 and 250-kD fragment) that were highly non-permissive
for neurite growth and fibroblast spreading (Caroni & Schwab, 1988). To generate a function-blocking antibody, the Schwab laboratory immunized mice with the rat CNS myelin 35-kD and 250-kD protein fractions, raised hybridoma cells, and screened all the supernatants for anti-myelin antibodies. The antibody that had the strongest myelin neutralizing properties was designated as IN-1 and led to neurite growth and fibroblast spreading in vitro (Caroni et al., 1988). A myelin protein homologue of NI-250 was then purified from bovine spinal cord myelin (bNI-220) and was also shown to exert a potent neurite outgrowth inhibitory effect, including the collapse of growth cones and the inhibition of fibroblast spreading (Spillman et al., 1988). The monoclonal antibody IN-1 was found to neutralize the inhibitory effects of bNI-220. The purification of the bNI-220 protein led to the identification of six peptide sequences that were used to isolate the gene encoding NI-220/250, and this gene was named Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

*The Nogo Gene and its Protein Products*

Alternative splicing and alternate promotor usage produces three protein isoforms from the Nogo gene, Nogo-A, -B, and –C, see Figure 1.1 (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). All three Nogo isoforms are assigned to the Reticulon protein family due to the presence of a shared COOH-terminus containing a reticulon homology domain (RHD). Therefore, the Nogo gene has also been named as Reticulon-4 (RTN4/nogo). This shared COOH-terminus is made up of two long hydrophobic domains that are separated by a hydrophilic short loop of 66 amino acids called Nogo-66. All three
isoforms of Nogo contain the Nogo-66 domain. The Nogo-66 domain in Nogo-A has been found to induce growth cone collapse in vitro (Oertle et al., 2003). Among reticulon proteins, there is no homology at the NH2–terminal, suggesting biologically diverse functions for this protein family. Specifically, Nogo-A and Nogo-B isoforms share a common NH2-terminus, which has been found to inhibit fibroblast spreading (Oertle et al., 2003). Finally, the Nogo-A isoform contains a unique specific region within the NH2-terminus called the Amino-Nogo domain, which inhibits neurite outgrowth, induces growth cone collapse, and inhibits fibroblast spreading (Oertle et al., 2003). The three active sites found in Nogo-A are exposed to the extracellular space when Nogo-A is in the cell membrane (GrandPre et al., 2000; Dodd et al., 2005).
Figure 2.1. Nogo protein isoforms. Modified with permission from Kempf et al., 2013.
**Figure 2.2 Nogo-A signaling cascade.** Modified with permission from Kempf et al., 2013.


*Localization of the Nogo Proteins*

The protein Nogo-A is highly enriched in the endoplasmic reticulum due to its endoplasmic reticulum retention signal, however, it is also highly expressed in mammalian oligodendrocytes, localized in the outer and innermost adaxonal myelin sheath (Huber et al., 2002). Nogo-A is also highly expressed in subpopulations of neurons during development, with a relative decrease in expression in adulthood (Josephson et al., 2001; Huber et al., 2002; Hunt et al., 2003; Meier et al., 2003). Additionally, beyond the CNS, Nogo-A is expressed in developing skin, skeletal muscle during differentiation, macrophages, testicular tissue, and in heart tissue (Huber et al., 2002; O’Neill et al., 2004; Fry et al., 2007; David et al., 2008; Pool et al., 2009; Bullard et al., 2008).

The protein Nogo-B is expressed in the central and peripheral nervous system as well as in peripheral tissues (Huber et al., 2002; Bullard et al., 2008). In the CNS, Nogo-B has been found to be involved in regulating vascular homeostasis and remodeling (Acevedo et al., 2004). Additionally, Nogo-B may play a role in hepatocyte proliferation, liver fibrosis, immune function, as well as cervical cancer metastasis (Tashiro et al., 2013; Gao et al., 2013; Di Lorenzo et al., 2011; Xiao et al., 2013).

Nogo-C is expressed in skeletal muscle and to lower levels in the brain and heart (Huber et al., 2002). The function of Nogo-C remains to be determined. Unlike Nogo-A, evidence supports little to no role of Nogo-B and Nogo-C taking part in neurite outgrowth inhibition (GrandPre et al., 2000).
Nogo-A Receptors and Intracellular Signaling Cascade

The Nogo-66 domain of all three Nogo isoforms signals through two receptors: the Nogo-66 receptor (NgR1) and the paired immunoglobulin-like receptor B (PirB), see Figure 1.2 (Fournier et al., 2001; He et al., 2003). NgR is a GPI-linked cell surface receptor protein that lacks an intracellular domain and therefore signals through co-receptors including p75 (Wang et al., 2002; Wong et al., 2002) or TROY/Taj (Park et al., 2005), and Lingo-1 (Mi et al., 2004). Given the growth arresting effect of Nogo-A signaling, including growth cone collapse and neurite outgrowth inhibition, it was hypothesized that the signaling cascade most likely acted on cytoskeletal dynamics (Gozenbach & Schwab, 2008). The signaling cascade of the Nogo-66 domain and the amino-Nogo domain has been found to involve multiple signaling mechanisms. The major pathway of signaling involves activation of the small GTPase RhoA and concomitant suppression of Rac1, which leads to growth inhibition via a ROCK pathway (Fournier et al., 2003; Niederost et al., 2002; Lehmann et al., 1999). Activation of RhoA is accomplished by its release from the inhibitory regulator Rho-guanine dissociation inhibitor (Yamashita et al., 2003). Next RhoA activates Rho Kinase (ROCK), with its ability to activate multiple effectors including Lin-11, Isl-1, and Mec 3 Kinase 1 (LIMK1) (Hsieh et al., 2006). Once activated, LIMK1 inactivates cofilin through phosphorylation, leading to F-actin polymerization, and causing growth cone stabilization and the inhibition of neurite outgrowth (Hsieh et al., 2006). The activation of the RhoA/ROCK pathway also leads to growth inhibition through microtubule disassembly mediated by CRMP2 and actomyosin contraction mediated by MLCII.
Additionally, the Nogo-66 domain has been found to signal via the paired immunoglobulin-like receptor B (PirB) which also signals through p75 (Atwal et al., 2008). Although much more is known about Nogo-66 signaling, the amino portion of Nogo-A is considered more important for neurite outgrowth inhibition and the putative Amino-Nogo receptor has yet to be discovered. Amino-Nogo is thought to also signal by activating the RhoA/ROCK pathway as well as activation of CREB and down-regulating the neuronal growth program.

Additionally, Nogo-A signaling has been found to increase intracellular levels of calcium (Bandtlow et al., 1993; Wong et al., 2002; Hasegawa et al., 2004), activate Protein Kinase C (PKC, Hasegawa et al., 2004; Sivasankaran et al., 2004), and activate Epidermal Growth Factor Receptor (EGFR, Koprivica et al., 2005). Interestingly, many downstream effectors activated by Nogo-A signaling are also activated by other myelin associated neurite growth inhibitors as well as chondroitin sulfate proteoglycans.

Additionally, various changes in gene and protein expression have been found after Nogo-A neutralization. Neutralization of Nogo-A by anti-Nogo-A antibodies (11C7 and 7B12) in rat hippocampal slice cultures led to changes in RNA transcripts in genes related to growth, extracellular matrix, cell adhesion, neurogenesis, GTPase signal transduction, and growth factors (Craveiro et al., 2008). Furthermore, unlesioned spinal cord samples from genetically modified mice lacking Nogo-A, exhibited changes in protein expression in proteins related to cytoskeleton, signaling, neuroprotection, metabolism, and transport (Montani et al., 2009). These gene and protein changes after Nogo-A neutralization provide insight into additional signaling cascades that may be
involved in Nogo-A signaling.

In summary, the protein Nogo-A has emerged as an important inhibitory molecule located in the CNS environment that acts to restrict the regenerative potential of neurons and stabilize existing neuronal circuits. With the discovery of Nogo-A, additional factors have been found that also restrict neuronal reorganization in the CNS and are detailed in the following section.

*Additional Molecules Contributing to the CNS Inhibitory Environment*

Although the protein Nogo-A is considered to be one of the most potent neurite outgrowth inhibitors, there are various molecules that inhibit neurite outgrowth after injury in the CNS. Additional inhibitory proteins found in myelin that contribute to the growth restrictive environment seen after injury are myelin associated glycoprotein (MAG, McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte and myelin glycoprotein (OMgp, Wang et al., 2002), and the repulsive axon guidance molecules Ephrin B3 (Benson et al., 2005), and Semaphorin 4D (Moreau-Fauvarque et al., 2003). Interestingly, MAG and OMgp also interact with NgR1 as well as PirB and it is thought that myelin inhibitors may signal through multiunit receptor systems and the contribution of receptor subunits may depend on the type of neuron (Giger et al., 2008). In addition to myelin proteins, chondroitin sulfate proteoglycans, extracellular matrix molecules, also contribute to the growth inhibitory environment of the adult CNS (Niederost et al., 1999; Schmalfeldt et al., 2000). With the discovery of CNS inhibitory molecules, the possibility of developing strategies to inhibit these molecules and improve
functional recovery in the human after brain and spinal cord injury is at last a possibility. If these strategies prove successful in clinical trials, the field of rehabilitative medicine will be revolutionized and brain and spinal cord injured patients will for the first time have hope for significant recovery.

Reorganizational Potential of the Aged CNS

As previously described, the adult CNS is much more limited in its capacity to repair itself after brain or spinal cord injury than the developing CNS or the adult PNS. However, some limited reorganization can occur and is the basis of some spontaneous recovery seen in patients. However, with age, the adult CNS becomes even less able to repair itself. For example, after brain damage due to stroke, functional recovery is diminished in the aged (Nakayama et al., 1994; Johnston et al., 2000) and it is possible that aging may reduce the ability of the lesioned brain to reorganize after brain and spinal cord injury. Animal studies have shown that after focal ischemic stroke in adult and aged rats, neurons in the peri-infarct cortex in the aged brain showed a greater degree of oxidative DNA and protein damage as well as a reduced induction of the heat shock protein (HSP) 70, a cell stress protective protein, as compared to the adult brain (Li et al., 2005). Additionally, another study showed that aged rats versus adult rats after focal ischemic stroke displayed an abnormal profile of growth promoting and inhibiting proteins. For example, protein levels of MAG and Ephrin A5 were increased at post-stroke weeks 1 and 2 in aged rats as compared to young adult rats (Li et al., 2006). These results indicate that the aged CNS may be more limited in its ability to repair itself than the adult CNS and
therefore therapeutic strategies may need to involve combinatorial therapies that can simultaneously stimulate a neuronal growth state and block multiple axonal growth inhibitors to induce functional recovery.

**NOGO-A BLOCKADE AFTER CNS INJURY**

Following the discovery of Nogo-A’s role in restricting neuronal plasticity in the adult and aged CNS, a flurry of investigation has been conducted over the last two decades to determine the therapeutic potential of inhibiting the protein Nogo-A in order to induce neuronal plasticity and thereby functional recovery after CNS injury. A variety of approaches have been used to block the function of Nogo-A including specific monoclonal antibodies directed against Nogo-A, Nogo-66 receptor blockers, and downstream signaling molecule inhibitors. The following three sections will outline the effectiveness of Nogo-A blockade to improve function in various CNS injury paradigms with a focus on using monoclonal antibodies to inhibit Nogo-A in a rat model of focal ischemic stroke.

*Functional Recovery and Neuroanatomical Plasticity after Nogo-A Neutralization after Spinal Cord Injury*

Following the discovery of Nogo-A as a myelin associated inhibitor, experiments were undertaken to determine whether blockade of Nogo-A function *in vivo* after spinal cord injury would lead to axonal regeneration and functional recovery. In the 1990s Schnell and Schwab used the monoclonal antibody IN-1 and delivered it to spinal cord-injured rats.
The antibody was delivered to the spinal cord-injured rats by implanting hybridoma cells that secreted IN-1 or a non-specific antibody into the brain. Rats treated with IN-1 displayed functional recovery and correlated with regeneration considerably past the lesion site. These exciting results have now been replicated in rats and primates by using local intrathecal pump infusions of recombinant IN-1 Fab fragments or highly purified Nogo-A specific monoclonal antibodies that recognize various domains in the Amino-Nogo segment of the Nogo-A protein.

Furthermore, these antibody treatment results have been extended by using other approaches to block the function of the protein Nogo-A in spinal cord injury, such as blocking the Nogo receptor (NgR). A competitive antagonist for the NgR receptor called NEP1-40 has been used to treat spinal cord-injured rats and resulted in axonal regeneration and improved recovery (GrandPre et al., 2002). Another NgR antagonist, called NgR(310)ecto-Fc, also produced improved axonal growth and recovery of function when administered to spinal cord-injured rats. Given the promising results shown after Nogo-A inhibition after spinal cord injury in rat and primate studies (Freund et al., 2006; Freund et al., 2009), a phase I clinical trial was recently completed evaluating the feasibility and safety of administering anti-Nogo-A antibodies intrathecally to acutely spinal cord injured patients. The trial reported no safety concerns and a phase II clinical trial is currently in preparation (Abel et al., 2011).

*Functional Recovery and Neuroanatomical Plasticity after Nogo-A Neutralization after Traumatic Brain Injury*
Based on the *in vivo* success of treating rats with spinal cord injury with various approaches that block the function of Nogo-A, the effectiveness of blocking Nogo-A after traumatic brain injury (TBI) was investigated. Using a lateral fluid percussion brain injury model, rats were treated with anti-Nogo-A immunotherapy or a control antibody 24 hours after injury. Using a test of motor function called the composite neuroscore, it was shown that there was no difference between the anti-Nogo-A antibody treated rats and the control antibody treated rats when tested 4 weeks post-treatment (Lenzlinger et al., 2005; Marklund et al., 2007). However, in these same experiments, rats that were treated with anti-Nogo-A antibody performed better than the control antibody treated rats on the Morris water maze task for spatial memory (Lenzlinger et al., 2005; Marklund et al., 2007). Additionally, rats that were treated with anti-Nogo-A antibody following TBI had a higher growth-associated protein-43 expression, an axonal growth marker, in the CA1 of the hippocampus than control antibody treated rats (Marklund et al., 2007). More studies are needed to address the effectiveness of anti-Nogo-A immunotherapy after TBI for improving sensorimotor deficits.

*Functional Recovery and Neuroanatomical Plasticity after Nogo-A Neutralization after Ischemic Stroke*

Many studies have also been performed evaluating the therapeutic potential of blocking Nogo-A in rodent models of ischemic stroke. The rodent model of middle cerebral artery occlusion (MCAO) typically damages the ipsilateral sensorimotor cortical area and rats are rendered incapable of accurately performing a skilled motor task that
involves reaching for, grasping, and bringing a pellet to their mouth with the impaired forelimb (Castro et al., 1972; Whishaw et al., 2000). These types of movements have been found to be dependent on intact cortico-spinal and cortico-rubral-spinal pathways (Castro et al., 1972; Whishaw et al., 2000). Using this model, the motor deficits seen after rodent ischemic stroke, like in humans, are generally permanent and limited spontaneous recovery occurs over time (Papadopoulos et al., 2002; Weissner et al., 2003; Seymour et al., 2005; Markus et al., 2005; Tsai et al., 2007; Tsai et al., 2011).

Studies mainly from our laboratory have evaluated the therapeutic potential of blocking the protein Nogo-A using function blocking antibodies (anti-Nogo-A immunotherapy) after ischemic injury in the rat. Adult rats underwent a middle cerebral artery occlusion and were immediately treated with the monoclonal antibody IN-1 that recognizes the Amino-Nogo domain. These rats were then tested on a skilled reaching task and it was shown that treatment with IN-1 led to recovery of 80% of pre-stroke skilled reaching ability while control antibody and stroke only rats recovered only 40% of their pre-stroke reaching ability (Papadopoulos et al., 2002). Evaluation of the stroke lesion in these animals showed that the lesions were confined to the cortical tissue with no subcortical involvement and the average hemispheric stroke size was between 8% and 12%, with no significant difference between groups. Therefore, no neuroprotective effect was seen with anti-Nogo-A immunotherapy. This study also displayed for the first time the successful application of anti-Nogo-A immunotherapy in rats with small to medium sized strokes. This profound functional recovery was also found to be associated with new axonal growth from the undamaged hemisphere. The unlesioned hemisphere was
found to form new cortico-efferent projections to the contralateral red nucleus (Papadopoulos et al., 2002).

Since immediate treatment following stroke is often not possible in the hospital, subsequent studies went on to evaluate the treatment time window that exists for the successful administration of anti-Nogo-A immunotherapy after ischemic stroke in adult rats. The efficacy of anti-Nogo-A immunotherapy administered 24 hours, 1 week, and 9 weeks post-stroke was evaluated in three different studies, using three different anti-Nogo-A antibodies: 7B12, IN-1, and 11C7 respectively (Weisser et al., 2003; Seymour et al., 2005; Tsai et al., 2011). All three antibodies recognize the Amino-Nogo domain, however 7B12 and 11C7 are more specific and recognize short amino acid sequences within the Amino-Nogo domain. Adult rats treated with these function blocking Nogo-A antibodies at each of the three time points recovered up to 70 to 78% of their pre-stroke skilled reaching ability while control antibody and stroke only animals recovered only 30 to 50% of their pre-stroke skilled reaching ability (Weisser et al., 2003; Seymour et al., 2005; Tsai et al., 2011). Functional recovery in these studies was also associated with neuroanatomical plasticity, and increases in cortico-efferent projections from the undamaged hemisphere were found crossing the midline to reinnervate the contralateral deafferented red nucleus (Seymour et al., 2005) and the contralateral cervical spinal cord (Weisser et al., 2003). Additionally, increased dendritic complexity was found in layer V pyramidal neurons within the undamaged homotopic sensorimotor cortex in adult rats treated with IN-1 at the time of stroke (Papadopoulos et al., 2006). Together, these studies support the use of anti-Nogo-A immunotherapy in adult rats with small to
medium sized strokes in the acute (immediate to 24 hours post-stroke), subacute (1 week post-stroke), and early chronic (9 weeks post-stroke) phases after ischemic stroke.

Given that stroke is more prevalent in human aged populations (Lloyd-Jones et al., 2009), it is important to test preclinical therapies in aged rodents. Therefore, anti-Nogo-A antibodies (7B12) were administered to aged rats 1 week post-stroke. Anti-Nogo-A immunotherapy improved functional recovery on the skilled reaching task in aged rats to the same magnitude as seen in previous studies, although the rate of recovery was slower and a longer time period for recovery was necessary as compared to adult rats (Markus et al., 2005). Anti-Nogo-A immunotherapy administered 1 week post-stroke has also been shown to improve cognitive impairment in aged rats as measured by the Morris Water Maze spatial memory task (Gillani et al., 2011). Anti-Nogo-A immunotherapy administered 1 week post-stroke has also been found to improve cognitive recovery from neglect after cortical aspiration lesion in adult rats (Brenneman et al., 2008).

Other experimental methods have been used to interfere in the Nogo-A-NgR signaling pathway after stroke in rodents. Various mechanisms can be used to inhibit the Nogo-66 receptor (NgR) including the NEP1-40 antagonist peptide, the NgR(310)Ecto-Fc IgG fusion protein that binds to Nogo-A, genetic knockout of the NgR gene, and RNAi mediated NgR knockdown. NEP1-40 is a Nogo-66 receptor antagonist peptide that competes for the NgR receptor with the Nogo-66 domain (GrandPre et al., 2002). Treatment with the NEP1-40 antagonist peptide improved skilled reaching after cortical stroke in rodents (Fang et al., 2010; Zai et al., 2011). Another mechanism for blocking NgR signaling is the use of a truncated soluble Nogo receptor fragment that binds the
Nogo-66 domain (Fournier et al., 2002). Rats treated with this IgG fusion protein after stroke displayed improved motor recovery in skilled reaching (Lee et al., 2004). Other ways of inhibiting the signaling mediated by NgR include adenovirus-mediated RNA interference knockdown of NgR and NgR genetic knockout mice. Knockdown of the NgR receptor by RNAi in rats after stroke at 24 hours or two weeks post-stroke lead to motor recovery on the Montoya’s Staircase skilled reaching task (Wang et al., 2010). NgR -/- mice also showed improved motor recovery after ischemic focal stroke as compared to NgR +/+ mice (Lee et al., 2004).

Although methods of interfering with NgR signaling have been shown to be effective in improving recovery after stroke in rodents, antibody based approaches for inhibiting the protein Nogo-A directly are thought to be more effective in inducing neuronal plasticity. This is due to several reasons. Firstly, the Nogo-A protein is thought to signal through three different receptors at minimum, which include NgR, PirB, and the proposed Amino-Nogo receptor. Antibody binding to Nogo-A may lead to direct and indirect inability (steric inhibition) of the protein to bind all three receptors at once. Secondly, antibody binding of Nogo-A leads to endocytosis and a decreased level of Nogo-A levels at the cell membrane (Joset et al., 2010). Finally, signaling through the Amino-Nogo domain and the unknown Amino-Nogo receptor is thought to lead to gene changes in the neuron that render it more responsive to growth promoting signals (Joset et al., 2010).

The following section will describe the pathophysiology, epidemiology, and recovery potential of human ischemic stroke. Afterwards, the unanswered questions
regarding the use of anti-Nogo-A immunotherapy to improve recovery of function in chronic large sized strokes will be discussed.

**STROKE**

**Introduction**

Cerebrovascular disease encompasses conditions that arise as a result of pathology affecting cerebral blood vessels. Stroke is defined as the sudden focal loss of brain function due to an interruption of cerebral blood flow. Following decreased blood flow, neurons begin to dysfunction due to the lack of oxygen and nutrients. Neurological functioning is compromised and the brain will become permanently damaged if blood flow is not reestablished as soon as possible. Strokes are either ischemic or hemorrhagic, depending on whether the compromised cerebral blood flow is caused by a blockage in a cerebral blood vessel or by rupture of a cerebral blood vessel respectively. Ischemic strokes caused by an occluded blood vessel are the most common type of stroke seen (87%), while the remainder of strokes are hemorrhagic (AHA, 2013). Most commonly, ischemic strokes occur due to thrombosis or emboli from a large vessel or the heart while hemorrhagic strokes often occur due to rupture of blood vessels due to hypertension or rupture of intracranial aneurysms (Biller, 2012).

**Prevalence and Incidence**

Over 6 million American adults living in the United States have suffered a stroke and it is estimated that an additional 795,000 people will experience a new or recurrent stroke each year (AHA, 2013). Globally, it is estimated that upwards of 60 million people
have suffered a stroke and yearly more than 16 million new stroke events will occur (Truelsen & Bontina, 2009). While the overall incidence of stroke is declining in many developed countries, the overall burden of stroke is increasing due to the aging population globally (WHO, Global Burden of Stroke, 2013).

Constellation of Symptoms Seen Post-Stroke

Symptoms of a stroke start suddenly and are dependent on the area of brain tissue that is affected. Commonly, stroke causes deficits across many modalities including motor, sensory, language, and cognitive domains, with the severity related to how extensive the stroke damage was (Kelly-Hayes et al., 2003). Due to the extensive deficits caused by stroke, it is considered to be the leading cause of serious long-term disability in the United States (Survey of Income and Program Participation, a survey of the US Bureau of the Census). Among ischemic stroke survivors over the age of 65, a large proportion still suffers from significant disabilities after 6 months post-stroke (Kelly-Hayes, 2003). Specifically, up to 80% experience motor dysfunction, including 50% who are suffering from hemiparesis and 30% who are unable to walk without assistance (Kelly-Hayes, 2003). Almost half of stroke survivors suffer from cognitive symptoms and 35% had depressive symptoms (Kelly-Hayes, 2003). Language troubles also plague stroke sufferers, with approximately 19% having aphasia (Kelly-Hayes, 2003). The most severely affected stroke survivors are dependent in activities of daily living (~26%) and are institutionalized in nursing homes (~26%) (Kelly-Hayes, 2003).
Treatment Strategies

Currently, the only approved treatment strategy for acute ischemic stroke is the use of recombinant tissue plasminogen activator (tPA) within the first 4.5 hours after stroke onset (Hacke et al., 2008). Unfortunately, multiple barriers exist for the widespread utilization of tPA in most stroke cases. Firstly, a large percent of stroke patients do not recognize the symptoms of stroke and therefore delay seeking treatment. According to one study, only 23.5% of stroke patients arrive at the emergency room within 3 hours of symptom onset, and only 4.3% end up receiving thrombolytic therapy (CASPR Investigators, 2005). Additionally, it is estimated that only 21% of U.S. counties have a hospital and this may also lead to delays in seeking treatment (CDC, 2008). Other factors limiting the use of tPA include delays in diagnosis, misdiagnoses, and adverse side effects. Once the short time window for tPA administration has passed, rehabilitative strategies are the only viable approaches in the subacute and chronic stages after stroke. Such therapy may include physical therapy, speech therapy, occupational therapy, and neuropsychological and cognitive therapy. Although real improvements in function are seen after rehabilitation, the magnitude of recovery is limited (Duncan et al., 2000). Additionally, neuroprotective therapies once held great promise in preclinical trials, however this class of drugs mainly failed in clinical trials (Cheng et al., 2004; Savitz et al., 2007; Ginsberg et al., 2007; Faden et al., 2007; Young et al., 2007). Therefore, it is important to investigate treatment strategies that can be used successfully after a stroke has already taken place to enhance recovery. The population of chronic stroke sufferers is large and optimism exists among neuroscientists for the development of neurorestorative
therapies, such as anti-Nogo-A immunotherapy, that can improve functional recovery after stroke by enhancing brain plasticity even in the chronic phases after stroke.

**Prognosis for Functional Recovery in Stroke**

For most patients, the majority of significant recovery occurs during the first 3-6 months after stroke (Skilbeck et al., 1983). Although limited in magnitude, further improvements may occur beyond 6 months and for many years if the stroke patient engages in stimulating and rehabilitative activities. Many factors play a role in the time course and magnitude of functional recovery after stroke. In general, the strongest prognostic factor for the magnitude of stroke recovery is the severity of the initial deficit. In addition, stroke severity affects the time course of recovery as well. Data from the Copenhagen Study of stroke survivors showed that 95% of mild stroke survivors reached their best recovery within 6 weeks post-stroke while moderate, severe, and very severe stroke patients reached their best recovery at 10, 15, and 13 weeks post-stroke respectively (Jorgensen et al., 1995a, 1995b).

**Enhanced Potential for Neuronal Plasticity Early After Stroke Injury**

Immediately after stroke, it is recognized that a critical time window opens that reinstates a molecular environment in the adult CNS that is more conducive to neuronal plasticity. The peri-infarct tissue that lies beyond the glial scar turns into a growth permissive zone where levels of growth inhibitory molecules are reduced and growth-promoting molecules are increased during the first month after stroke in adult rats.
transient increase in neurotrophic factors such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) are seen after focal ischemia in ipsilateral non-ischemic areas (Speliotes et al., 1996). Furthermore, hyperexcitability exists in the tissues ipsilateral and contralateral to the infarct, though dissipating over several weeks (Buckkremer-Ratzmann et al., 1996). Finally, recovery after brain injury can be delayed by pharmacological prevention of hyperexcitability during the first 3 weeks after ischemia, but not during later time points (Schaller et al., 1986). Therefore, treatments that enhance neuronal plasticity such as rehabilitation and restorative therapies may be more efficacious when applied early after stroke (within the first four weeks in adult rats) than when applied later. Research in rats exposed to rehabilitation support this hypothesis. Biernaske et al. exposed adult rats after stroke to enriched environments and rehabilitative reach training starting at 5 days, 14 days, or 30 days after focal ischemia. The effect of enriched rehabilitation was found to be time dependent. Animals that were exposed to enriched rehabilitation beginning at 5 days post-stroke showed a statistically greater improvement in skilled reaching than rats that began the enriched rehabilitation at 14 days post-stroke. Furthermore, rats that began the rehabilitation scheme at 30 days post-stroke did not display any improved motor performance as compared to control animals. Changes in dendritic complexity in the homotopic contralesional sensorimotor cortex were also time dependent and only animals that underwent the enriched rehabilitation paradigm at 5 days post-stroke displayed increases in dendritic complexity in layer V pyramidal neurons (Biernaske et al., 2004). These results support the view that
the remaining ipsilesional and contralesional cortical tissues are more responsive to rehabilitation early after stroke. Therefore, in humans and rodents, it is likely that spontaneous neuronal plasticity will be most likely to occur in the early weeks after stroke.

**Mechanisms of Stroke Recovery**

Early spontaneous recovery after stroke in humans (within hours to days) is attributed to the resolution of edema and the reperfusion of the ischemic penumbra (Dombovy et al., 1991). Recovery seen over the course of weeks, months, and years after stroke is considered to be the result of spontaneous neural circuit reorganization (Carmichael et al., 2006; Murphey et al., 2009), with neurogenesis and angiogenesis (Thored et al., 2007; Hermann et al., 2009) potentially playing a role as well. Through rehabilitation, the stroke-injured brain is able to relearn tasks, and other undamaged areas of the brain are thought to take over the lost functions. With the advent of functional neuro-imaging techniques such as functional Magnetic Resonance Imaging (fMRI), Positron Emission Tomography (PET), Electroencephalography (EEG), and Magnetoencephalography (MEG), extensive investigations of the neural correlates of motor recovery after stroke have been evaluated at the systems level. Various motor areas in both the damaged (ipsilesional) hemisphere and the undamaged (contralesional) hemisphere have been implicated (Chollet et al., 1991; Grefkes et al., 2008; Weiller et al., 1992).

In the damaged hemisphere, increased task related neural activity has been found
using fMRI in the spared adjacent tissue around the infarct and the primary sensorimotor cortex, as well as in secondary motor areas such as the premotor cortex and the supplementary cortex (Chollet et al., 1991; Cramer et al., 2003; Johansen-Berg, et al., 2002). Increased activation has also been found in the ipsilateral cerebellum (Chollet et al., 1991).

Cortical reorganization has also been seen in the undamaged hemisphere. Increased neural activation related to movement of the affected hand has been seen in the contralesional sensorimotor cortex as well as in secondary motor areas such as the premotor cortex and the supplementary motor cortex, and the ipsilateral cerebellum (Chollet et al., 1991; Rehme et al., 2010).

The factors leading to increased activation of ipsilesional versus contralesional motor areas and which pattern of activation leads to better recovery is controversial. Feydey et al. showed that the nature of the lesion affects cortical reorganization and may dictate which hemisphere is recruited in recovery mechanisms. They found that in strokes that affected most of the primary motor cortex, it was more likely for the contralesional hemisphere to show increased activation (Feydey et al., 2002). Conversely, strokes that mostly spared the primary motor cortex were more likely to show increased activation in the ipsilesional hemisphere (Feydey et al., 2002). Additionally, studies have shown that activity in bilateral premotor areas and in contralesional primary motor cortex correlate with more severe motor impairment (Loubinoux et al., 2007; Marshal et al., 2009; Ward et al., 2003; Ward et al., 2004). A recent meta-analysis evaluated the current literature on motor related neural activity after stroke and found that better motor performance was
associated with greater activation in the ipsilesional primary motor cortex (Rehme et al., 2012). Acutely after stroke, neural activity is enhanced in both hemispheres and is correlated with stroke recovery, while over the course of 12 months post-stroke, good recovery is correlated with the return of ipsilesional activation patterns (Ward et al., 2003; Tombari et al., 2004; Rehme et al., 2010).

Summary

Currently, the only FDA approved treatment for stroke is thrombolysis. However, most stroke patients do not receive this therapy and are left with permanent and profound deficits. A treatment that could restore function long after the acute phases after stroke have passed would greatly decrease disability among stroke survivors. Anti-Nogo-A immunotherapy has been shown to be successful in restoring lost functions after small and medium sized strokes in adult and aged rats when applied immediately or up to 9 weeks post-stroke. However, it is not known whether anti-Nogo-a immunotherapy is effective in adult rats with large strokes. Additionally, it is not known how long the treatment window exists for the administration of anti-Nogo-A immunotherapy in chronic stroke-impaired rats. Therefore, this dissertation proposes to examine the treatment time window and efficacy of anti-Nogo-A immunotherapy in the early and late chronic phases after ischemia in adult rats with large sized strokes.
CHAPTER THREE
METHODOLOGY AND EXPERIMENTAL DESIGN

INTRODUCTION

The experiments outlined in this dissertation involve testing the hypothesis of whether blocking the function of Nogo-A after chronic moderate to very severe stroke in a rodent model of focal ischemic infarction will induce functional sensorimotor recovery. In the clinic, there is a tendency towards pessimism regarding the expected success of interventions, whether pharmacological or rehabilitative, to be successful in chronic stroke. A similar pessimistic outlook exists for the success of interventions for severe stroke, with many questioning the appropriate use of resources to try to rehabilitate this low likelihood recovery population. We decided to assess whether treatment with anti-Nogo-A immunotherapy in the chronic phases after stroke can lead to functional recovery in adult rats with moderate to very severe chronic sensorimotor deficits. This chapter will outline the experimental design, rationale, and methods used for this dissertation.

Methodological Considerations in Preclinical Stroke Studies

Over the past decade many therapeutic agents and strategies were tested in preclinical studies and found to be efficacious in small animal models of ischemia. Billions of dollars later, none of these treatments except tPA, were found to be effective
in human trials (Turner et al., 2013). In general, it is thought that various experimental design issues in preclinical studies may have contributed to the translation failure of neuroprotective strategies. In 1999, Dr. Marc Fisher and Dr. Gary Houser of Massachusetts University conceived of assembling a select group of the world’s leading scientists to meet and discuss issues that were critical for the development of drugs and devices for stroke treatment. The recommendations of this group are known as the Stroke Therapy Academic Roundtable Preclinical Recommendations (STAIR) and updates to their statements are issued on a periodic basis (Fisher et al., 1999; Fisher et al., 2009). Furthermore, recent meta-analyses have shown that when quality scores are calculated for preclinical studies using the STAIR criteria, there is a troubling negative association between studies with low methodological quality and large estimates of treatment efficacy (Horn et al., 2001). Clearly, problems with preclinical study design are contributing factors in the failure of “bench to bedside translation”. Therefore, the most recent recommendations of the STAIR committee as well as recommendations from key reviews on the topic of preclinical design quality will be discussed as applicable throughout this methodology chapter (Fisher et al., 1999; Dirnagl et al., 2006; Fisher et al., 2009; Kahle & Bix, 2012). For an overview of specific recommendations for the design of preclinical studies and how the experiments in this dissertation adhere to those recommendations, refer to Table 3.1.

Additional factors that complicate the evaluation of treatment effects in both preclinical and human clinical trials include the suboptimal reporting of a priori
inclusion/exclusion criteria as well as post-randomization exclusions. The gold standard for avoiding these types of problems is to design studies with strict a priori inclusion/exclusion criteria as well as to use an intention-to-treat (ITT) paradigm for the analysis of the data. Intention-to-treat paradigms involve analyzing all the data from subjects as they were randomized, regardless of study protocol or treatment delivery deviations. This approach is considered to provide the highest level of evidence regarding the treatment efficacy since investigator bias under the disguise of well intentioned post-randomization exclusions is not allowed to influence the data (Fisher et al., 1999). The use of this type of analysis is highly recommended in human randomized clinical trials (FDA Guidelines, 1988). Additionally, this type of analysis may give a better estimate of what the treatment efficacy would be in actual clinical use as deviations from treatment paradigms are frequent. As of yet however, not many preclinical studies adhere to this type of analysis. Therefore, in order to adhere to similar levels of stringency seen in human clinical trials, the data in this dissertation were first analyzed using an intention-to-treat paradigm. Given that the intention-to-treat analysis may provide very conservative estimates of treatment efficacy, a second analysis was done that excluded animal subjects that majorly deviated from the experimental protocol. This analysis is known as a per-protocol analysis (PP), and is often conducted with human clinical trial data as well. PP paradigms may lead to treatment estimates that are inflated and may be more reflective of optimal conditions and not typical efficacy. In human clinical trial literature, the results from these types of analyses are considered to have a higher chance of specified and unspecified bias due to the loss of pure randomization procedures.
Therefore, if a study conducts post-randomization exclusions, the potential of this treatment to improve recovery must be evaluated in light of both an ITT and a PP analysis. The results from this project will be expressed from both of these types of analyses to provide an accurate picture of treatment efficacy. Finally, reporting of dissertation study parameters and results was based on specific guidelines described in the Consolidated Standards of Reporting Trials (CONSORT) and the Animal Research: Reporting In Vivo Experiments (ARRIVE) checklists (Schultz, et al., 2010; Kilkenny et al., 2010). These checklists were used to improve the reporting in this dissertation.

**Experimental Design and Timeline**

See Figure 3.1 for a description of experimental design and timeline. As described in CHAPTER ONE, the experimental overview and hypothesis chapter, Specific Aim 1 evaluated the spontaneous sensorimotor recovery profile of all the animal subjects that underwent a focal ischemic stroke. This aim was designed like a prospective observational cohort study to determine the relationship between sensorimotor recovery across three different behavioral tasks during the acute and chronic phases after stroke induction. Furthermore, retrospective analysis of sensorimotor recovery and its correlation to stroke size was performed using histological sections. Post-stroke data was collected from all animal subjects for a total of 8 weeks post-stroke (2 months). A subset of animals from the original set was examined for a total of 24 weeks post-stroke (6 months), see Figure 3.1.
Specific Aim 2 evaluated anti-Nogo-A immunotherapy as a treatment to improve sensorimotor function in adult rats suffering from chronic stroke with moderate to very severe deficits. This aim was designed as a randomized, single blind, preclinical trial evaluating treatment efficacy at two different chronic administration time points, approximately 2 and 6 months post-stroke. This was done to evaluate the treatment window for anti-Nogo-A immunotherapy in the chronic stage following ischemia in moderate to very severely impaired rats. All data was evaluated using an intention-to-treat paradigm as well as a per-protocol paradigm. The animals used in the ITT and PP analyses are shown in Table 3.2. The animals excluded from the PP analysis are shown in Table 3.3 and a description of these exclusions is listed in Table 3.4.

Aim 3 involved post-mortem evaluation of neuroanatomical plasticity following anti-Nogo-A immunotherapy in the brains of treated rats, specifically evaluating dendritic complexity of layer V pyramidal neurons located in the perilesional and contralesional caudal forelimb motor cortex.

All animals (n = 75) underwent focal ischemic stroke. Post-stroke data was collected for n = 66 animals. After a period of 8 weeks, n = 35 animals (according to inclusion/exclusion criteria) were randomized into three balanced groups, taking into account the severity of their stroke deficit. The three experimental groups treated at 9 weeks post-stroke consisted of: (1) middle cerebral artery occlusion (MCAO)/anti-Nogo-A immunotherapy (n = 14), MCAO/control antibody (n = 12), and MCAO only (n = 9). A subset of the original n = 66 animals, namely n = 7 animals were used to collect post-
stroke data for a total of 24 weeks. These animals were then randomized into three groups. The three experimental groups treated at 25 weeks (6 months) post-stroke consisted of: (1) middle cerebral artery occlusion (MCAO)/anti-Nogo-A immunotherapy (n = 4), MCAO/control antibody (n = 2), and MCAO only (n = 2).

**Animal Subjects**

*Animal Subjects Background.* The selection of species, strain, and even vendor is an extremely important parameter in the design of preclinical studies and in the potential success of translating preclinical treatment results to clinical trials in humans. Studies have shown that different rat strains and even the same strains from different vendors display remarkable differences in regards to behavioral performance, evolution of injury after lesion models, and recovery responses to treatment interventions (O’Bryent et al., 2011; Oliff et al., 1995; Oliff et al., 1996). Therefore when evaluating a treatment intervention during the preclinical phase it is important to maintain the same parameters as closely as possible to previous work in order to properly compare across studies. In keeping with this premise, the previous work in the Kartje laboratory using anti-Nogo-A immunotherapy after stroke has mostly focused on using male adult (2-3 month old) Long Evans black-hooded rats to evaluate recovery potential (Seymour et al, 2005; Papadopoulos et al., 2006; Tsai et al., 2007; Gillani et al, 2010; Tsai et al., 2011). Consequently, the experiments in this dissertation were conducted using the Long Evans black-hooded rat strain (2-3 months of age) to maintain experimental homogeneity. This outbred strain was initially selected for our laboratory experiments due to the rats’
temperament being very amenable for behavioral training. Additionally, Long Evans black-hooded rats have been shown to display superior skilled reaching ability, larger forelimb movement representations in the motor cortex, and lower cortical stimulation intensities needed to initiate forelimb movements as compared to other rat strains (Vandenberg et al., 2002). All these factors have lead to the continued use of this rat strain in our laboratory as a model species for the evaluation of sensorimotor function after cerebral injury. Although the use of this strain to evaluate the efficacy of anti-Nogo-A immunotherapy in chronic severe stroke in this dissertation is considered prudent in regards to facilitating comparison across similar studies, it is also important to note that once a particular hypothesis has been tested in this setting of homogenous methodology, the next step in evaluating a treatment for bench to bedside translation is evaluating its efficacy across various heterogenic parameters such as rat strain, age, sex, health status, ischemic lesion model, and ultimately different species such as larger mammals and nonhuman primates. With this approach, preclinical to clinical trial translation failure can be minimized and the robustness of a particular treatment strategy can be evaluated.

**Animal Subjects Description.** All experiments described in this dissertation were approved by the Institutional Animal Care and Use Committee of Hines Veterans Affairs Hospital under IACUC protocol # H10-028. IACUC documentation is provided in Appendix A of this dissertation. A total of 75 adult male Long Evans black-hooded rats (2 months of age at the start of the study) were used. All animals, strain ID HsdBLU:LE, were ordered from the Harlan Laboratories animal facility, Barrier 217, in Indianapolis.
This was done in order to maintain the same genetic background in the animals, as animals were ordered in overlapping cohorts. Animals were housed in standard cages (2 rats per cage) with a 12-hour light/dark cycle in a fully accredited animal care facility at Hines Veteran Affairs Hospital. Due to aggressive fighting, some rats had to be separated and housed individually (n = 10). Rats had access to fresh water ad libitum and were fed standard laboratory chow (Harlan 2018 Rodent Diet). Rats were food restricted to 95% of their predicted weight by age at the start of the experiments. Rats were fed approximately 12 grams of standard rat chow each at the beginning of the study (2 month old rats weighing 250-300 grams each) and increased over time to approximately 16-20 grams per rat (7-16 month old rats weighing 400-500 grams). All animals were number coded and investigators conducting behavioral testing and neuroanatomical analyses were blinded to the treatment condition throughout the experiment.

Behavioral Training and Testing

Skilled Forelimb Reaching Task

Reaching Task Background. Skilled forelimb movements involve the ability to reach for an object and manipulate it using the digits. These movements are thought to originate from general food-handling behavior (Ivanco et al., 1996) and are displayed by various mammals including rats, primates, and humans. The level of homology across skilled forelimb movements such as reaching for food is quite striking when comparing rodents and humans as shown in Figure 3.2 (Iwaniuk et al., 2000; Whishaw et al, 1992;Sacrey et
al., 2009; Klein et al., 2012). These similarities can be explained by the morphological homology across mammalian species that includes similarities in skeletal and muscular structure, as well as comparable neural control of motor movement (Iwaniuk et al., 2000). Due to this fact, this natural behavior has become the focus of a select number of behavioral tests meant to measure movement deficits after various insults to the central nervous system in preclinical studies. One such test is the skilled forelimb-reaching task. This task is a sensitive skilled motor task designed to test voluntary skilled forelimb function in normal as well as brain damaged rats. It has been shown that the execution and coordination of skilled forelimb movements in this task are dependent on the synergism of multiple descending cortico-efferent pathways that primarily include the corticospinal tract originating from both the rostral and caudal forelimb motor cortex, the rubrospinal tract, the tectospinal tract, as well as the reticulospinal tract (Kennedy, 1990; Neafsey et al., 1986; Naus et al., 1985; Akintude and Buxton, 1992). Damage to any of these tracts will lead to impairment of skilled forelimb reaching performance. Additionally, damage to the dorsolateral striatum impairs forelimb reaching as well (Whishaw et al., 2007). Expectantly, various forms of neurological injury, including focal ischemic stroke in the rat, have produced acute and chronic deficits in skilled forelimb reaching due to damage to the various motor systems involved in skilled motor control (Markus et al., 2005; Papadopoulos et al., 2009). Therefore, this task is considered to be a good model of skilled movement in the rat that can be used to evaluate therapies aimed at improving manual dexterity in humans.
Reaching Task Description. Although reach to eat behavior in rats is a natural and spontaneous behavior, rats must be trained to perform the skilled reaching task. Training begins with food restricting the rats as stated previously. This is done to motivate the rats to reach for a food object, a round sucrose pellet (45 mg; Bilaney Consultants, Frenchtown, NJ). Rats were trained within approximately 2-4 weeks. Rats were placed in the reaching apparatus in pairs during the first 1-3 days of training to decrease anxiety during the habituation period. The reaching apparatus is a Plexiglas chamber (30 x 36 x 30 cm) with an oval opening on one wall (1.5 x 3 cm). Underneath the oval opening there is a shelf where sugar pellets are placed on a grid (see Figure 3.3). Rat pairs were given two 10-minute trials per day (morning/afternoon). The trials consisted of placing 10-15 pellets near the oval opening in order to entice the rats to reach. Once both rats began reaching through the oval opening for sugar pellets, they were separated and singly trained. Training continued daily (Monday through Friday), for two 10-minute trials per day. At this time sugar pellets were placed near the opening, one at a time, to train rats to grasp the pellets with precision. Once rats began showing a limb preference and started grasping the pellets successfully, the pellet was moved slightly farther from the opening and lateral, since Long Evan black-hooded rats are documented to be able to reach out at a considerable distance and tend to reach to the side, instead of straight out. Placing the pellet laterally also dissuades the rat from using their non-preferred forelimb. The rats were slowly challenged to reach for a pellet that was located at a final distance of 1 cm away from the opening (see Figure 3.3). Rats were trained daily, until a success score of 15 out of 20 pellets was obtained for three consecutive days. Once the rat successfully
completed this criterion, it was deemed to be “at baseline”.

**Reaching Task Analysis.** A successful pellet was defined as a pellet that was grasped by the rat with the preferred forelimb off the shelf, pulled back inside the reaching apparatus, and placed in the mouth without being dropped. Rats naturally grasp food with both forelimbs while eating, however some use only their preferred forelimb during eating. Therefore, the contribution of the non-preferred forelimb during eating was not quantified, as long as the pellet was eaten from the preferred forelimb and was not dropped. Additionally, rats typically eat while sitting on their hindlimbs, with their forelimbs lifted against gravity. However, some rats at baseline eat with their forelimbs resting on the ground and move their head down to their paws. Therefore, a successful reach was defined as grasping a pellet and bringing it to the mouth with the forelimbs either held up against gravity or with forelimbs resting on the floor. As a caveat, rats did display increased prevalence of non-preferred forelimb assisted eating, as well as decreased ability to hold forelimbs against gravity post-stroke. However, due to the complexity of testing these behaviors, these changes were not quantified, only noted. The types of errors that led to non-successful pellets were also noted.

During evaluation post-stroke (specific aim 1) and post-treatment (specific aim 2), rats were tested once a day (20 pellet trial), 1-5 days a week. The exact number of testing sessions per week during the different parts of the experiment are summarized in Table 3.5.
Inclusion/Exclusion Criteria:

*Inclusion/exclusion criteria were based on performance in the skilled reaching task only.

A Prior Inclusion Criteria. Rats that were trained on the skilled reaching task to a criterion of 15/20 successful pellets, 3 days in a row, were included in the study and proceeded to undergo ischemic stroke surgery. All animals were trained to this criterion.

A Priori Exclusion Criteria. Following stroke surgery, rats were tested on the skilled forelimb-reaching task weekly. At either 8 or 24 weeks post-stroke, rats were assessed for a new post-stroke baseline, through three days of skilled reaching testing. Any rats that scored more than 10/20 successful pellets were excluded from Aim 2 and Aim 3 experiments, namely treatment with anti-Nogo-A immunotherapy.

Post-Randomization Exclusion Criteria. Additional exclusion criteria were deemed necessary for the PP analysis. For an in depth description of these exclusions, refer to Table 3.4. Briefly, animals were excluded from the PP analysis due to antibody pump technical problems, behavioral abnormalities that interfered with assessment of skilled forelimb reaching score, animals displaying no functional spontaneous recovery post-stroke prior to treatment initiation (success score = 0/20), and severe (> 80%) subcortical striatal damage.
Ladder Rung Walking Task

Walking Task Background. The ladder rung-walking task is a test used to assess skilled walking, limb placement, and limb co-coordination (Metz et al., 2009; Metz and Whishaw, 2002). This task has been found to be sensitive to acute and chronic deficits seen after various motor system lesions, including different models of rat stroke including MCAO (Rieck-Burchardt et al., 2004; Farr et al., 2006; Papadopoulos et al., 2009; Gemerasca Mestriner et al., 2013). Through the use of qualitative and quantitative measures, this task is able to determine subtle losses of movement capacity as well as assess performance during post-lesion recovery periods.

Walking Task Description. The horizontal ladder apparatus consists of two, 1-meter Plexiglas panels connected with irregularly placed metal rungs (minimum inter rung distance of one cm, rung diameter of 3 mm, Fig. 3.4). Animal cages were placed underneath each side of the horizontal ladder, one clean cage at the start and a home cage at the end. Each rat was placed individually in the clean cage and allowed to climb up onto the ladder and to cross the ladder. The rat was motivated to cross the ladder in order to reach their home cage. Rats were habituated to this task over the course of three consecutive days, with one daily 10 minute session or until each rat crossed the ladder three times. Once the rats were habituated to the apparatus, they were videotaped while crossing the horizontal ladder for three trials. The animals were tested using the skilled horizontal ladder walking task across three time-points during the experiments outlined in this dissertation: at baseline (prior to stroke), 8 or 24 weeks post-stroke (prior to
treatment), and at 20 or 32 weeks post-stroke (final testing week post-treatment).

*Walking Task Analysis.* Qualitative and quantitative performance on the ladder rung walking task was assessed using the videotaped trials. Although both forelimb and hindlimb function can be analyzed using this task, only the preferred forelimb (determined by the skilled reaching task) was evaluated. Qualitative analysis of forelimb placement involves analysis of videos frame-by-frame and scoring the foot placements according to a 7-category foot fault scale developed by Metz and Whishaw (2003). The foot fault scoring scale is described in Table 3.6. Briefly, a foot placement is assigned a score of 0 when the limb completely misses the rung and a fall occurs (total miss), a score of 1 when the limb slips off the rung and causes a fall (deep slip), a score of 2 when the limb slips off the rung but the animal is able to maintain balance and keeps stepping (slight slip), a score of 3 when the limb was placed on a rung but moved to another rung before weight bearing (replacement), a score of 4 when the limb was either aimed for one rung and placed on another or when a limb was repositioned on the same rung (correction), a score of 5 when the limb was placed on a rung using either the wrist or digits of the forelimb (partial placement), and a score of 6 when the mid portion of the palm of the limb was placed on the rung with full weight bearing support (correct placement). When more than one error occurred, the lowest score was used. The final score was expressed as the mean foot fault score per step. Quantitative analysis of the skilled horizontal ladder-walking task involved counting the number of foot fault errors that occurred while crossing the ladder. The errors were based off the foot fault scoring
scale and were defined as foot faults with scores 0, 1, and 2, effectively anytime the forelimb slipped off the rung or missed it completely. Foot faults were expressed as the # of foot faults per 10 steps (based on three crossings).

**Bilateral Tactile Adhesive Removal Task**

*Adhesive Removal Background.* Contralateral tactile sensory neglect commonly occurs after cortical or striatal injury in humans and over time the complete sensory neglect can dissipate, leaving a patient with residual tactile extinction deficits (Benton et al., 1952; Benton et al., 1972). A patient with tactile extinction will be able to perceive unilateral tactile stimulation however simultaneous bilateral tactile stimulation will mask the ability of the patient to perceive stimulation on the affected side. However, with time, this tactile extinction deficit can also improve and patients are often left with “obscuration deficits”, where the perception of the contralateral stimulus is reduced. This evolution from complete contralateral tactile sensory neglect to obscurcation has also been demonstrated in rats (Schallert et al., 1984). The bilateral tactile adhesive removal task is a sensitive test for discriminating these types of sensorimotor dysfunctions in rodents, and was originally described by Schallert et al. (1982; 1983). This task, as described by Dr. Schallert was originally based on the human clinical sensory exam used to test sensory neglect, which is called the Double Simultaneous Stimulation Test (DSS). In the tactile DSS exam, patients are presented with tactile sensory stimulation unilaterally and then bilaterally to the same location on each side of the body. This test allows the physician to determine if sensory loss is located in the primary somatosensory cortex as evidenced by
the patient denying unilateral sensation or if there is damage in the higher level sensory association cortex as evidenced by the patient losing the ability to discriminate sensation during bilateral stimulation. The rodent version of this test is a good translational functional outcome assessment as it is directly comparable to the human version of the same test.

**Adhesive Removal Description.** Sticky paper sheets with adhesive backing (Staples, product # 490429) were used to manually cut out small 0.5 inch by 0.5 inch square adhesive stickers (161 mm²). Rats were well handled before attempting this task. Each rat was taken out of its home cage and was held by one investigator while another investigator placed two adhesive stickers on the forelimbs, one on the palmar surface of each forepaw, covering the whole palm and most of the digits, see Figure 3.5. The order of placement (right versus left) was randomized for every trial. Additionally after sticker placement, both forelimbs with the stickers were simultaneously pressed firmly before the trial started. Quickly, before the rat could attempt to remove the stickers, the rat was placed inside the skilled reaching apparatus for the duration of the trial. Before data acquisition started, rats were given one training day consisting of four trials to habituate the rats to the testing procedure. Then the animals were tested using the bilateral tactile adhesive removal task across three time-points during the experiments outlined in this dissertation: at baseline (prior to stroke), at 8 or 24 weeks post-stroke (prior to treatment), and at 20 or 32 weeks post-stroke (final testing week post-treatment).

**Adhesive Removal Analysis.** Each trial consisted of a maximum of 240 seconds (4
minutes). Rats typically contact the stickers with their mouths and removed them using their teeth. During the trial, it was noted which forelimb sticker was touched with the mouth first and which was removed first. The time it took for each forelimb sticker to be contacted (contact latency) and removed (removal latency) was also recorded for each forelimb using four separate stopwatches. Four trials were given per session until a >75% forelimb bias was found, defined as which forelimb sticker was contacted using the mouth first. If a greater than 75% bias for either forelimb was not found, an additional trial was given. The inter-trial rest period lasted 2-3 minutes.

**Magnitude of Sensory Asymmetry Testing**

*Asymmetry Testing Description.* Once a forelimb bias has been found in the bilateral tactile adhesive removal task, the magnitude of sensory asymmetry between the forelimbs can be quantified. The test is conducted by progressively increasing the size of the sticker on the impaired forelimb and decreasing the size of the sticker on the non-impaired forelimb by an equal amount (20.2 mm$^2$) until the rat reverses its bias from the non-impaired forelimb to the impaired forelimb. There are seven levels of magnitude ratios (Schallert et al., 2000; Schallert and Whishaw, 1984). The reversal of this original bias represents the magnitude of sensory asymmetry score and a higher score indicates a greater degree of somatosensory impairment. This testing has been shown to be very sensitive to the extent of brain damage in various lesion models (Schallert et al., 1982; Schallert et al., 1983; Schallert et al., 1984; Schallert et al., 1986; Barth et al., 1990).
Asymmetry Testing Analysis. As described above, there are 7 levels of magnitude differences that each rat is tested with. Testing begins at level 3, and depending on whether the rat takes off the sticker on the impaired or non-impaired forelimb, testing will continue with levels higher or lower. For example, at level 3 if the rat has not reversed its bias for the non-impaired forelimb, the rat will be tested with the level 4 sticker sizes. At this point, if the rat contacts the impaired forelimb first, it will receive a score of 3.5, signifying that the rat switches bias from the non-impaired forelimb to the impaired forelimb somewhere between level 3 and level 4. In addition to the seven levels, if the original bias is towards the impaired forelimb (i.e. at baseline) the scores are given a positive sign, if however the bias is towards the non-impaired forelimb (i.e. post-stroke) the scores are given a negative sign.

Stroke Surgery

Stroke Procedure Background. Human clinical stroke is a heterogeneous disorder with various etiologies, presentations, and recovery profiles. However, 80% of human strokes are ischemic in nature and typically affect the middle cerebral artery territory (AHA, 2013). Therefore, a majority of preclinical studies model human stroke by occluding the middle cerebral artery in the rat. The rat, although not perfect, is a good model for human focal ischemic stroke due to similarities in cerebral vasculature (Macrae et al., 1992), genomics (Ginsberg et al., 1996), and post-stroke deficits (Whishaw et al., 1992). However, when evaluating recovery data post-intervention in the rat it is important to
note that the rodent brain, among other differences, is lissencephalic and has a higher grey matter to white matter ratio than does the human brain.

**Stroke Procedure Description.** A focal ischemic stroke was induced by using the middle cerebral artery occlusion (MCAO) method as described in Chen et al. (Chen et al., 1986), and as in our previous work (Papadopoulos et al., 2002; Markus et al., 2005, Seymour et al., 2005; Papadopoulos et al., 2006; Tsai et al., 2007; Gillani et al., 2009; Tsai et al., 2011). See Figure 3.6 for a surgical view of the MCAO procedure. Rats were anesthetized with isoflurane inhalant anesthesia (3% in oxygen) and body temperature was maintained during the entire procedure. Temperature was maintained using a rectal probe and automatic heating pad during the MCAO procedure and during the transient CCA occlusion temperature was maintained manually with a heating pad and was manually checked using a rectal thermometer. Animals’ core body temperature were maintained at normal physiological ranges, between 99 to 101 degrees F. The bilateral common carotid arteries (CCA) were isolated from an anterior cervical incision and the common carotid artery ipsilateral to the intended ischemic stroke hemisphere was permanently ligated using a 5-0 chromic gut suture. Rats were then placed in a stereotaxic frame and a 2 cm vertical incision was made between the eye and ear and the temporalis muscle was retracted. A burr hole was made to expose the middle cerebral artery (MCA) and it was permanently occluded by cauterization and then transected with microscissors. Lastly, the contralateral CCA was temporarily occluded for 45 minutes using an aneurysm clip. After the aneurysm clip was removed, the surgical wound was
closed and animals were warmed using a heating pad until awake. Animals were monitored during the post-op period until fully awake, and able to eat and drink. If animals showed signs of severe lethargy post-stroke, a 1 cc saline injection was given to protect against dehydration and animals were given standard rat chow dissolved in water to facilitate feeding. Animals were tested for either 8 or 24 weeks to determine sensorimotor deficits prior to antibody infusion as described below.

**Antibody Intracerebroventricular Infusion**

*Antibody Infusion Background.* An intracerebroventricular antibody infusion approach was used in these experiments. This approach is considered to be a clinically viable approach as such delivery systems are already used in humans to administer anti-spastic agents such as baclofen for dystonia and opiates to treat intractable cancer pain (Fleischhack et al., 2005). Previous work from our laboratory has shown that intrathecal injection of anti-Nogo-A antibodies also induces sensorimotor recovery after ischemic stroke in rodents. Therefore, application of anti-Nogo-A antibody is clinically feasible, either through intracerebroventricular infusion or intrathecal delivery. In fact, a phase I clinical trial, testing the safety of intrathecal delivery of anti-Nogo-A immunotherapy in spinal cord injury patients recently was completed and there have been minimal side effects to this treatment.

*Antibody Infusion Description.* After the post-stroke testing period, either 8 or 24 weeks post-stroke, rats were randomized to either stroke only, anti-Nogo-A antibody, or control
antibody treatment groups (see experimental timeline Figure 3.1) with careful attention to balance pre-stroke deficit from post-stroke week 8 on the skilled reaching task. Antibody infusion was initiated at the beginning of either 9 weeks or 25 weeks post-stroke. Rats were placed in a stereotaxic frame and a midline incision was made in the scalp. A burr hole on the same side as the stroke lesion exposed the cortex. A cannula was placed into the lateral cerebral ventricle at coordinates 1.3 mm lateral, 0.8 mm posterior, and 3.8 mm ventral (relative to bregma) and secured to the skull with cyanoacrylate gel. Through a mid-scapular incision an Alzet osmotic mini-pump (model 2ML2; Duract Corporation, Cupertino, CA, USA) was implanted subcutaneously posterior to the scapulae and connected to the cannula with polyethylene tubing. Either purified mouse monoclonal anti-Nogo-A antibody (11C7; IgG1) or control antibody (anti-BRDU, IgG1) was infused at a rate of 15-micrograms/ hour (2.5 mg/ml) for two weeks, after which the animals are again anesthetized and the pumps removed.

**Perfusion and Golgi-Cox Staining**

*Golgi-Cox Method Background.* In 1873 the Italian physician and scientist, Camillo Golgi, discovered what was then called the “black reaction” to stain whole neurons and effectively ushered in a new era of neuroscience discovery. The black reaction became known as the Golgi stain and was improved and used by Ramon y Cajal to study the morphology of neurons and to the eventual birth of the neuron doctrine. Since then, this method has been adapted and used by countless investigators studying neuronal morphology as well as changes to neuronal morphology following various insults.
**Golgi-Cox Method Description.** At either 21 or 33 weeks post-stroke (one week after the end of sensorimotor testing), rats were overdosed with pentobarbital (100mg/kg, i.p.) and transcardially perfused with 0.9% saline and 10,000 U heparin/liter. The brains were removed and immersed whole in Golgi-Cox solution (Glaser et al., 1981) for two weeks. The brains were then coronally sectioned at 200 microns on a vibratome, mounted on 2% gelatinized slides and reacted as described by Gibb and Kolb (Gibb et al., 1998). Slides were coded to blind the experimenters to antibody treatment group. Refer to Figure 3.7 for Golgi-Cox method overview.

**Stroke Lesion Analysis**

**Method Description.** Golgi-Cox stained coronal brain sections were quantitatively analyzed (+4.7 to -5.2 mm from bregma according to Paxinos and Watson, 1998) using a computer-interfaced imaging system (NIH Image) by the method described in Kawamata et al., 1999) (total area of the intact contralateral hemisphere – total area of the intact ipsilateral hemisphere, and multiplied by the total distance between sections). Stroke size was expressed as a percentage of the intact contralateral hemispheric volume, see Figure 3.8.

**Neuroanatomical Analysis**

**Description of Tracing Neurons.** Layer V pyramidal neurons were located within the caudal forelimb area (CFA) region of the unlesioned motor cortex and the perilesional area of the lesioned motor cortex with the aid of an atlas, see Figure 3.9 (Paxinos &
Watson, 2005) and according to previous electrophysiological studies (Neafsey et al., 1986). Criteria for inclusion in the analyses were that the neuron had to be well impregnated, unobstructed by other dendrites, blood vessels or glia cells, and that the dendritic arborization were intact and visible in the plane of the section.

**Neuronal Morphology Analysis.** An average of 6 apical and basilar dendritic trees of layer V pyramidal neurons were traced using Neurolucida software and Leica DM 4000B with a 40x objective. Dendritic trees were analyzed for dendritic length and number of branch segments using the branched structure analysis tool of Neurolucida. Branches emanating from the apical shaft were considered first order as well as branches originating from the cell body (basilar) were considered first order, see Figure 3.10.

**Statistical Analysis for Behavioral Data**

**Skilled Forelimb Reaching Task.** An alpha value of < 0.05 was considered statistically significant for all behavioral data. Skilled reaching data, including post-stroke spontaneous recovery data and anti-Nogo-A immunotherapy data from both ITT and PP datasets, was analyzed initially using a repeated measures analysis of variance (RM ANOVA), as has been done in previous work and most recently in Tsai et al, 2010. However, there are many disadvantages to using the RM ANOVA in the analysis of longitudinal data, most important of which are its inability to analyze data sets with missing data points across time, its assumption that measured values across time points are independent with no correlation, its rigid variance-covariance structure, and its focus
on estimation of group mean trends without information about how specific individuals change across time. A more robust way to analyze longitudinal data is to use non-linear mixed effects modeling to gather information about recovery curves including initial severity, rate of recovery, and final magnitude of recovery reached at the plateau. The biological response to a treatment is often nonlinear and therefore the most accurate way to analyze this type of data is to use a bespoke mathematical equation that will match the data. Therefore, the statistical software SAS was used to run the “nlmixed proc” procedure to generate a non-linear model of the reaching data. The equation that was used for modeling of the reaching data was an exponential function expressed by $\pi = \theta_1 + (\theta_2 - \theta_1) \times \exp(-\theta_3 \times (t-8))$, where $\pi$ signifies probability of success (# of successful pellets out of 20), $\theta_1$ signifies the upper asymptote (final magnitude of success score reached at plateau), $\theta_2$ signifies the y-intercept (initial pre-treatment stroke severity), and $\theta_3$ signifies the rate (rate of recovery). For a graphical display of the nlmixed proc equation, refer to Figure 3.11.

Skilled horizontal ladder walking task. Slips per ten steps were analyzed using a RM ANOVA.

Bilateral adhesive removal task/Magnitude of sensory asymmetry testing. Sensory contact and removal latencies were analyzed using a RM ANOVA.

Neuroanatomical Data. An alpha value of $< 0.05$ was considered statistically significant for all neuroanatomical data. All statistical analyses were performed with
SPSS 17.0 (SPSS, Chicago, IL). Total stroke lesion size was evaluated using a one way ANOVA. Dendritic plasticity measurements were analyzed using a one way ANOVA.
Experimental Design and Timeline

(A) Experimental Timeline

(B) Experimental Overview

Figure 3.1. Experimental Design and Timeline
Comparison of dissertation experimental design and preclinical design recommendations.

<table>
<thead>
<tr>
<th>Recommendation</th>
<th>Dissertation Experiments</th>
<th>Previous Work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomization</td>
<td>Yes; Randomization based on stroke deficit severity pre-treatment.</td>
<td>-</td>
</tr>
<tr>
<td>Blinded Surgery Procedure</td>
<td>Yes; Animals were randomized post surgery.</td>
<td>-</td>
</tr>
<tr>
<td>A priori inclusion/exclusion criteria/ report excluded animals</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Analyze as randomized (ITT)</td>
<td>Yes; ITT and PP analysis performed.</td>
<td>-</td>
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<tr>
<td>Monitor physiological parameters</td>
<td>Yes; Temperature controlled during MCAO</td>
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<td>A priori power analysis</td>
<td>Yes</td>
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<tr>
<td>Use CIs, do not use SEMs</td>
<td>Yes; Both were used</td>
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<td>Professional advice on statistics</td>
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<td>Replicate key findings</td>
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<td>Report mortalities</td>
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<td>Report exact details on animal (sub)strains</td>
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<td>Report sponsorship, conflict of interest</td>
<td>Yes; Anti-Nogo-A antibody was received from Novartis as a gift.</td>
<td>-</td>
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<tr>
<td>Define SOPs (including quality checks)</td>
<td>Yes; As defined in ACORP Details.</td>
<td>-</td>
</tr>
<tr>
<td>Drug (clinically relevant treatment window and delivery route)</td>
<td>Yes; Chronic treatment time point (9 weeks or 25 weeks post-stroke in moderate to severe stroke deficit. ICV delivery.</td>
<td>Intrathecal delivery (Tsai et al., 2007); Established treatment window 24 hours to 9 weeks post-stroke in mild-moderate stroke deficit (Weissner et al. 2003; Seymour et al., 2005; Tsai et al., 2010)</td>
</tr>
<tr>
<td>Blinded drug administration</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>Blinded outcome evaluation</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Use of aged, diabetic, or hypertensive animals</td>
<td>No</td>
<td>Aged rats + stroke, treated at 1 week post-stroke (Markus et al., 2005); Adult Spontaneously Hypertensive Rats (SHR) treated 24 hours post-stroke (Weissner et al., 2003)</td>
</tr>
<tr>
<td>Assessment of both infarct and functional outcome</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Outcome assessment in the chronic phase (7-30 days)</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Dose-response curve performed</td>
<td>No</td>
<td>5 mg versus 1.65 mg (total received) anti-Nogo-A antibody more effective after MCAO in SHR (Weissner et al., 2003)</td>
</tr>
<tr>
<td>Initial rodent studies, then consider gyrencephalic species</td>
<td>Rat study only</td>
<td>Evidence of functional recovery and sprouting shown in primate animals treated with anti-Nogo-A antibodies after spinal cord injury + cortical lesions using ibotenic acid (Fouad et al., 2004; Freund et al., 2006, 2007, 2009); Primates + stroke yet to be tested</td>
</tr>
<tr>
<td>Permanent MCAO then transient MCAO</td>
<td>Permanent MCAO model used only</td>
<td>Transient MCAO not evaluated yet; Other stroke models not evaluated yet</td>
</tr>
<tr>
<td>Using ARRIVE guidelines for reporting in vivo experiments</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Using CONSORT guidelines for reporting RCTs</td>
<td>Yes</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1. Recommendations for improved preclinical trial quality.

Recommendations are summarized from various recommendation statements and reviews regarding preclinical study quality (Fisher et al., 1991; van der Worp et al., 2005; Dirnagl, 2006; Kahle & Bix, 2012; Fisher et al., 2009).
List of Animal Numbers used in ITT and PP Analysis

<table>
<thead>
<tr>
<th>Analysis</th>
<th>11C7</th>
<th>Control Antibody</th>
<th>Stroke Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITT (treatment at 9 weeks post-stroke)</td>
<td>09, 14, 32, 34, 47, 48, 51, 56, 64, 67, 72, 76, 84, 89 (n = 14)</td>
<td>27, 31, 41, 45, 49, 52, 53, 59, 74, 77, 78, 82 (n = 12)</td>
<td>11, 16, 32, 55, 60, 61, 68, 80, 88 (n = 9)</td>
</tr>
<tr>
<td>PP (treatment at 9 weeks post-stroke)</td>
<td>09, 14, 34, 47, 51, 56, 76 (n = 7)</td>
<td>31, 41, 45, 49, 52, 59, 74, 77, 78, 82 (n = 10)</td>
<td>11, 16, 32, 55, 61, 68, 80, 88 (n = 8)</td>
</tr>
<tr>
<td>ITT (treatment at 25 weeks post-stroke)</td>
<td>06, 18, 19, 20 (n = 4)</td>
<td>12, 13 (n = 2)</td>
<td>17, 24 (n = 2)</td>
</tr>
<tr>
<td>PP (treatment at 25 weeks post-stroke)</td>
<td>06, 18, 19 (n = 3)</td>
<td>12, 13 (n = 2)</td>
<td>17, 24 (n = 2)</td>
</tr>
</tbody>
</table>

Table 3.2. List of animals used in the two different analyses used for evaluation of anti-Nogo-A immunotherapy in chronic stroke. An ITT and PP data analysis paradigm was used for evaluating anti-Nogo-A immunotherapy treatment effects in animals treated at 9 or at 25 weeks post-stroke.
## PP Analysis Exclusions

<table>
<thead>
<tr>
<th>Rat ID</th>
<th>Treatment Timepoint</th>
<th>Treatment Group</th>
<th>Pump Disconnect</th>
<th>Absolute Deficit</th>
<th>Severe FL Switching</th>
<th>Severe Striatal Damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP32</td>
<td>9 weeks</td>
<td>11C7</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP48</td>
<td>9 weeks</td>
<td>11C7</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP64</td>
<td>9 weeks</td>
<td>11C7</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP67</td>
<td>9 weeks</td>
<td>11C7</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP72</td>
<td>9 weeks</td>
<td>11C7</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP84</td>
<td>9 weeks</td>
<td>11C7</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>KP89</td>
<td>9 weeks</td>
<td>11C7</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP20</td>
<td>25 weeks</td>
<td>11C7</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP27</td>
<td>9 weeks</td>
<td>Control Ab</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP53</td>
<td>9 weeks</td>
<td>Control Ab</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KP54</td>
<td>9 weeks</td>
<td>Stroke Only</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>KP60</td>
<td>9 weeks</td>
<td>Stroke Only</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3. Exclusion categories for animals excluded in PP analysis.
## Detailed Descriptions of Post-Randomization Exclusions for Per-Protocol Analysis

<table>
<thead>
<tr>
<th>Exclusion Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pump Disconnect</td>
<td>Tubing connecting cannula to osmotic pump was disconnected at removal from animal. Final volume of antibody delivered intracerebroventricularly is unknown. Full treatment regimen not administered.</td>
</tr>
<tr>
<td>Absolute Deficit</td>
<td>During the reaching task, the rat was never able to grasp a pellet successfully during any post-stroke testing time point (post-stroke + post-treatment). This type of rat is unable to perform the reaching task and it is assumed that no recovery of motor function as tested by the reaching task is possible (supported by data).</td>
</tr>
<tr>
<td>Severe FL Switching</td>
<td>After stroke, rat changes forelimb preference and this extreme reliance on the non-impaired forelimb leads to inaccuracy in testing the impaired forelimb (forelimb affected by stroke). Extreme reliance on non-impaired forelimb leads to maladaptive changes in strategies used in reaching such as: posture, aiming, two-handed reaching, learned non-use, learned bad-use. Performance score is deemed inaccurate with a large signal to noise ratio.</td>
</tr>
<tr>
<td>Severe Striatal Damage</td>
<td>Rats with ischemic necrosis leading to a complete loss of the dorsolateral striatum have an inability to initiate forelimb movements and typically show little improvement (ref). Rats displaying grasps in the air for pellets instead of on the shelf typically have striatal damage (ref) and therefore are to be excluded.</td>
</tr>
</tbody>
</table>

Table 3.4. Detailed Descriptions of Post-Randomization Exclusions for Per-Protocol Analysis.
**Comparisons in Rat and Human Reaching**

<table>
<thead>
<tr>
<th>Movement Component</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orientation towards food</td>
<td>Olfaction</td>
<td>Vision</td>
</tr>
<tr>
<td>Advance</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Pronation of paw/hand over food</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Grasp</td>
<td>Whole paw grasp</td>
<td>Pincer grasp (between thumb and index finger)</td>
</tr>
<tr>
<td>Supination of paw/hand with retraction, rotation at wrist, placement of food in mouth</td>
<td>Similar</td>
<td>Similar</td>
</tr>
</tbody>
</table>

**Figure 3.2. Comparisons between limb movements made by normal rats and humans during a reach for food task.** Information in figure modified from Sacrey et al., 2009 and Whishaw et al., 1992.
Success Score: # Successful

**Figure 3.3. Diagram of skilled reaching task.** Rats are trained to reach for sugar pellets placed on a ledge. The number of successful pellet is attained when the rat reaches out with its preferred forelimb, grasps the pellet, pulls the forelimb back inside and brings the sugar pellet to the mouth without dropping it.
### Number of Skilled Reaching Testing Sessions per Time Point

<table>
<thead>
<tr>
<th>Experimental Time Point</th>
<th># of Sessions (days per week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>3</td>
</tr>
<tr>
<td>Post-Stroke Weeks 1-7 or 1-23</td>
<td>1</td>
</tr>
<tr>
<td>Pre-Treatment Baseline</td>
<td></td>
</tr>
<tr>
<td>(post-stroke week 8 or 24)</td>
<td>3</td>
</tr>
<tr>
<td>Treatment Weeks</td>
<td></td>
</tr>
<tr>
<td>(post-stroke weeks 9-20 or 25-32)</td>
<td>3-5</td>
</tr>
</tbody>
</table>

Table 3.5. Number of Skilled Reaching Testing Sessions per Time Point
Figure 3.4. Diagram of Skilled Horizontal Ladder Walking Task. A normal rat can easily traverse the horizontal ladder with little to no errors (1 slip or less). After brain injury, the number of slips and errors increases.
Foot Fault Scoring Scale

<table>
<thead>
<tr>
<th>Category</th>
<th>Points</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Miss</td>
<td>0</td>
<td>Forelimb completely misses a rung; a fall occurs</td>
</tr>
<tr>
<td>Slight Slip</td>
<td>1</td>
<td>Forelimb slips off rung with weight bearing; a fall occurs</td>
</tr>
<tr>
<td>Deep Slip</td>
<td>2</td>
<td>Forelimb slips off with weight bearing; no fall occurs</td>
</tr>
<tr>
<td>Replacement</td>
<td>3</td>
<td>Forelimb placed on a rung but then replaced on another</td>
</tr>
<tr>
<td>Correction</td>
<td>4</td>
<td>Forelimb aimed for one rung but placed on another or repositioned on the same rung</td>
</tr>
<tr>
<td>Partial Placement</td>
<td>5</td>
<td>Forelimb placed on rung with either wrists or digits of forelimb</td>
</tr>
<tr>
<td>Correct Placement</td>
<td>6</td>
<td>Mid portion of palm of forelimb is placed on rung with full weight bearing</td>
</tr>
</tbody>
</table>

*Table information modified from Metz et al., 2009

Table 3.6. Overview of foot fault scoring system. The table describes the scoring system based on assigning points (0 to 6) for each foot fault category. Information in the table is modified from Metz et al., 2009.
Bilateral Adhesive Removal Task

Figure 3.5. Diagram of bilateral adhesive removal task.
**MCAO Procedure**

- Dissect contralateral CCA
- Untied suture left as marker

- Dissect Ipsilateral CCA
- Permanent occlusion with suture ligation

- Craniotomy to expose MCA
- Permanent occlusion of MCA using suture ligation

- Transient 45-minute occlusion of contralateral CCA

**Figure 3.6. MCAO procedure.** Procedure shown involves a Fisher 344 rat, however the procedural steps are performed identically in either Fisher 344 or Long Evans black- hooded rats. Pictures compliments of Daniel Sheperd.
Golgi-Cox Method for Staining Neuronal Morphology

Sacrifice study animals → Place whole brain into Golgi-Cox Solution 14 days → Coronal sections at 200 μm Golgi Reaction

Dendritic Morphology Analysis
Neurolucida Software

Neurons filled with crystallized precipitate

Figure 3.7. Golgi-Cox staining procedure.
Analysis of Stroke Volume and Lesion Location

Lesion Volume % = \frac{(\text{Non-lesioned ROI Volume} - \text{Lesioned ROI Volume})}{\text{Non-lesioned ROI Volume}} \times 100

Figure 3.8. Analysis of Stroke Volume and Lesion Location
Selection of Neurons

Figure 3.9. Location of selected neurons.
Analyzing Dendritic Morphology

Figure 3.10. Dendritic Analysis. (A) Representative neuron morphology. (B) Example of branching pattern and numbering scheme.
Non-Linear Mixed Effects Modeling of Skilled Reaching

Figure 3.11. Graphical display of equation used for non-linear mixed effects modeling of skilled reaching data.

Non-Linear Equation =

\[ \pi = \theta_1 + (\theta_2 - \theta_1) \times \exp(-\theta_3 \times (\text{time} - 8)) \]
CHAPTER FOUR

TIME COURSE OF SPONTANEOUS SKILLED REACHING RECOVERY POST-STROKE

ABSTRACT

Following human clinical stroke, spontaneous recovery of function is limited but does occur. The majority of spontaneous recovery of motor function occurs within 3 month post-stroke, with small improvements in function seen up to 6 months post-stroke and beyond. Severity at initial stroke onset is considered to be the best prognostic factor in determining recovery after stroke in humans. Patients with mild to moderate stroke deficits immediately after stroke improve faster and better than patients with severe stroke deficits. New therapeutic strategies are commonly tested in rat models of ischemic stroke, including the middle cerebral artery occlusion model. In order to increase the successful translation of preclinical findings to human trials it is important to model human stroke appropriately in the rodent models used in preclinical studies. Therefore, prior to testing the efficacy of anti-Nogo-A immunotherapy in the chronic phase after stroke in adult male rats, we set out to evaluate and compare the spontaneous motor recovery profiles in adult rats after stroke to that of humans after stroke. Adult male rats underwent a middle cerebral artery occlusion and were evaluated for motor recovery in skilled reaching performance for 8 weeks and 24 weeks post-stroke. On average, rats showed a profound impairment on the skilled reaching task at post-stroke week 1. Similar
to humans, animals displayed different recovery profiles based on initial deficit severity immediately after stroke. Overall, spontaneous recovery was seen in the first few weeks after stroke, with no improvements seen beyond 4 weeks (one month) post-stroke, even in animals evaluated out to 24 weeks post-stroke (6 months). Similar to the recovery profile seen in humans, the majority of spontaneous motor recovery occurs early post-stroke with essentially little to no improvements seen beyond 6 months post-stroke.
INTRODUCTION

Neuronal death and damage following ischemic stroke leads to permanent deficits in motor, sensory, language, and cognitive function. The potential burden of disability post-stroke is very large and with the world’s population aging, the magnitude of disability and loss of function is likely to increase exponentially (WHO, 2011; United Nation, 2001).

Treatment options post-stroke are fairly limited, and often the patient is faced with limited options for improving recovery after stroke. One mechanism of improved function after stroke is due to spontaneous recovery. Although limited, spontaneous recovery occurs to varying degrees following stroke. Numerous studies have shown that initial clinical severity after stroke is the strongest prognostic factor for spontaneous clinical recovery after stroke in humans (Jorgensen et al., 1995; Jorgensen et al., 1996; Jorgensen et al., 2000). Overall, most spontaneous recovery occurs before 6 months post-stroke. In the Copenhagen study (Jorgensen et al., 2000), it was estimated that 80% of patients reach their best neurological recovery within 4.5 weeks, with varying time profiles based on initial severity post-stroke. Therefore, clinical stroke recovery profiles vary from patient to patient based on initial clinical severity, potential for spontaneous recovery, individual potential for neuroplasticity, and other various factors. Generally, it is thought that the majority of spontaneous recovery plateaus by 6 months post-stroke leaving a patient with little options for further improved recovery in the chronic phase.

Hence, there is a need for the development of therapeutics options that can improve functional recovery post-stroke in the chronic stable phase after spontaneous
recovery has ended. One such promising therapy is anti-Nogo-A immunotherapy (Tsai et al., 2010). By inhibiting the protein Nogo-A, the regenerative potential of neurons is enhanced, and it is possible to induce reorganization of neural circuits, leading to functional recovery. In order to improve the translatability of preclinical findings using this therapy to human clinical trials it is important to model human stroke appropriately in preclinical studies using this therapy. Therefore, before the application of anti-Nogo-A immunotherapy in the chronic phase after stroke, we set out to evaluate the time course of spontaneous sensorimotor recovery in the adult rat after stroke up to 24 weeks post-stroke (6 months) and to compare it to the recovery patterns seen in human stroke recovery. Rats underwent a stroke procedure by permanent occlusion of the middle cerebral artery and sensorimotor recovery was assessed weekly for 8 or 24 weeks post-stroke using the skilled forelimb reaching task.
RESULTS

Spontaneous recovery of skilled reaching success score over 24 weeks post-stroke

Rats were trained to reach a baseline criterion of 15 successful pellets out of 20 on the skilled forelimb reaching task. Then skilled forelimb reaching recovery was assessed for a total of 8 weeks post-stroke in a cohort of 66 rats and was assessed for an additional 24 weeks post-stroke for a subset of the former cohort, in 7 rats (see Figure 4.1A and 4.1B). Analysis of the first 8 weeks post-stroke showed a significant main effect of time during the post-stroke recovery phase, Figure 4.1A (p < 0.001, repeated measures ANOVA). Initially, all animals exhibited a profound decrease in skilled reaching performance at 1 week post-stroke (p < 0.001). On average, rats improved until post-stroke week 2 (0.019) and then reached a recovery plateau until post-stroke week 7. Reaching success was significantly different between post-stroke week 7 and week 8.

Analysis of the smaller cohort assessed out to 24 weeks post-stroke (Figure 4.1B) showed that there was also a significant main effect of time on skilled reaching performance (p <0.033). Following stroke, there was a profound decrease in skilled reaching performance at post-stroke week 1 (p < 0.001). There was also a significant difference between skilled reaching performance between post-stroke week 1 and post-stroke week 4 (p = 0.022). Further statistically significant improvements in skilled reaching performance were not seen beyond post-stroke week 4.
**Recovery profiles based on initial skilled reaching severity at post-stroke week 1**

Animals were categorized into four stroke severity deficit groups based on the skilled reaching score at post-stroke week 1, Figure 4.2A. Figure 4.2B shows the scoring criteria used to categorize animals to mild, moderate, severe, and very severe stroke deficit groups.

Repeated measures ANOVA showed that there was a significant interaction between stroke severity category and skilled reaching recovery over time (p = 0.001). On average across all deficit categories, post-stroke recovery plateaued by post-stroke week 4. Bonferroni post-hoc testing showed that recovery profiles between the mild and moderate categories was not significantly different across time (p = 0.203). However, the recovery profiles of the severe and very severe deficit groups were different from the either the mild or moderate recovery group and different from each other (all p values < 0.01) (see Figure 4.2A).

Recovery profiles for animals in each stroke severity category were analyzed separately to determine the time course of recovery. Since the recovery profiles of mild and moderate stroke severity animals was found to be non-significant, these two categories were combined to evaluate the time course of recovery. Post-stroke, animals in the combined mild and moderate deficit category did not exhibit a significant change over time in their skilled reaching performance after stroke (p = 0.085, repeated measures ANOVA).

Animals in the severe stroke category displayed a change in skilled reaching recovery over time (p < 0.001, repeated measures ANOVA). Skilled reaching
performance improved from post-stroke day 3 over time up to post-stroke week 3 (p < 0.001). No significant improvements were seen beyond post-stroke week 3 (p > 0.05).

Animals in the very severe category showed no improvements across time and were never able to grasp and bring any pellets to the mouth on any attempt out of 20 during the skilled reaching task.

**Body weight of rats post-stroke**

The body weights of rats post-stroke across the 4 stroke severity deficit categories was compared. There was no different between the weight of rats in any stroke deficit severity group across time (p = 0.577, Repeated Measures ANOVA), see Figure 4.3.

**No correlation between skilled reaching performance and post-stroke body weight**

Rats were food restricted in order to provide motivation for performing the skilled reaching task. Therefore it was evaluated whether there was a correlation between weight and success score at baseline or post-stroke week 8 across all rats post-stroke. There was no significant correlation between either baseline weight (p = 0.091, Pearson’s Correlation) or post-stroke weight (p = 0.901, Pearson’s Correlation) with performance on skilled reaching.

**Positive correlation between initial skilled reaching impairment and final reaching recovery**
The correlation between skilled reaching success score at early time points post-stroke was correlated with final reaching recovery at post-stroke week 8 (see Table 4.1). Post-stroke severity at post-stroke week 1 (p < 0.01, Pearson’s Correlation) and post-stroke week 4 (p < 0.01, Pearson’s Correlation) was strongly correlated with post-stroke week 8 recovery.

**No difference in skilled reaching performance at baseline based on skilled reaching forelimb preference**

Rats were trained to perform the skilled reaching task to a criterion of 15 successful pellets out of 20. Most rats were trained to this criterion within 2-4 weeks. Rats exhibit a forelimb preference when trained on the skilled reaching task. The majority of rats displayed a purely single forelimb preference (right or left) on the skilled reaching task (78%) while a minority displayed ambidextrous reaching patterns (22%), Figure 4.4A. Based on these categories, 49% of rats had a right forelimb preference, 29% had a left forelimb preference and 22% were ambidextrous. A chi squared test of the distribution forelimb preferences among the rats indicated that the distribution was significantly different (p = 0.019). A larger proportion of rats displayed a single right forelimb preference than a left preference or ambidexterity. Ambidextrous rats were not excluded from the study since it was possible to elicit a largely single forelimb reach by placing the sugar pellet very far lateral. Including ambidextrous rats, a significantly larger proportion of the total, 66% of rats displayed primarily a right forelimb preference, while 34% of rats displayed a largely left forelimb preference (p = 0.009). Neither primary forelimb
preference nor ambidexterity influenced performance on the skilled reaching task at baseline, Figure 4.4.B. There were no differences in mean skilled reaching performance across animals with different forelimb preferences (p = 0.731, one-way ANOVA).

**No difference in skilled reaching performance at post-stroke week 8 based on baseline forelimb preference**

The final skilled reaching recovery score at post-stroke week 8 was evaluated by original forelimb preference (right, left, ambidextrous right, and ambidextrous left) at baseline. There was no difference between the scores at post-stroke week 8 on the skilled reaching task based on original forelimb preference determined at training, Figure 4.5 (p = 0.510, one-way ANOVA).

**Increased prevalence of forelimb preference change post-stroke and a correlated decrease in skilled reaching performance**

Following stroke, there was an increase in the reliance of animals using the unimpaired forelimb to reach for pellets in the skilled reaching task, Figure 4.6A. The percent of animals with solely a right forelimb preference dropped to 28% after stroke versus a baseline value of 49%. Likewise, the percent of animals with solely a left forelimb preference decreased to 18% after stroke compared with a baseline value of 29%. The percentage of animals displaying ambidextrous reaching patterns increased to 53% after stroke compared with a baseline value of 22%.

Due to the increased prevalence of animals using the unimpaired forelimb after stroke, we evaluated whether animals with a change in forelimb preference had an effect
on recovery of skilled reaching ability at post-stroke week 8. First we evaluated whether there was a difference between animals with a right or left preference within switching categories, i.e. animals that never relied on the unimpaired forelimb, animals that were ambidextrous from baseline, and animals that began to rely on the unimpaired forelimb after stroke. There was no difference in post stroke week 8 success scores among animals with right or left forelimb preferences that never used the unimpaired forelimb (p = 0.323), were ambidextrous from baseline (p = 0.348), or that developed a stroke preference for the unimpaired forelimb (p = 0.473). Since there was no effect of original right or left forelimb preference and switching status, it was evaluated whether overall there was a difference in skilled reaching success score at post-stroke week 8 across animals that never switched (right and left preferences combined), animals that were ambidextrous from baseline (right and left preferences combined) or animals that developed a new preference for the unimpaired forelimb (right and left preferences combined). Switching status had a significant effect on post-stroke week 8 success score (p = 0.004). There was no difference between animals that never switched and those that were ambidextrous from baseline (p = 0.066). The skilled reaching scores at post-stroke week 8 were also not significantly different between the ambidextrous group and the group that developed a new forelimb preference (p = 1.0). However, there was a significant difference in the skilled reaching success score at post-stroke week 8 between animals that never switched and animals that developed a new forelimb preference after stroke (p = 0.006), Figure 4.6B.
Figure 4.1. Spontaneous recovery of skilled reaching success score over 24 weeks post-stroke. (A) shows how skilled reaching performance changed over the course of 8 weeks post-stroke. Initially, there was a significant decrease in the skilled reaching success score at 1 week post-stroke (p < 0.001). The success score improved until post-stroke week 2 (p = 0.019) with no further improvements until post-stroke week 8 (p = 0.001). (B) shows how skilled reaching performance changed over the course of 24 weeks post-stroke in a subset of animals. Similarly to the larger cohort, there was a significant decrease in the skilled reaching success score at 1 week post-stroke (p < 0.001). Furthermore, there was an improvement in success score from week 1 to week 4 post-stroke (p = 0.022), with no further improvements seen at post-stroke week 8 or 24. *p < 0.05, **p < 0.01, ***p < 0.001 comparing the current week to the previous week, repeated measures ANOVA. Error bars denote ± standard error of the mean.
Figure 4.2. Spontaneous skilled reaching recovery based on initial severity at post-stroke week 1. Animals were categorized into four stroke severity groups based on the skilled reaching score attained at post-stroke week 1. (A) shows the recovery profiles of animals based on mild, moderate, severe, and very severe deficit categories. The recovery profiles of the mild and moderate stroke groups were statistically insignificant (p = 0.203, repeated measures ANOVA). However, recovery profiles for the severe and very severe stroke groups were significantly different from that of the mild and moderate stroke groups (p < 0.01) and different from each other (p < 0.01). Animals within the mild and moderate stroke deficit categories displayed no significant improvement of reaching success score over time (p = 0.085). Animals with a severe stroke deficit improved until post-stroke week 3 (p < 0.001). No improvements were seen in the very severe deficit animals. (B) shows the criteria used to categorize animals into mild, moderate, severe, and very severe stroke deficit groups. Error bars denote ± standard error of the mean.
Figure 4.3. **Body weight of rats post-stroke.** Rats that underwent stroke were weighed once a week starting at week 0 (pre-stroke) and lasting through week 8 post-stroke. Weight was considered as a measure of health and simultaneously as an indirect measure of motivation to perform the food restriction based skilled reaching task. There was no difference in the body weight of rats across any stroke deficit category (mild, moderate, severe, very severe) across time ($p = 0.577$). Error bars denote ± standard error of the mean.
Correlation between Weeks Post-Stroke and Success Score

<table>
<thead>
<tr>
<th>Time Point</th>
<th>PSWk1</th>
<th>PSWk4</th>
<th>PSWk8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSWk1</td>
<td>--</td>
<td>0.649**</td>
<td>0.753**</td>
</tr>
<tr>
<td>PSWk4</td>
<td>0.649**</td>
<td>--</td>
<td>0.798**</td>
</tr>
<tr>
<td>PSWk8</td>
<td>0.753**</td>
<td>0.798**</td>
<td>--</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01

Table 4.1. Pearson’s correlations between skilled reaching success scores across different post-stroke weeks.
Figure 4.4. The effect of forelimb preference on skilled reaching success score at baseline. At the start of training, all rats use both forelimbs to make largely unsuccessful attempts for pellets. With training, most rats begin to show a forelimb preference and pellet retrieval improves. (A) Once baseline criteria are attained, the majority of rats (78%) show a single forelimb preference on the skilled reaching task, with a minority displaying ambidextrous reaching patterns (22%). (B) There were no differences in mean skilled reaching performance across animals with different forelimb preferences (p = 0.731). Error bars denote ± standard error of the mean. FL = forelimb, Ambi = ambidextrous
Skilled Reaching Performance at Post-Stroke Week 8 based on Baseline Forelimb Preference

Figure 4.5. Relationship between baseline forelimb preference and post-stroke week 8 reaching score. Forelimb preference determined at baseline (right, left, ambidextrous right, ambidextrous left) had no effect on the final skilled reaching success score at post-stroke week 8 (p = 0.510). Error bars denote ± standard error of the mean. FL = forelimb, Ambi = ambidextrous
Figure 4.6. Increased reliance on unimpaired forelimb and correlated decrease in skilled reaching performance. (A) Following stroke, the number of animals using unimpaired forelimb to reach for sugar pellets during the skilled forelimb reaching task increased by 31%. (B) There was a significant difference in the skilled reaching success score at post-stroke week 8 in animals that never changed forelimb preference and animals that changed forelimb preference after stroke (p = 0.006). Animals that changed preference after stroke and used their unimpaired forelimb as well as their original reaching forelimb had a lower average skilled reaching score at post-stroke week 8 than animals that did not use their unimpaired forelimb during reaching. Note: attempts with the unimpaired forelimb were not counted in the success score. Only attempts with the training defined ‘preferred limb’ were counted towards the success score. Error bars denote ± standard error of the mean. **p < 0.01
DISCUSSION

In this study we have demonstrated that spontaneous sensorimotor recovery takes place in adult rats post-stroke as measured by the skilled forelimb reaching task. The time course and magnitude of recovery is related to initial stroke deficit severity at post-stroke week 1. Overall, animals displayed spontaneous sensorimotor recovery on the skilled reaching task within one month (4 weeks) post-stroke with no further improvements in skilled reaching performance out to 6 months (24 weeks) post stroke. Interestingly, the time course of recovery of adult animals post-stroke is similar to reports of human post-stroke recovery. Therefore, the rat model of ischemic stroke (MCAO) is a valid model that can be used to study treatment effects in the acute and chronic phases after stroke.

Overall, in rats that were evaluated for 8 weeks post-stroke, the rats displayed a large decrease in skilled reaching ability post-stroke at week 1. Spontaneous recovery of function was seen up to week 2 post-stroke. Beyond the second week post-stroke, no further statistically significant improvements in reaching ability were seen up to post-stroke week 7. However, a statistically significant increase in skilled reaching performance was also seen from post-stroke week 7 to post-stroke week 8. It is likely that this improvement in function, after a total of 5 weeks of plateau in skilled reaching success score is related to a change in the frequency in testing. Animals were tested once a week during post-stroke weeks 1-7 and were tested three times during post-stroke week 8. Therefore, the increase in the pellet score likely represents a practice effect and not true recovery. A similar recovery pattern was found in the smaller subset of animals that
were evaluated for a total of 24 weeks. Rats displayed a large decrease in skilled reaching performance at post-stroke week 1, with improvement in skilled reaching score occurring between post-stroke week 1 and post-stroke week 4. Statistically significant improvements were not seen beyond post-stroke week 4 until the end of the assessment of spontaneous recovery at post-stroke week 24. Overall, the majority of improvements seen post-stroke take place within two weeks post-stroke with an unequivocally stable deficit seen at 6 months post-stroke. The time course for spontaneous recovery of skilled forelimb reaching in these experiments is supported by previous studies in our lab that used a stroke only group as a control group during other anti-Nogo-A immunotherapy experiments. On average, spontaneous skilled reaching improvements were seen in stroke only animals up to 2-4 weeks post-stroke, mirroring the results in the present experiments (Papadopoulos et al., 2002; Seymour et al., 2005; Tsai et al., 2007).

In humans, the most valid predicting factor for stroke recovery is the magnitude of initial stroke severity. Therefore, we evaluated if initial stroke deficit as measured by the skilled reaching task affected the recovery profile of adult rats post-stroke. A strong correlation between success score at early post-stroke weeks (week 1 and 4) was found with the recovery success score at post-stroke week 8. Therefore, it is likely that initial stroke deficit is a factor in the prognosis of stroke recovery in rats. To further assess the relationship between recovery and initial deficit in rats we categorized the rats into 4 stroke deficit categories based on post-stroke week 1 (mild, moderate, severe, and very severe) and analyzed the corresponding recovery profiles.
Animals in the mild deficit category (n = 5) displayed a small statistically insignificant improvement from day 3 post-stroke to post-stroke week 1. No further improvements over time were seen up to post-stroke week 8. Since the deficits in this group were so mild, improvements post-stroke were not detectible possibly due to a ceiling effect. This has also been demonstrated in human stroke recovery as well (Duncan et al., 1992). Patients with very mild post-stroke deficits display very little to no measureable improvements over time, and any improvements that do occur do so within the first month post-stroke. Since this population has near normal use of the forelimb (rat) or upper extremity (human) post-stroke it is less likely to show significant improvements in function. Due to the low level of deficits and no recovery seen in the mild deficit category, animals in this deficit category would be unlikely to show improvements in recovery due to treatment interventions or rehabilitation.

The recovery profile of animals in the moderate deficit category was statistically indistinguishable from the recovery profile seen in animals with mild initial deficits. However, it is possible that due to the low number of animals in this group (n = 6) there was not enough statistical power to distinguish between the recovery profiles of mild and moderate deficit animals. Animals in the moderate deficit category displayed lower reaching success scores at post-stroke week 1 than the mild deficit category, but over time, their success scores improved and at post-stroke week 7 and 8 became indistinguishable from that of the mild deficit category. Animals in the moderate deficit category start from a greater post-stroke deficit and although statistically insignificant,
display an increased rate of recovery over the first weeks post-stroke, eventually reaching the same overall recovery plateau as the mild deficit animals.

Animals in the severe deficit category (n = 43) show a slow and steady rate of improvement from post-stroke day 3 to post-stroke week 3. From post-stroke week 3 to post-stroke week 7 no statistically significant improvements area seen. A statistically significant increase in skilled reaching success score is seen from post-stroke week 7 to post-stroke week 8. As discussed previously, this is likely due to the change in testing frequency from week 7 to week 8. The severe deficit animals were shown to be the most sensitive to this increase in testing frequency among the deficit categories. Additionally, primarily among the severe and very severe deficit category animals, we observed an increase in animals switching their forelimb preference post-stroke and relying on the unimpaired forelimb during skilled reaching. Although, attempts with the unimpaired forelimb were not counted as an attempt, the use of the unimpaired forelimb may have affected the ability of the rat to successfully grasp the pellet with its impaired forelimb. Comparison of post-stroke week 8 success scores in animals that had no change in their forelimb preference post-stroke to animals that changed their forelimb preference to the unimpaired forelimb post-stroke showed that there was a statistically significant decrease in the mean success score at post-stroke week 8. There have been reports in the literature that large cortical lesions are more likely to cause a change in forelimb preference (Castro-Alamancos et al., 1991) and therefore would support our finding of increased forelimb preference change among animals in the severe and very severe deficit
categories. The decrease in the mean recovery success score in the animals with forelimb preference change may have be due to the switching behavior interfering in obtaining successful pellets with the impaired forelimb, or it may have been simply a symptom of the greater stroke severity in these animals that leads to the lower success score and higher prevalence of switching behavior.

Animals in the very severe deficit category \((n = 12)\) immediately after stroke were not able to successfully grasp and place a pellet in the mouth during the skilled reaching task. However, the rats did attempt to obtain pellets, and sometimes would drag the pellet into the cage and drop it or would knock it away. This group of animals showed absolutely no change in reaching performance over the course of 8 weeks. This group may be the hardest to rehabilitate and may not show improvements in skilled reaching even with rehabilitation or restorative treatments. Patients with very little to no recovery post-stroke have also been reported in stroke literature (Prabhakaran et al., 2007; Stinear et al., 2012). This absolute deficit population may not show traditional improvements in sensorimotor function but may instead show improvements in ancillary measures such as spasticity or shoulder pain (Stinear et al., 2012).

Given that animals in the mild and moderate deficit categories showed recovery up to 65\% of baseline performance it is likely that treating this group of animals with anti-Nogo-A immunotherapy may not show statistically detectible improvements due to a ceiling effect. Since the severe stroke category only showed recovery up to 25\% of baseline performance, it may be more likely that treating these animals with anti-Nogo-A immunotherapy would show an effective improvement in function because there would
be more room for anti-Nogo-A immunotherapy effects to be seen. Finally, the very severe stroke category could be treated with anti-Nogo-A immunotherapy, however this group may be too severely damaged to show improvement.

One of the potential limitations to the interpretation of recovery profiles based on initial severity is the unequal distribution of rat numbers within the different deficit categories. The mild and moderate deficit categories each had approximately 5 rats while the severe category had 43 rats and the very severe category had 12 rats. The low number of animals in the mild and moderate deficit categories may have led to the inability to detect statistical differences between these two recovery groups. Additionally, since the distribution of deficits post-stroke was so heavily localized in the ‘severe’ deficit category, it is possible that there are additional clusters of recovery trajectories within this category. Further analysis of this data could use initial severity post-stroke as well as stroke size and location in order to predict recovery clusters. Additionally, since post-stroke recovery in rats and humans has been shown to progress non-linearly (Jorgensen et al., 1995), the data could be analyzed using a non-linear model with initial severity and stroke lesion characteristics as covariates in order to further characterize spontaneous recovery post-stroke in adult rats.

In conclusion, animals undergoing ischemic focal stroke undergo a short spontaneous recovery time course, up to 4 weeks depending on severity of initial deficit, and do not show further improvements over time up to post-stroke week 24 (6 months) on the skilled forelimb reaching task. Therefore, when treatments or rehabilitative
strategies are initiated after 1 month post-stroke in adult rats, regardless of initial severity, any improvements seen are likely to be due to the therapy and not spontaneous recovery.
CHAPTER FIVE

SENSORIMOTOR RECOVERY AND DENDRITIC PLASTICITY AFTER CHRONIC TREATMENT DELAY USING ANTI-NOGO-A IMMUNOTHERAPY IN THE ADULT RAT WITH MODERATE TO VERY SEVERE STROKE DEFICITS.

ABSTRACT

Yearly, more than 700,000 people in the United States suffer from a new or recurrent stroke and the majority are left with permanent life changing impairments. Therefore, the development of strategies to enhance neural repair following stroke is of utmost clinical importance. One promising new approach is to inhibit the Nogo-A protein, a myelin associated protein that acts to restrict neuronal plasticity in the adult central nervous system. A previous study from our laboratory showed that inhibition of protein Nogo-A at 9 weeks post-stroke in rats with mild to moderate deficits resulted in sensorimotor recovery and was associated with axonal plasticity (Tsai et al., 2010). The current study was designed to further evaluate the treatment time window for the administration of anti-Nogo-A immunotherapy in the chronic period following stroke. We aimed to examine the extent functional recovery in rats with moderate to very severe chronic sensorimotor deficits. Adult rats were trained on the skilled forelimb reaching task and subsequently underwent a unilateral permanent middle cerebral artery occlusion. Rats received anti-Nogo-A antibody, control antibody, or no treatment at one of two delayed treatment time points: 9 weeks post-stroke (subchronic) or 25 weeks post-stroke.
(chronic), approximately corresponding to 2 and 6 months post-stroke. Sensorimotor performance was assessed up to 12 weeks following treatment initiation and was evaluated using an intention-to-treat and per-protocol analysis. Following testing, rats were sacrificed and brain tissue was processed and stained to evaluate dendritic morphology using the Golgi-Cox method. Layer V pyramidal neurons in the perilesional caudal forelimb motor cortex and layer II/III and layer V pyramidal neurons in the contralesional caudal forelimb motor cortex were evaluated for dendritic tree complexity. Both intention-to-treat and per-protocol analysis showed that anti-Nogo-A immunotherapy administered at either 9 or 25 weeks post-stroke resulted in improved sensorimotor recovery on the skilled forelimb reaching task but no significant recovery was seen in sensory function or skilled walking. The improved performance on the skilled reaching task was not associated with dendritic changes in layer V pyramidal neurons in the perilesional caudal forelimb motor cortex or layer II/III or layer V pyramidal neurons in the contralesional caudal forelimb motor cortex. Our finding of improved sensorimotor recovery even when treatment was started 25 weeks post-stroke demonstrates the promising therapeutic potential for anti-Nogo-A immunotherapy to treat stroke sufferers long after the initial ischemic damage has taken place.
INTRODUCTION

Stroke is a leading cause of death and the primary cause of serious long-term
disability in the United States (AHA, 2013; CDC, 2005). Neuronal damage subsequent to
the interruption of blood supply in the brain leads to the profound sensorimotor,
linguistic, and cognitive deficits seen following stroke. Stroke is classified into two main
categories, ischemic or hemorrhagic. The majority of strokes are ischemic in nature
(87%), while the rest are either intracerebral hemorrhages (10%) or subarachnoid
hemorrhages (3%) (Woo et al., 1999). It is estimated that there are over 6 million stroke
survivors living in the United States at the present time and over 700,000 new or
recurrent strokes take place each year (AHA, 2013). Given these statistics, it is clear that
the potential burden of disability seen after stroke is profound. In fact, 80% of ischemic
stroke survivors over the age of 65 have hemiparesis or cannot walk without assistance.
Additionally troubling is the fact that up to 26% of these ischemic stroke survivors are
institutionalized in a nursing home and lose all ability for independent living (Kelly-
Hayes et al., 2003).

Unfortunately, treatment options following ischemic stroke are limited, and
therefore exacerbate the magnitude of disability seen after stroke. Acutely after ischemic
stroke, the only therapeutic option is to use thrombolytic therapy to lyse the blood clot
causing the ischemia. However, the use of tissue plasminogen activator (tPA) is only
effective when used within 4.5 hours of stroke onset, and its efficacy at promoting
improvement of function is greatest when applied early in the 4.5-hour time window
(Hacke et al., 2008). Given the short treatment time window of tPA therapy, only a small
percentage of patients (4.3%) receive the drug (CASPR Investigators, 2005). With the majority of ischemic stroke patients not receiving thrombolytic therapy, patients can only hope for recovery of function through spontaneous recovery mechanisms and their potential augmentation through stroke rehabilitation.

Given the fact that spontaneous recovery is the main mechanism available for recovery after stroke, it is important to take a pragmatic view of its efficacy. Although real gains in function can occur through spontaneous recovery, in general, the recovery potential after moderate to very severe strokes is generally limited even with the addition of rehabilitative approaches. This is due to the limited ability of the adult mammalian central nervous system (CNS) to engage in restorative regeneration and neuroplasticity after injury. The lack of widespread regeneration and neuroplasticity in the CNS after injury is in part related to the inhibitory environment that surrounds neurons. The main components of this growth restrictive environment include inhibitory proteins that are associated with myelin. The most potent of these myelin inhibitory proteins is Protein Nogo-A (Gozenbach and Schwab, 2008). Protein Nogo-A is expressed on the membrane of oligodendrocytes and restricts the outgrowth of neurites after CNS injury (Caroni et al., 1988; Caroni Schwab, 1988; Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Therefore, the neutralization of Nogo-A has become a fascinating and active avenue for preclinical and clinical investigation as a therapeutic approach to improving CNS regeneration and neuroplasticity and thereby functional recovery after CNS injury.

Previous studies from our laboratory have shown that inhibition of the protein Nogo-A after focal ischemic stroke in the adult rat immediately and one week post-stroke
results in significant recovery of skilled reaching ability. This improved recovery was associated with axonal and dendritic plasticity in the contralesional sensorimotor cortex (Papadopoulos et al., 2002; Seymour et al., 2005; Papadopoulos et al., 2006). In order to properly model human stroke, anti-Nogo-A immunotherapy was also tested in aged rats one week post-stroke. The skilled reaching recovery results mirrored the effects seen in adult rats, albeit with a slower rate of improvement (Markus et al., 2005). Most recently, our lab began investigating the use of anti-Nogo-A immunotherapy in chronic stroke. A small set of animals with mild to moderate stroke deficits were treated with anti-Nogo-A immunotherapy 9 weeks post-stroke and evaluated for skilled reaching recovery. Tsai et al. (2010) showed that adult rats treated at 9 weeks post-stroke had a similar rate and magnitude recovery profile as those treated at one week post stroke. The reaching recovery seen in these animals was also associated with enhanced corticorubral axonal sprouting from the contralesional forelimb motor cortex to the deafferented red nucleus. These results opened up the possibility of anti-Nogo-A immunotherapy being a powerful treatment even in the chronic phases after ischemic injury and set the stage for further investigation of recovery of function after chronic stroke following anti-Nogo-A immunotherapy.

The following experiments described in this chapter set out to further evaluate the effective treatment time window for anti-Nogo-A immunotherapy chronically after stroke in adult rats. Additionally, the efficacy of anti-Nogo-A immunotherapy has been evaluated in rats with mild to moderate stroke deficits and small to medium stroke sizes primarily involving the cortex. Therefore, we also set out to evaluate the efficacy of anti-
Nogo-A immunotherapy in adult rats with moderate to very severe chronic sensorimotor deficits and medium to large stroke sizes that include cortical and subcortical damage. Rats were treated with anti-Nogo-A immunotherapy at either 9 weeks (subchronic time point) or 25 weeks (chronic time point) post-stroke. Treatment efficacy was evaluated using an intention-to-treat and a per-protocol analysis.
METHODS

General methods common to both aims 1 and 2 are described in chapter 3.

Methods specific to the experiments outlined in this chapter (Aim 2 experiments) are described here.

A priori inclusion criteria

All rats (n = 75) were trained to successfully reach 15 out of 20 pellets, for three consecutive days and then all rats underwent a MCAO procedure. After stroke, all surviving rats (n = 66) were tested on the skilled reaching task once a week to assess for spontaneous recovery for a total of 8 weeks or 24 weeks prior to treatment group randomization (see chapter 4 on spontaneous recovery (Aim 1)). All remaining rats were included in the study if they were able to reach with their impaired forelimb through the opening aperture in the skilled reaching apparatus, and at least touch the pellets on some attempts out of 20 total.

A priori exclusion criteria

Rats were excluded if a score of > 10/20 pellets was attained during any testing session post-stroke, weeks 1-8 for the subchronic delay treatment cohort and weeks 1-24 for the chronic treatment delay cohort. A total of 23 out of 66 rats (35%) rats were excluded from the study with a score > 10/20 pellets.
Randomization

Based on the skilled reaching deficit at post-stroke week 8 (subchronic treatment cohort) or post-stroke week 24 (chronic treatment cohort), animals were randomized across three stroke groups: Stroke/Anti-Nogo-A Ab, Stroke/Control Ab or Stroke Only. Treatment was initiated at the beginning of week 9 for the subchronic treatment delay cohort and at the beginning of week 25 for the chronic treatment delay cohort.

Description of the intention-to-treat (ITT) dataset for the subchronic treatment delay cohort (treatment at 9 weeks post-stroke)

In the intention-to-treat (ITT) dataset, all animals regardless of experimental protocol deviation were included in the final skilled reaching analysis as randomized. The subchronic treatment cohort was analyzed as both an ITT and a PP dataset. The ITT dataset for the subchronic treatment delay cohort is composed of the following treatment groups: Stroke/Anti-Nogo-A Ab (n = 14); Stroke/Control Ab (n = 12); Stroke Only (n = 10).

Description of the Per-Protocol (PP) dataset for the subchronic treatment delay cohort (treatment at 9 weeks post-stroke)

The per-protocol (PP) dataset includes all animals from the subchronic treatment delay ITT dataset that adhered to the experimental protocol without major deviations. Experimental protocol deviations that led to post-randomization exclusions included treatment pump disconnection upon removal (2 rats), absolute deficit animals that never grasped a pellet and scored a 0 out of 20 throughout the experiments (6 rats), severe
behavioral compensation using the unimpaired forelimb to reach for pellets and the resultant inability to obtain an accurate score for the impaired forelimb (2 rats), and severe striatal damage (1 rat). The PP dataset for the subchronic treatment delay cohort is composed of the following treatment groups: Stroke/Anti-Nogo-A Ab (n = 7), Stroke/Control Ab (n = 10), Stroke Only (n = 8).

Description of the dataset for the chronic treatment delay cohort (treatment at 25 weeks post-stroke)

The chronic delay treatment cohort (treatment initiated at 25 weeks post-stroke) had a minimal number of animals, thereby making an ITT vs. PP distinction obsolete. However, there were no animals with pump disconnections, no absolute deficit animals included, no severe behavioral abnormalities, or severe striatal damage in any animals that were randomized to this treatment time point (total n = 7). The dataset for the chronic treatment delay cohort is composed of the following treatment groups: Stroke/Anti-Nogo-A Ab (n = 3), Stroke/Control Ab (n = 2), Stroke Only (n = 2). The two control groups, animals treated with control antibody and stroke only animals, were combined in order to perform statistical calculations given the small number of animals in this dataset.
RESULTS

Body weight of adult rats at baseline, post-stroke, and post-treatment.

At the beginning of the experiments, rats were approximately 2 months of age and weighed approximately 250 grams. There was no difference in overall weight between the stroke groups in either the subchronic treatment cohort or the chronic treatment cohort at: arrival prior to food restriction, after skilled reaching training/food deprivation prior to the stroke procedure, prior to treatment initiation, or at the conclusion of the study (p > 0.05).

Morbidity and mortality associated with experimental procedures

Following stroke surgery (n = 75), a total of 9 rats (12%) died either during or within 24 hours of the stroke procedure.

Stroke lesion size did not differ across stroke groups in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke.

Total stroke lesion volume was measured for all animals and expressed as a % of the intact contralateral hemisphere. In the cohort of animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke, there were no significant difference between the stroke sizes between stroke groups within the ITT dataset (p = 0.775, one-way ANOVA), Figure 5.1A. ITT stroke lesion volumes were found to be 27.7 % ± 1.9 in the Stroke/Anti-Nogo-A Ab group, 29.6 % ± 1.9 in the Stroke/Control Ab group, and 28.4 % ± 2.3 in the Stroke only group, Figure 5.1A.
In the PP dataset, there was also no significant difference between the stroke sizes between stroke groups (p = 0.809, one-way ANOVA), Figure 5.1B. PP stroke lesion volumes were found to be 27.2 % ± 3.0 in the Stroke/Anti-Nogo-A Ab group, 29.2 % ± 2.2 in the Stroke/Control Ab group, and 27.1 % ± 2.7 in the Stroke only group, Figure 5.1B.

The overall stroke size in both the ITT and PP datasets was similar and therefore post-randomization exclusions did not affect the average stroke size (p = 0.957, one-way ANOVA).

Topological location of the stroke lesion included major damage to the caudal forelimb motor area, varying levels of damage to the rostral forelimb motor area, and minimal to moderate damage to the striatum, Figure 5.2.

**Stroke lesion size did not differ across stroke groups in animals treated with anti-Nogo-A immunotherapy at 25 weeks post-stroke.**

There was no significant difference between the stroke sizes across the anti-Nogo-A antibody treated or control stroke groups (p = 0.279), Figure 5.3. Stroke lesion volumes were found to be 23.7 % ±4.0 in the Stroke/Anti-Nogo-A Ab group and 35.2 % ± 8.2 in the combined control group (Control Ab + Stroke Only), Figure 5.3. Topological location of the stroke lesion included major damage to the caudal forelimb motor area, varying levels of damage to the rostral forelimb motor area, and minimal to moderate damage to the striatum, Figure 5.2.
Improved skilled reaching performance in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT and PP analyses).

ITT Analysis of Skilled Reaching Recovery following treatment with anti-Nogo-A antibody at 9 weeks post-stroke with a General Linear Model Repeated Measures ANOVA

To test whether treatment with anti-Nogo-A immunotherapy at 9 weeks post-stroke can improve sensorimotor function in rats with moderate to very severe stroke deficits, skilled reaching performance was assessed across 12 weeks post-treatment initiation (20 weeks post-stroke). Figure 5.4 shows the individual reaching recovery scores of each animal in the ITT dataset across treatment type starting at post-stroke week 7 (two weeks prior to treatment) and ending with post-stroke week 20 (the final testing week following treatment initiation).

Skilled reaching was first analyzed using a general linear model repeated measures ANOVA. Repeated measures analysis of animals treated at 9 weeks post-stroke (ITT) showed no statistically significant effect of treatment group upon reaching score across time (p = 0.235), Figure 5.5A. However, inspection of the recovery profiles indicated that the anti-Nogo-A Ab group had a trend towards a small improvement in final reaching performance. Figure 5.5B shows a linear regression applied to the recovery profiles of each treatment group showing that the two control groups, Stroke/Control Ab and Stroke Only, have a nearly horizontal recovery slope while the Stroke/Anti-Nogo-A Ab group shows a larger recovery slope, though modest. We therefore decided to evaluate whether there existed a significantly different mean linear rate of recovery across the different treatment groups. The mean linear recovery rate was quantified
starting from post-stroke week 7 and ending with post-stroke week 20. We decided to use both post-stroke weeks 7 and 8 as the pre-treatment stroke impairment baseline to account for skilled reaching practice effects caused by the change in testing frequency from post-stroke week 7 to post-stroke week 8 and beyond. The mean linear rate of recovery (Figure 5.5C) was found to be significantly different between the different treatment groups (p = 0.039), one-way ANOVA. Bonferroni post-hoc testing showed that the mean linear rate of recovery in the anti-Nogo-A antibody treated group was significantly greater than the recovery rate in animals treated with control antibody (p = 0.045) but not significantly different from stroke only animals that received no treatment (p = 0.234).

ITT Analysis of Skilled Reaching Recovery following treatment with anti-Nogo-A antibody at 9 weeks post-stroke with a Non-Linear Mixed Effects Logistic Regression Model

A non-linear mixed effects logistic regression model was used to fit a 3 parameter binomial logistic exponential function to the ITT recovery profiles in each treatment group. The exponential function is represented by \( P(t) = \theta_1 + (\theta_2 - \theta_1)e^{(-\theta_3(t-7))} \),

where \( P \) = probability of success, \( t \) = time, \( \theta_1 \) = recovery asymptote, \( \theta_2 \) = initial deficit (y-intercept), and \( \theta_3 \) = exponential recovery rate. This statistical method was used to analyze the recovery profiles of skilled reaching ability in post-stroke animals treated with anti-Nogo-A antibody.

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\(^1\) Rats were tested once a week during post-stroke weeks 1-7 in order to maintain skilled reaching ability without introducing motor rehabilitation. During post-stroke week 8 it was decided to test the rats 3 days in a row in order to re-establish an accurate post-stroke impairment baseline. However, this change in testing frequency introduced practice effects and we observed a significant improvement in skilled reaching score across all groups from post-stroke week 7 to week 8.
chosen to further analyze the recovery of animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT dataset) due to the increased ability of non-linear modeling methods to handle longitudinal data with its inherent complexity without violating statistical assumptions. For a discussion of linear versus non-linear handling of the longitudinal data in this dissertation please refer to chapter 3.

Figure 5.6A shows the fitted group-level logistic recovery functions for each treatment group based on the non-linear model. The initial deficit severity (post-stroke week 7), modeled by the parameter $\theta_2$ (Figure 5.6B), was shown to be the same across all three treatment groups ($p = 0.317$). Fitted functions including all parameter estimates were found to be statistically identical for the two control groups, Stroke/Control Ab and Stroke Only. Specifically, the exponential recovery rate and final skilled reaching recovery asymptote was found to be statistically the same across both control groups. Analysis of the exponential recovery rate between the two control groups and the anti-Nogo-A antibody treated group shows that the control animals improve faster than the anti-Nogo-A antibody group. However, the control groups reach their maximal recovery by post-stroke week 9 and show very little improvement beyond that time point. In contrast, the anti-Nogo-A antibody treated animals improve slowly but continue to improve over time and reach their maximal recovery asymptote at approximately post-stroke week 20 and beyond (theoretically), as modeled by the function. The anti-Nogo-A antibody treated animals reach a statistically greater final recovery magnitude of 33.31% success than the final recovery magnitude of approximately 20% success reached by
either the control antibody treated animals or the stroke only animals (p < 0.001 and p < 0.01 respectively). Parameter estimates and associated significance level contrasts are shown in Table 5.1 and 5.2 respectively for the non-linear modeling of animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT).

PP Analysis of Skilled Reaching Recovery following treatment with anti-Nogo-A antibody at 9 weeks post-stroke with a General Linear Model Repeated Measures ANOVA

Post-randomization exclusions were applied to the ITT dataset and the PP dataset was created. Figure 5.7 shows the individual reaching recovery scores of each animal in the PP dataset across treatment type starting at post-stroke week 7 and ending with post-stroke week 20.

Mirroring the statistical handling of the ITT data, we analyzed the PP skilled reaching recovery response to anti-Nogo-A immunotherapy initiated at 9 weeks post-stroke by using a general linear model repeated measures ANOVA. Repeated measures analysis of animals treated 9 weeks post-stroke (PP) showed a statistically significant interaction between time and treatment group for reaching success score attained (p = 0.004), Figure 5.8A. However, Bonferroni post-hoc testing did not show any statistically significant pairwise comparisons (p > 0.05). Figure 5.8B shows a linear regression applied to the recovery profiles of each treatment group showing that the two control groups, Stroke/Control Ab and Stroke Only, have a nearly horizontal recovery slope while the Stroke/Anti-Nogo-A Ab group shows a larger recovery slope and final magnitude. We therefore decided to evaluate whether there existed a significantly
different mean linear rate of recovery across the different treatment groups. The mean linear recovery rate was quantified starting from post-stroke week 7 and ending with post-stroke week 20. The mean linear rate of recovery (Figure 5.8C) was found to be significantly different between the different treatment groups (p < 0.01), one-way ANOVA. Bonferroni post-hoc testing showed that the mean linear rate of recovery in the anti-Nogo-A antibody treated group was significantly greater than the recovery rate in animals treated with control antibody (p < 0.01) and significantly greater from stroke only animals that received no treatment (p < 0.01).

**PP Analysis of Skilled Reaching Recovery following treatment with anti-Nogo-A antibody at 9 weeks post-stroke with a Non-Linear Mixed Effects Logistic Regression Model**

The same non-linear mixed effects logistic regression model that was used for the ITT dataset was fit to the PP dataset to evaluate the efficacy of anti-Nogo-A immunotherapy in animals treated 9 weeks post-stroke. Figure 5.9A shows the fitted group-level logistic recovery functions for each treatment group based on the non-linear model. The initial deficit severity (post-stroke week 7), modeled by the parameter \( \theta_2 \) (Figure 5.9B), was shown to be the same across all three treatment groups (p = 0.317). Fitted functions including all parameter estimates were found to be statistically identical for the two control groups, Stroke/Control Ab and Stroke Only. Specifically, the exponential recovery rate and final skilled reaching recovery asymptote was found to be statistically the same across both control groups. Analysis of the exponential recovery rate between the two control groups and the anti-Nogo-A antibody treated group shows
that the control animals improve faster than the anti-Nogo-A antibody group. However, the control groups reach their maximal recovery by post-stroke week 9 and show very little improvement beyond that time point. In contrast, the anti-Nogo-A antibody treated animals improve slowly but do not approach their maximal recovery asymptote until post-stroke week 20 and beyond (modeled projection). The anti-Nogo-A antibody treated animals reach a statistically greater final recovery magnitude of 55.75% than the final recovery magnitude of approximately 25% reached by either the control antibody treated animals or the stroke only animals (p < 0.001). Parameter estimates and associated significance level contrasts are shown in Table 5.3 and 5.4 respectively for the PP non-linear modeling of animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke.

**Correlation of Total Stroke Lesion Volume and Final Skilled Reaching Recovery in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT and PP datasets)**

Due to the variability in stroke lesion size among animals treated with anti-Nogo-A immunotherapy we evaluated whether stroke size was correlated with final skilled reaching recovery success score (post-stroke week 20). Figure 5.10B shows a scatterplot of the correlation between stroke lesion volume % and final success score for both the ITT and PP datasets across treatment groups. A negative although insignificant correlation was found between total stroke volume and final skilled reaching recovery score in both the ITT and PP datasets (p > 0.05). When the same data was expressed as the stroke volume lesion % versus the final pellet difference (post-stroke week 20 – post-
stroke week 8) virtually no association was seen between stroke size and improvement across all treatment groups in both the ITT and PP dataset (p > 0.05), Figure 5.10C.

**Correlation of % damage based on lesion location and the Final Skilled Reaching Recovery in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke.**

Percent damage was measured for the rostral forelimb motor cortex, the caudal forelimb motor cortex, and the striatum based on coordinates found in Paxinos and Watson (2005) as well as Neafsey et al. (1986). A small subset of the ITT animal dataset was used for this preliminary analysis: 4 animals treated with anti-Nogo-A antibody, 3 animals treated with control antibody, and 3 animals that received no treatment. First we evaluated the correlation between the success score at post-stroke week 20 and % damage in the three brain areas outlined above. In the animals treated with anti-Nogo-A immunotherapy, there was a negative correlation between all three brain areas and final reaching recovery score, however only the correlation between damage in the rostral forelimb motor cortex was found to be significantly correlated with final reaching score at post-stroke week 20 (r = -0.997, n = 4, p = 0.003). In animals treated with either control antibody or no treatment (combined control group), there was a negative correlation between all three brain areas and final reaching recovery score, however only the correlation between damage in the rostral and caudal forelimb motor cortex was found to be significantly correlated with final reaching score at post-stroke week 20 ((r = -0.873, n = 6, p = 0.023)(r = -0.817, n = 6, p = 0.047) respectively). Next we evaluated how the strength of the correlations change between the % damage of the three brain
areas and the final pellet difference. In animals treated with anti-Nogo-A immunotherapy, the relationship between % damage in the rostral forelimb area and final pellet improvement showed an insignificant though negative correlation ($r = -0.785$, $n = 4$, $p = 0.215$), Figure 5.11B. In animals treated with control antibody or no treatment, there was virtually no association seen between damage in the rostral forelimb motor cortex, the caudal forelimb motor cortex, or the striatum and the final pellet difference, Figure 5.11B.

No sensory recovery in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT and PP datasets)

Recovery of somatosensory function was evaluated using the bilateral adhesive removal task. The time necessary to contact the sticker on the impaired forelimb and the time to remove the sticker from the impaired forelimb was measured. There was no difference between treatment groups in the time necessary for animals to contact and remove the sticker on the impaired forelimb ($p = > 0.05$, Figure 5.12).

No skilled walking improvement in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT and PP datasets)

Recovery of skilled walking ability was evaluated using the horizontal ladder skilled walking task. There were no significant difference between treatment groups in the mean skilled walking movement score ($p = >0.05$, Figure 5.13A) or in the number of slips per ten steps while traversing the horizontal ladder ($p = >0.05$, Figure 5.13B).
Improved skilled reaching performance in animals treated with anti-Nogo-A immunotherapy at 25 weeks post-stroke (“PP”).

Animals were treated at 25 weeks post-stroke with either anti-Nogo-A antibody, control antibody, or no treatment. Skilled reaching performance was assessed for this dataset for 9 weeks post-treatment initiation (post-stroke weeks 25 through 32).

A general linear model repeated measures ANOVA was used to analyze the skilled reaching recovery response to anti-Nogo-A immunotherapy initiated at 25 weeks post-stroke. Repeated measures analysis of animals showed a statistically significant interaction between time and treatment group for reaching success score attained (p = 0.004), Figure 5.14A. However, Bonferroni post-hoc testing did not show any statistically signification pairwise comparisons (p > 0.05). Figure 5.14B shows a linear regression applied to the recovery profiles of each treatment group showing that the combined control group, Stroke/Control Ab and Stroke Only, has a nearly horizontal recovery slope while the Stroke/Anti-Nogo-A Ab group shows a larger recovery slope and final magnitude. We therefore decided to evaluate whether there existed a significantly different mean linear rate of recovery across the different treatment groups. The mean linear recovery rate was quantified starting from post-stroke week 23 and ending with post-stroke week 32. The mean linear rate of recovery (Figure 5.14C) was found to be significantly different between the different treatment groups (p < 0.01), one-way ANOVA. Due to the small number of animals in this dataset, non-linear analysis was not applied.
No Change in Dendritic Complexity of Layer V Pyramidal Neurons within the Perilesional Caudal Forelimb Motor Cortex

Following the completion of sensorimotor testing of animals treated with anti-Nogo-A immunotherapy at 9 weeks, animals were perfused and processed for analysis of neuronal morphology using the Golgi-Cox method. The animals included for the analysis of neuroanatomical plasticity were not distinguished based on the behavioral ITT or PP datasets. Intact, non-ischemic Layer V pyramidal neurons in the medial perilesional cortex within the rostrocaudal coordinates of the caudal forelimb motor cortex were chosen for analysis. Neurons were selected within 2 mm of the ischemic border. The number of branches in different branch orders and the dendritic length in different branch orders was analyzed separately for each layer V neuron’s apical and basilar dendritic tree. Inhibition of protein Nogo-A resulted in no discernable effect on the complexity of the apical arbors of perilesional layer V neurons as evidenced by no significant differences across treatment groups in terms of the number of apical branches across branch order, total number of apical branches, dendritic length across branch order, or total dendritic length (p > 0.05, Figures 5.15). Inhibition of the protein Nogo-A also resulted in no discernable effect on the complexity of the basilar arbors of perilesional layer V neurons as evidenced by no significant differences across treatment groups in terms of the number of basilar branches across branch order, total number of basilar branches, dendritic length across branch order, or total dendritic length (p >0.05, Figures 5.16).

No Change in Dendritic Complexity in Layer II/III or Layer V Pyramidal Neurons within the Contralesional Caudal Forelimb Motor Cortex
The brains of animals treated at 9 weeks post-stroke with anti-Nogo-A antibody, control antibody, or no treatment were also analyzed for dendritic complexity changes in the contralesional caudal forelimb motor cortex. Pyramidal neurons in layers II/III and V were selected for analysis. Inhibition of protein Nogo-A resulted in no discernable effect on the complexity of the apical arbors of contralesional layer II/III or layer V pyramidal neurons located in the caudal forelimb motor cortex as evidenced by no significant differences across treatment groups in terms of the number of apical branches across branch order, total number of apical branches, dendritic length across branch order, or total dendritic length, Figures 5.17 and 5.19. Inhibition of protein Nogo-A also resulted in no discernable effect on the complexity of the basilar arbors of contralesional layer II/III or layer V pyramidal neurons located in the caudal forelimb motor cortex as evidenced by no significant differences across treatment groups in terms of the number of basilar branches across branch order, total number of basilar branches, dendritic length across branch order, or total dendritic length, Figures 5.18 and 5.20.
Figure 5.1. Stroke lesion size did not differ across stroke groups in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke. Total stroke lesion volume was measured for all animals and expressed as a % of the intact contralateral hemisphere. (A) No significant difference in the stroke size was found across stroke groups in the ITT dataset (p = 0.775, one-way ANOVA). ITT stroke lesion volumes were found to be 27.7 % ± 1.9 in the Stroke/Anti-Nogo-A Ab group, 29.6 % ± 1.9 in the Stroke/Control Ab group, and 28.4 % ± 2.3 in the Stroke only group. (B) No significant difference was found in stroke size across stroke groups in the PP dataset (p = 0.809, one-way ANOVA). PP stroke lesion volumes (PP) were found to be 27.2 % ± 3.0 in the Stroke/Anti-Nogo-A Ab group, 29.2 % ± 2.2 in the Stroke/Control Ab group, and 27.1 % ± 2.7 in the Stroke only group.
Figure 5.2. Diagram of stroke lesion topology. Diagram displays the differences in lesion territory between a large and small stroke size. The large lesion (approximately 40% stroke size) damages most the rostral-caudal hemispheric axis including damage to the rostral forelimb motor area, caudal forelimb motor area, and the dorso-lateral striatum. The small stroke lesion (approximately 15% stroke size) damages a more limited portion of the hemisphere including primarily the caudal forelimb motor cortex with minimal damage to the rostral forelimb motor cortex and dorso-lateral striatum. Topologies of small and large lesions are similar in both the treatment delayed cohort (treatment at 9 weeks post-stroke) and the prolonged treatment delay cohort (treatment at 25 weeks post-stroke).
Stroke Lesion Size in animals treated with anti-Nogo-A immunotherapy at 25 Weeks Post-Stroke Stroke Lesion Size

**Figure 5.3.** Stroke lesion size did not differ across stroke groups in animals treated with anti-Nogo-A immunotherapy at 25 weeks post-stroke. In the cohort of animals treated with anti-Nogo-A immunotherapy at 25 weeks post-stroke (prolonged treatment delay), there was no significant difference between the stroke sizes across the two stroke groups ($p = 0.279$, t-test). Stroke lesion volumes were found to be 23.7 % ± 4.0 in the Stroke/Anti-Nogo-A Ab group and 35.2 % ± 8.2 in the combined control group (Control Ab + Stroke Only).
Figure 5.4. shows the reaching recovery scores of each animal in the ITT dataset across time based on (A) treatment with anti-Nogo-A antibody (B) treatment with control antibody and (C) no treatment. Each plot shows the individual mean recovery score at each week as well as the group mean recovery score at each week.
Figure 5.5. Treatment analysis with anti-Nogo-A immunotherapy, control Ab, or no treatment analyzed using a general linear model repeated measures ANOVA. (A) Skilled reaching performance was analyzed at baseline, post-stroke, and post-treatment using a repeated measures ANOVA. There were no differences in rats across stroke groups in pre-stroke success scores. Following stroke surgery, rats across all stroke groups develop a large impairment in skilled reaching that persists across time. Following treatment initiation, there was no significant effect of treatment between groups on skilled reaching performance. (B) shows the linear recovery rate following treatment. The linear regression is shown using post-stroke scores at week 7 and 8 as the initial scores prior to treatment initiation. The Stroke/Anti-Nogo-A Ab group shows a statistically significant linear rate of recovery as compared to the Stroke/Control Ab group (p = 0.045) but not as compared to the Stroke Only Group (p = 0.234), one-way ANOVA). (*p < 0.05)
Non-linear Modeling of Skilled Reaching Recovery in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT Dataset)

Figure 5.6. Improved skilled reaching performance (ITT) following anti-Nogo-A immunotherapy in adult rats with chronic severe stroke deficits using nonlinear modeling. (A) shows the fitted group-level logistic recovery profiles for each treatment group based on the non-linear model. Animals in the Stroke/Anti-Nogo-A Ab group showed an improved recovery profile as compared to the Stroke/Control Ab and Stroke Only groups (B) Initial deficit severity at post-stroke week 7 was the same across all groups, as shown by the Θ2 parameter (p > 0.05). (C) The Stroke/Anti-Nogo-A Ab group showed a greater final recovery than either the Stroke/Control Ab or Stroke Only group, as given by the Θ1 parameter (p < 0.001 and 0.01 respectively). (D) Analysis of the exponential recovery rate shows that the Stroke/Anti-Nogo-A Ab group had a slower rate of recovery over a longer timeframe, while the Stroke/Control Ab and Stroke Only groups had a faster recovery rate over a shorter timeframe. Error bars denote ± standard error of the estimate (** p < 0.01, *** p < 0.001).
Parameter Estimates from Non-linear Model of Skilled Reaching Recovery at 9 weeks post-stroke (ITT Dataset)

A. Parameter estimates for recovery asymptote

<table>
<thead>
<tr>
<th>Group</th>
<th>$\theta_1$ Estimate</th>
<th>Standard Error</th>
<th>Lower Confidence Level</th>
<th>Upper Confidence Level</th>
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B. Parameter estimates for initial deficit severity

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<tr>
<th>Group</th>
<th>$\theta_2$ Estimate</th>
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<th>Upper Confidence Level</th>
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C. Parameter estimates for recovery rate

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<tr>
<th>Group</th>
<th>$\theta_3$ Estimate</th>
<th>Standard Error</th>
<th>Lower Confidence Level</th>
<th>Upper Confidence Level</th>
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<td>Stroke Only</td>
<td>0.08982</td>
<td>0.02111</td>
<td>0.04836</td>
<td>0.1313</td>
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</table>

Table 5.1. The estimates, standard error, and lower/upper confidence level intervals for the parameters ($\theta_1$, $\theta_2$, $\theta_3$) of the non-linear functions fitted to the Stroke/Anti-Nogo-A Ab, Control Ab, and Stroke Only groups are listed above in A, B and C respectively.
### A. Contrast comparisons for recovery asymptote

<table>
<thead>
<tr>
<th>$\theta_1$ Estimate</th>
<th>Group\Group</th>
<th>Anti-Nogo-A Ab</th>
<th>Control Ab</th>
<th>Stroke Only</th>
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### B. Contrast comparisons for initial deficit severity

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<tr>
<th>$\theta_2$ Estimate</th>
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<th>Anti-Nogo-A Ab</th>
<th>Control Ab</th>
<th>Stroke Only</th>
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<td>Control Ab</td>
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### C. Contrast comparisons for recovery rate

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**Table 5.2: Significance levels for parameter estimate contrasts between treatment groups.** Hypothesis testing was performed by comparing the full non-linear model with all 9 parameters to reduced versions of the original model with certain parameters held constant. The skilled reaching outcome variable is binomial (success or no success) and therefore the dataset is modeled by a $\chi^2$ distribution. Fitted models are contrasted by using $\chi^2$ likelihood based ratio tests.
Individual Animal Skilled Reaching Recovery following Anti-Nogo-A Ab, Control Ab, or no treatment Administered at 9 Weeks Post-Stroke (PP Dataset)

Figure 5.7. shows the reaching recovery scores of each animal in the PP dataset across time based on (A) treatment with anti-Nogo-A antibody (B) treatment with control antibody and (C) no treatment. Each plot shows the individual mean recovery score at each week as well as the group mean recovery score at each week.
Figure 5.8. Analysis of improved skilled reaching recovery (PP) following treatment with anti-Nogo-A immunotherapy using a general linear model repeated measures ANOVA. (A) Following treatment initiation, a significant time x treatment group interaction (weeks post-stroke) was found for skilled reaching recovery (p = 0.004), however Bonferroni post hoc testing revealed no statistically significant pairwise comparisons. Repeated measures ANOVA analysis of percent change from post-stroke week 24 (B) also showed a significant time x treatment interaction, with statistically significant improved recovery for animals treated with anti-Nogo-A immunotherapy starting at post-stroke week 26 and lasting until the end of treatment (error bars omitted for clarity). The Stroke/Anti-Nogo-A Ab group showed a significantly larger mean linear rate of recovery (C) as compared to the Stroke/Control Ab and Stroke Only Groups (p < 0.001) (F(2,24) = 24.789, p < 0.001, one-way ANOVA).
Non-linear Modeling of Skilled Reaching Recovery in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke (PP Dataset)

Figure 5.9. Improved recovery in skilled reaching performance (PP) following anti-Nogo-A immunotherapy. (A) shows the fitted group-level logistic recovery profiles for each treatment group based on the non-linear model. Animals in the Stroke/Anti-Nogo-A Ab group showed an improved recovery profile as compared to the Stroke/Control Ab and Stroke Only groups. Initial deficit severity at post-stroke week 7 (B) was the same for all groups (p > 0.05). The Stroke/Anti-Nogo-A Ab group showed a greater recovery asymptote (C) than either the Stroke/Control Ab or Stroke Only group (p < 0.001). Analysis of the exponential recovery rate (D) revealed that the Stroke/Anti-Nogo-A Ab group had a slower rate of recovery over a longer timeframe, ultimately resulting in a higher recovery asymptote, while the Stroke/Control Ab and Stroke Only groups had a faster recovery rate over a shorter timeframe, but ultimately a lower recovery asymptote. Error bars denote ± standard error of the estimate. *** p < 0.001, comparison of Stroke/Anti-Nogo-A Ab group against both Stroke/Control Ab and Stroke Only groups.
Parameter Estimates from Non-linear Model of Skilled Reaching Recovery (PP Dataset treated at 9 weeks post-stroke)

A. Parameter estimates for recovery asymptote

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<tr>
<th>Group</th>
<th>$\theta_1$ Estimate</th>
<th>Standard Error</th>
<th>Lower Confidence Level</th>
<th>Upper Confidence Level</th>
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B. Parameter estimates for initial deficit severity

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C. Parameter estimates for recovery rate

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Table 5.3. The estimates, standard error, and lower/upper confidence level intervals for the parameters ($\theta_1$, $\theta_2$, $\theta_3$) of the non-linear functions fitted to the Stroke/Anti-Nogo-A Ab, Control Ab, and Stroke Only groups are listed above in A, B and C respectively (PP Analysis).
Parameter Estimate Contrasts (PP treated at 9 weeks post-stroke)

A. Contrast comparisons for recovery asymptote

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<tr>
<th>$\theta_1$ Estimate</th>
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<th>Control Ab</th>
<th>Stroke Only</th>
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B. Contrast comparisons for initial deficit severity

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<th>$\theta_2$ Estimate</th>
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C. Contrast comparisons for recovery rate

<table>
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Table 5.4. Significance levels for parameter estimate contrasts between treatment groups (PP Dataset). Hypothesis testing was performed by comparing the full non-linear model with all 9 parameters to reduced versions of the original model with certain parameters held constant. The skilled reaching outcome variable is binomial (success or no success) and therefore the dataset is modeled by a $\chi^2$ distribution. Fitted models are contrasted by using $\chi^2$ likelihood based ratio tests.
Figure 5.10. Correlation between total stroke volume and reaching recovery in the ITT and PP datasets. (A) shows the total stroke lesion volume. (B) shows a negative correlation although insignificant between the final reaching score and the total stroke lesion volume in both ITT and PP datasets. (C) shows the lack of an association between reaching recovery and stroke lesion volume when the final reaching score is expressed as the percent improvement from pre-treatment (post-stroke week 8).
Correlation of Lesion Location and Final Reaching Recovery

Figure 5.11. Correlation between lesion location and final reaching recovery. (A) shows the negative correlation between final reaching success score post-treatment (post-stroke week 20) and damage in the caudal forelimb motor cortex, rostral forelimb motor cortex, and the striatum in both the Stroke/Anti-Nogo-A Ab group and the control group. When controlling for initial pre-treatment severity by using percent change from pre-treatment, only the correlation between rostral forelimb motor cortex and final improvement percent was found to be correlated in animals treated with anti-Nogo-A immunotherapy.
Somatosensory Recovery following treatment with anti-Nogo-A immunotherapy at 9 weeks post-stroke.

Figure 5.12. No somatosensory recovery following treatment with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT + PP Dataset). (A and A’) There was no difference across groups for the time it took the rats to contact the sticker on the impaired forelimb in either the ITT or PP dataset respectively (p > 0.05). (B and B’) There was no difference across groups for the time it took the rats to remove the sticker on the impaired forelimb in either the ITT or PP dataset respectively (p > 0.05).
Recovery of Skilled Walking in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke

Figure 5.13. No skilled walking recovery following treatment with anti-Nogo-A immunotherapy at 9 weeks post-stroke (ITT + PP Dataset). (A and A’) There was no difference across groups for the movement score across time in either the ITT or PP dataset respectively (p > 0.05). (B and B’) There was no difference across groups in the number of foot slips across time in either the ITT or PP dataset respectively (p > 0.05).
Mean Skilled Reaching Recovery following treatment Administered at 25 Weeks Post-Stroke ("PP" Dataset)

Figure 5.14. Analysis of improved skilled reaching recovery ("PP") following treatment using Generalized Linear Model statistics. (A) Following treatment initiation, there was a significant interaction between time x treatment across groups on skilled reaching performance but individual time point analysis did not show significantly different means. Percent change from post-stroke week 24 (B) also showed a significant time x treatment interaction with improved recovery for animals treated with anti-Nogo-A immunotherapy starting at post-stroke week 26 and lasting until the end of treatment (error bars omitted for clarity). The Stroke/Anti-Nogo-A Ab group shows a statistically significant linear rate of recovery (C) as compared to the Stroke/Control Ab group (p =0.016), one-way ANOVA). *p< 0.05, **p < 0.001
Figure 5.15. **Apical dendritic tree morphology.** Apical tree morphology was quantified for number of branch points by branch order and branch length by branch order. (A) Treatment with anti-Nogo-A immunotherapy had no effect on the number of dendritic branch points across branch order ($p > 0.05$, one-way ANOVA). (A’) Treatment with anti-Nogo-A immunotherapy had no effect on the total number of branches in the apical dendritic tree ($p > 0.05$, one-way ANOVA). (B) Treatment with anti-Nogo-A immunotherapy had no effect on the apical branch length across branch orders ($p > 0.05$, one-way ANOVA). (B’) Treatment with anti-Nogo-A immunotherapy had no effect on the total apical dendritic length ($p > 0.05$, one-way ANOVA).
Basilar Dendritic Complexity of Layer V Pyramidal Neurons Located in the Perilesional Caudal Forelimb Motor Cortex

Figure 5.16. Basilar dendritic tree morphology. Basilar tree morphology was quantified for number of branch points by branch order and branch length by branch order. (A) Treatment with anti-Nogo-A immunotherapy had no effect on the number of dendritic branch points across branch order (p > 0.05, one-way ANOVA). (A’) Treatment with anti-Nogo-A immunotherapy had no effect on the total number of branches in the basilar dendritic tree (p > 0.05, one-way ANOVA). (B) Treatment with anti-Nogo-A immunotherapy had no effect on the basilar branch length across branch orders (p > 0.05, one-way ANOVA). (B’) Treatment with anti-Nogo-A immunotherapy had no effect on the total basilar dendritic length (p > 0.05, one-way ANOVA).
Fig. 5.17. Apical dendritic tree morphology. Apical tree morphology was quantified for number of branch points by branch order and branch length by branch order. (A) Treatment with anti-Nogo-A immunotherapy had no effect on the number of dendritic branch points across branch order (p > 0.05, one-way ANOVA). (A’) Treatment with anti-Nogo-A immunotherapy had no effect on the total number of branches in the apical dendritic tree (p > 0.05, one-way ANOVA). (B) Treatment with anti-Nogo-A immunotherapy had no effect on the apical branch length across branch orders (p > 0.05, one-way ANOVA). (B’) Treatment with anti-Nogo-A immunotherapy had no effect on the total apical dendritic length (p > 0.05, one-way ANOVA).
Figure 5.18. Basilar dendritic tree morphology. Basilar tree morphology was quantified for number of branch points by branch order and branch length by branch order. (A) Treatment with anti-Nogo-A immunotherapy had no effect on the number of dendritic branch points across branch order (p > 0.05, one-way ANOVA). (A’) Treatment with anti-Nogo-A immunotherapy had no effect on the total number of branches in the basilar dendritic tree (p > 0.05, one-way ANOVA). (B) Treatment with anti-Nogo-A immunotherapy had no effect on the basilar branch length across branch orders (p > 0.05, one-way ANOVA). (B’) Treatment with anti-Nogo-A immunotherapy had no effect on the total basilar dendritic length (p > 0.05, one-way ANOVA).
Apical Dendritic Complexity of Layer V Pyramidal Neurons Located in the Contralesional Caudal Forelimb Motor Cortex

Figure 5.19. **Apical dendritic tree morphology.** Apical tree morphology was quantified for number of branch points by branch order and branch length by branch order. (A) Treatment with anti-Nogo-A immunotherapy had no effect on the number of dendritic branch points across branch order (p > 0.05, one-way ANOVA). (A’) Treatment with anti-Nogo-A immunotherapy had no effect on the total number of branches in the apical dendritic tree (p > 0.05, one-way ANOVA). (B) Treatment with anti-Nogo-A immunotherapy had no effect on the apical branch length across branch orders (p > 0.05, one-way ANOVA). (B’) Treatment with anti-Nogo-A immunotherapy had no effect on the total apical dendritic length (p > 0.05, one-way ANOVA).
Basilar Dendritic Complexity of Layer V Pyramidal Neurons Located in the Contralesional Caudal Forelimb Motor Cortex

Figure 5.20. **Basilar dendritic tree morphology.** Basilar tree morphology was quantified for number of branch points by branch order and branch length by branch order. (A) Treatment with anti-Nogo-A immunotherapy had no effect on the number of dendritic branch points across branch order (p > 0.05, one-way ANOVA). (A’) Treatment with anti-Nogo-A immunotherapy had no effect on the total number of branches in the basilar dendritic tree (p > 0.05, one-way ANOVA). (B) Treatment with anti-Nogo-A immunotherapy had no effect on the basilar branch length across branch orders (p > 0.05, one-way ANOVA). (B’) Treatment with anti-Nogo-A immunotherapy had no effect on the total basilar dendritic length (p > 0.05, one-way ANOVA).
Comparison of anti-Nogo-A immunotherapy efficacy across different treatment initiation time points and ages of animals

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<th>% Improv. at 12 Weeks Post-Treatment</th>
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<tr>
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<td>--</td>
<td>--</td>
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Table 5.5. Comparison of anti-Nogo-A immunotherapy efficacy across different treatment initiation time points and ages of animals.
DISCUSSION

This present study demonstrates that there exists an extended treatment window for the use of anti-Nogo-A immunotherapy to improve sensorimotor function in adult rats with chronic moderate to very severe stroke deficits. Adult rats that were treated with anti-Nogo-A antibodies at either 9 (~ 2 months) or 25 (~ 6 months) weeks post-stroke displayed an improvement in skilled forelimb reaching performance but exhibited no change in skilled walking or sensory function. Furthermore, the improvement in skilled forelimb reaching performance was not associated with changes in dendritic tree complexity in layer V pyramidal neurons located in the perilesional caudal forelimb motor cortex or in layers II/III and V pyramidal neurons located in the contralesional caudal forelimb motor cortex.

In order to determine an unbiased efficacy rate for anti-Nogo-A immunotherapy in animals with moderate to very severe stroke deficits treated at 9 weeks post-stroke, the data was first evaluated using an intention-to-treat analysis and then using a per-protocol analysis. An intention-to-treat (ITT) procedure was used whereby all randomized animals, regardless of experimental protocol deviations, were used in the final analysis. Although, this approach may lead to conservative estimates of efficacy, it provides the most valid assessment of meaningful differences across groups and is considered the gold standard approach to data analysis in human clinical trials (Day et al., 2008; FDA, 1988). The ITT dataset included all animals that scored 10 out of 20 pellets or less on the skilled forelimb reaching task, and could be stratified by deficit severity. Our ITT population included a range of animals with moderate stroke deficits (36%), severe deficits (42%),
and very severe deficits (22%). We found that this population when treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke had a statistically significant improvement in the final recovery asymptote (probability of success out of 20 trials) as compared to both of the control groups. Animals treated with anti-Nogo-A antibody had a final recovery asymptote of 33.31% ± 2.0, compared to animals treated with control antibody (20.64% ± 1.0), and stroke only animals (21.09% ± 1.0). When adjusting the recovery percent by initial deficit pre-treatment (post-stroke week 8), anti-Nogo-A antibody treated animals displayed an improvement in skilled forelimb reaching performance by 21.89%, while animals treated with control antibody improved by only 6.94% and stroke only animals improved by only 12.11%. However, of note, only animals with moderate to severe deficits improved after anti-Nogo-A immunotherapy. Animals in the very severe category, i.e. those that never grasped a pellet in the timeframe between their stroke and prior to treatment, showed no improvement on the skilled reaching task after treatment. Therefore, these results show the effectiveness of anti-Nogo-A immunotherapy in the early chronic stage after stroke (9 weeks post-stroke) in adult rats with moderate to very severe stroke deficits, but also highlight the inability of anti-Nogo-A immunotherapy to result in recovery of skilled forelimb reaching when there is no residual ability to move the forelimb prior to treatment. To our knowledge, the present study is the first report of using anti-Nogo-A immunotherapy to improve sensorimotor function after stroke at a clinically relevant chronic time point and in a population that includes rats with severe and very severe stroke deficits and
corresponding large stroke sizes. The positive results from the present study support the perspective that the brain can respond to treatment interventions in the chronic phase after stroke and also in the context of severe stroke damage.

However, given the fact that the ITT treatment efficacy estimates were based on a dataset that included animals that deviated from the experimental protocol, we set out to see how the efficacy of anti-Nogo-A immunotherapy is affected by post-randomization exclusions. Animals were excluded for various reasons. Firstly, animals were excluded if the antibody intracerebroventricular pump was found to be disconnected upon removal, thereby potentially supplying a suboptimal therapeutic dose of antibody to the animal. Secondly, animals were excluded when displaying very severe reliance on using the unimpaired forelimb during the skilled reaching task. These animals primarily used their “good” forelimb for reaching for pellets leading to very inaccurate success score estimations for the impaired forelimb. Thirdly, animals were excluded if they displayed an “absolute deficit” phenotype where post-stroke, no pellets were ever successfully grasped and brought to the mouth. Fourthly, rats were excluded with very severe subcortical damage (complete damage of dorso-lateral striatum). Our PP results showed that once these animals were excluded from the study, the efficacy rate of anti-Nogo-A immunotherapy in animals treated at 9 weeks post-stroke improved. Although the mean total stroke size per treatment group did not change between the ITT and PP datasets, the distribution of stroke deficit severity at time of treatment in the population was affected. Our PP population on average included animals with moderate stroke deficits (43.5%), and severe deficits (56.5%), with no animals displaying very severe deficits (0%).
Animals treated with anti-Nogo-A antibody had a final recovery asymptote of 55.75% ± 3.0, compared to animals treated with control antibody (24.45% ± 1.0), and stroke only animals (26.10% ± 1.0). When adjusting the recovery percent by initial deficit pre-treatment, anti-Nogo-A antibody treated animals displayed an improvement in skilled forelimb reaching performance by 47.03%, while animals treated with control antibody improved by only 8.51% and stroke only animals improved by only 14.75%. Therefore, the results from the PP analysis show that when animals with chronic moderate to severe stroke deficits are treated at 9 weeks post-stroke with anti-Nogo-A immunotherapy, recovery from initial impairment will be 35% greater than that of controls. These PP results suggest that in a population of rats that received the entire dose of anti-Nogo-A antibody, had no behavioral abnormalities during testing procedures, did not have very severe deficits or severe subcortical damage, we can expect a greater improvement of sensorimotor function after treatment with anti-Nogo-A immunotherapy at 9 weeks after stroke than suggested by the ITT efficacy rates.

As mentioned above, comparison of the ITT and PP datasets shows that among moderate to very severe stroke deficits, and correspondingly moderate to very large stroke sizes, not all animals will recover and not all will recover the same amount with anti-Nogo-A antibody treatment. Based on the ITT and PP results, the mean efficacy rate for anti-Nogo-A immunotherapy above controls when administered at 9 weeks post-stroke may vary anywhere from 12% to 35%. The efficacy rates of anti-Nogo-A immunotherapy in this present study may be related to an animal’s individual total stroke
size, total damage to particular areas important for skilled reaching (rostral forelimb area, caudal forelimb area, striatum), specified and unspecified behavioral characteristics, experimental error, and unknown factors. Therefore we evaluated the correlation between total stroke size and recovery in both the ITT and PP datasets. Although we found a significant correlation between the total stroke lesion size and final reaching success score (out of 20), we found no correlation between the total stroke size and the final absolute increase in pellets obtained in the skilled reaching task. Hence the relationship between the total stroke lesion size and treatment induced improvement is complex. This complex relationship has been seen in human stroke studies as well. Studies have shown conflicting results, with some showing significant correlations between total stroke lesion size and upper extremity recovery (Beloosesky et al. 1995; Van Everdingen et al., 1998), while others have shown little to no correlation between total stroke lesion size and upper extremity recovery (Pantano et al., 1996; Miyai et al., 1997). Interestingly, using both total stroke lesion size and lesion size based on location seems to be more strongly correlated with recovery potential (Chen et al., 2000). Therefore we decided to perform a preliminary location specific stroke lesion analysis to gain a greater understanding between anti-Nogo-A immunotherapy induced improvements and lesion size and location. We evaluated, in a small subset of the animals (n=4), the correlation between the final absolute increase in pellets obtained after treatment and the % damage in the rostral forelimb motor cortex, caudal forelimb motor cortex, and striatum. Although not statistically significant, we found a strong inverse correlation between the damage in the rostral forelimb motor cortex and the final improvement in the number of pellets obtained
at the end of the study in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke. Based on these preliminary results, this might suggest that the residual ipsilesional rostral forelimb motor cortex may also play a role in the recovery seen after anti-Nogo-A immunotherapy and underscore the importance of detailed lesion location analysis. Future analyses will complete detailed lesion based analysis on all animals in the ITT and PP datasets.

In addition to evaluating skilled reaching recovery after anti-Nogo-A immunotherapy at 9 weeks post-stroke, we evaluated recovery in sensory function and skilled walking. Results from these two tasks showed that the improved skilled forelimb reaching recovery did not translate to improvements seen in sensory function and skilled walking. Sensory function, tested by the use of the bilateral adhesive removal task, has been tested after anti-Nogo-A immunotherapy after a corticospinal tract (CST) lesion and after a traumatic brain injury. In the first study, adult rats were given unilateral CST lesions at the medulla oblongata and sensory function was tested using the bilateral adhesive removal task. Animals treated with anti-Nogo-A antibodies (IN-1) showed a profound recovery of sensory function (Thalmair et al., 1998). In contrast, another study showed no improvement in sensory function, as measured by the bilateral adhesive removal task, in animals treated with anti-Nogo-A antibodies (11C7) after traumatic brain injury (Lenzlinger et al., 2005). Therefore, sensory recovery after anti-Nogo-A immunotherapy may be related to lesion type and location. Additionally, the task used to test sensory function, the bilateral adhesive removal task, has been shown to be highly variable and requires a large number of subjects to show significant differences
(Whishaw & Kolb., 2005). The task sensitivity can also be affected by the type of stickers used since different levels of adhesiveness can affect the difficulty of the task. It is possible the sensitivity of our task was low and our sample size was too small to pick up any meaningful differences across groups. Regarding skilled walking, at the chronic time points assessed, there were very mild deficits seen after stroke on this task at pre-treatment and therefore there was little room for improvement after treatment. The lack of large deficits on this task may be related to the spinal cord’s ability to mediate locomotion with little input from the sensorimotor cortex (Dietz et al., 2003; Forssberg et al., 1975). The discrepancy between improved recovery seen in the skilled forelimb reaching task and the little improvement seen in both the bilateral adhesive removal task and the skilled horizontal ladder walking task also underscore the complex relationship mediating the transfer of task related improved skills. The topic of whether trained skills can be translated to an untrained task is controversial, with both supporting evidence (Multon et al., 2003; Engesser-Cesar et al., 2007) and conflicting evidence (Edgerton et al., 1997; De Leon et al., 1998; Grasso et al., 2004). In the case of this study, rats were trained on the skilled forelimb reaching task prior to stroke injury and then during the treatment phase, were tested on the task almost daily. In contrast, the bilateral adhesive removal task and the skilled ladder horizontal ladder walking task do not require training, and simply require habituation to the testing environment. Furthermore, the rats in this present study were tested on these two tasks only four times during the study (baseline, pre-treatment, post-treatment time 1, post-treatment time 2), therefore negating any training effects that could have helped stimulate recovery. Finally, since the neural
strategy used for the movements in our three tasks differ, it might be less likely for the recovery of function on one task to be converted to the recovery of function in another task (Singh et al., 2010).

Next we evaluated skilled reaching recovery after treatment with anti-Nogo-A immunotherapy at 25 weeks post-stroke. These results are based on a small dataset ($n = 7$). All animals in this dataset were analyzed as randomized, however since the animals selected for this time point did not have any protocol deviations as seen in the 9 week treated group, the results could be considered as a PP analysis, in order to compare the results to the 9 week time point. Nonlinear modeling was not possible with this dataset due to the small number of animals. Repeated measures ANOVA showed anti-Nogo-A antibody treated animals had a significant interaction between time and treatment as compared to the control treated animals. However, the study was not properly powered for post-hoc testing to show a significant difference between control treated and anti-Nogo-A antibody treated animals at any given time point. However, we did find that the linear mean rate of improvement was significantly greater in the animals treated with anti-Nogo-A antibodies. Additionally, rats treated with anti-Nogo-A immunotherapy at 25 weeks post-stroke showed a similar final magnitude of improvement as the animals treated with anti-Nogo-A immunotherapy at 9 weeks (PP analysis). Although the final magnitude of recovery was similar, the animals treated at 25 weeks had a faster rate of recovery than the animals treated at 9 weeks post-stroke. This difference in recovery rates may be due to the smaller lesion size in the animals treated with anti-Nogo-A immunotherapy at 25 weeks versus the lesion size in animals treated at 9 weeks.
Numerous reports from our laboratory have investigated whether treatment with anti-Nogo-A immunotherapy at various time points post-stroke is efficacious for improving sensorimotor recovery in skilled reaching function (for a summary refer to Table 5.5). Treatment with anti-Nogo-A immunotherapy immediately and up to 1 week post-stroke in adult rats with mild to moderate stroke deficits (~10% stroke size) has been found to improve recovery beyond control therapy by 30-35% within 8 weeks of treatment initiation (Papadopolous et al., 2002; Seymour et al., 2005). A similar final recovery magnitude was also found in aged animals treated with anti-Nogo-A immunotherapy however, it took longer for the animals to reach this final recovery, up to post-stroke week 14 (Markus et al., 2005). A recent study by Tsai et al. (2010) treated adult rats with mild to moderate stroke deficits (~16% stroke size) at 9 weeks post-stroke and also found a similar rate and final magnitude of recovery as adult rats treated one week post-stroke, implying that recovery from anti-Nogo-A immunotherapy is independent from the differences between the post-ischemic brain at one week versus 9 weeks post-stroke. Our study set out to further analyze the efficacy of anti-Nogo-A immunotherapy in the chronic phases after stroke. As discussed previously, the animals treated at 9 weeks and 25 weeks post-stroke used in our study were considered to have moderate to very severe chronic stroke deficits and had an average stroke size of 27% and 19% respectively. In the animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke, the final improvement in the recovery asymptote according to the non-linear modeling in the PP analysis, was determined to be ~35%, similar to the Tsai study. However, it is important to note that the animals did not near this final recovery
magnitude until 12 weeks post-stroke. Given that the experimental parameters of the study were identical to the Tsai et al. (2010) study, it is likely that the stroke lesion size and/or location played a role in the delayed recovery rate seen in this study. The recovery results from the animals treated at 25 weeks post-stroke support the relationship between stroke lesion size/location and recovery since these animals had a nearly identical magnitude and rate of improvement as the animals treated at 9 weeks post-stroke in Dr. Tsai’s study. Not surprisingly, the stroke lesion size in the animals treated at 25 weeks post-stroke was smaller (~19%) than the lesion size of animals treated at 9 weeks post-stroke in this study (~27%). Since overall we do not see a decrease in potency of anti-Nogo-A immunotherapy chronically after stroke, it is likely that other factors such as lesion size and location are playing some role in the different recovery rates seen after treatment.

Following cortical lesions, various areas directly and remotely connected to the damaged cortex are thought to undergo spontaneous neuroanatomical structural plasticity (Murphy et al., 2009). Additional supporting evidence for this idea comes from previous work in our laboratory showing an increase in dendritic complexity in layer V pyramidal neurons in the contralesional sensorimotor cortex following treatment with anti-Nogo-A immunotherapy one week post-stroke (Papadopoulos et al., 2006). Therefore, we decided to investigate whether the improved skilled reaching recovery seen after administration of anti-Nogo-A immunotherapy at 9 weeks post-stroke in the adult rat was correlated with structural changes in neurons located within the contralesional caudal forelimb motor cortex.
We found no evidence of increased dendritic complexity in either layer II/III or layer V pyramidal neurons in the contralesional caudal forelimb motor cortex. This lack of apparent dendritic structural plasticity following anti-Nogo-A immunotherapy may be due to several reasons. Following large-scale damage to the motor cortex, rats often display a change in preferred forelimb use in the skilled reaching task (Castro-Alamancos et al., 2003). Given this change in preference, rats will try to use the unimpaired forelimb to obtain sugar pellets during skilled reaching. Without directly inhibiting the use of the unimpaired forelimb through the application of some sort of constraint, it is likely that the rat will over time acquire improved skill training in this forelimb, even with the best attempts at disallowing reaching attempts with this forelimb. Therefore, it is likely that this inadvertent skilled reach training may lead to increased dendritic complexity in the contralateral sensorimotor cortex innervating this forelimb, as has been shown with skilled reaching training (Kolb et al., 2008). However, since this is the same sensorimotor cortex being evaluated for treatment-induced changes in plasticity, any treatment effects may be obscured. In the previous study by Papadopoulos et al. (2006) that found increased dendritic plasticity in the contralesional sensorimotor cortex, the adult rats were not trained on the skilled reaching task and therefore forelimb switching was not a confounding factor in that study. It is possible that in our study, one reason for the lack of apparent changes in dendritic complexity in the contralesional caudal forelimb motor cortex may be due to the potential increase in basal level plasticity seen in this area due to the widespread use of the unimpaired forelimb among most rats post-stroke. Furthermore, the total number of apical and basilar branches and the total apical and basilar dendritic
length in layer V pyramidal neurons in the contralesional sensorimotor cortex in stroke only animals in this present study are much higher than the averages seen in stroke only animals seen in the study by Papadopoulos (Papadopoulos et al., 2006). The neuroanatomical data also supports the possibility that skilled use of the unimpaired forelimb in the animals in our study increased the basal complexity of layer V pyramidal neurons in the contralesional sensorimotor cortex. Additionally, the larger stroke size seen in our study as compared to the Papadopoulous study could have also increased the basal level of dendritic complexity as there is a direct relationship between increasing stroke size and increased dendritic complexity in the contralesional caudal forelimb motor cortex (Biernkaske et al., 2004). Furthermore, the treatment antibody used in the two studies was different. In the present study, the monoclonal antibody used was 11C7, a specific antibody raised against an 18-amino Nogo-A peptide to the rat sequence of amino acids 623-640. However in the Papadopolous study, hybridoma cells secreting IN-1 antibody were used. The use of hybridoma cells could have had the additional effect of secreting growth factors that may have enhanced the plasticity response in the animals treated with IN-1 in the Papadopoulous study. Another difference between the two studies is that the animals were treated at different times post-stroke, either one week post-stroke or 9 weeks post-stroke. Finally, dendrites were also evaluated much later post-treatment in this present study than in the Papadopoulous study. The dendrites were evaluated for plasticity more than 12 weeks post-treatment initiation in our study while in the Papadopolous study, dendrites were evaluated 6 weeks post-treatment. It is possible that an increase in dendritic plasticity early after treatment could have undergone
pruning, such as seen in mammalian neurological development (Rakic & Bourgeous, 1986) so that by 12 weeks post-treatment, dendritic changes may not have been detectible. In summary, a plasticity response in the neurons within the contralesional caudal forelimb motor cortex may have been masked due to a potential higher level of dendritic complexity due to forelimb switching, coupled with the a pruning response and therefore not detectible by our evaluation. Conversely, it is possible that anti-Nogo-A immunotherapy at 9 weeks post-stroke does not enhance plasticity in pyramidal neurons found within layers II/III and V of the contralesional caudal forelimb motor cortex and dendritic changes in other brain areas related to sensorimotor function, such as the contralesional rostral forelimb motor cortex or contralesional striatum, may account for the improved sensorimotor function seen in our study.

There have been conflicting reports of increased plasticity in the perilesional area after stroke. Brown and colleagues found an increased number of spines on layer V pyramidal neurons that were located within 200 microns of the infarct border in adult rats after photothrombotic stroke (Brown et al., 2008). In direct conflict with this study are the results from Mostany and colleagues that used in vivo two-photon imaging in adult mice and found no large scale dendritic plasticity of layer V pyramidal neurons located in the peri-infarct cortex (Mostany et al., 2011). However, functional imaging studies in humans have also supported the role of the perilesional cortex in stroke recovery (Dong et al., 2007). Therefore, we decided to investigate the effect of anti-Nogo-A immunotherapy administered at 9 weeks post-stroke on dendritic plasticity in the perilesional caudal forelimb motor cortex. We found no change in dendritic complexity
in layer V pyramidal neurons located within the perilesional caudal forelimb motor cortex. The lack of dendritic plasticity in the perilesional area may due to various reasons. Since we did not analyze dendritic spine number and morphology it is possible that spine dynamics could have played a role in the improved sensorimotor function seen after anti-Nogo-A immunotherapy in our experiments. Additionally, dendritic rearrangements may be related to the distance of the neuron from the ischemic border (Brown et al., 2008). Since in our study we included neurons within 2 millimeters from the ischemic border, it is possible that only neurons close to the ischemic border (200 microns) could have undergone reorganization. Additionally, dendritic pruning could have also played a role in the perilesional cortex and hence our evaluation time point could have missed the dendritic rearrangements. Finally, dendritic changes could have occurred in other brain areas such as the ipsilesional rostral forelimb area and the ipsilesional striatum.

Due to the lack of dendritic plasticity in either perilesional or contralesional areas of the caudal forelimb motor cortex in animals treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke, it is possible that behavioral improvement is occurring due to other mechanisms that do not involve the dendritic arbors. In the study by Tsai and colleagues (2010), animals with mild to moderate stroke deficits treated with anti-Nogo-A immunotherapy at 9 weeks post-stroke displayed skilled reaching recovery that was correlated with enhanced corticorubral axonal sprouting from the contralesional forelimb motor cortex to the deafferented red nucleus. Therefore, it is likely that axonal sprouting from the undamaged corticospinal tract is also the source of the improvements seen in our
study. Other areas that may be undergoing axonal sprouting and may be playing a role in the improvements seen in our study include axonal sprouting from callosal fibers of the undamaged hemisphere to the rostral forelimb motor cortex, callosal fibers in the undamaged hemisphere to the ipsilesional dorso-lateral striatum, undamaged corticospinal tract axonal sprouting to the basilar pontine nuclei, and undamaged corticospinal tract axonal sprouting in the spinal cord. Additionally, it is possible that anti-Nogo-A immunotherapy could have enhanced the electrophysiological characteristics or biochemical functioning of neurons in neural circuits important for sensorimotor function.

In conclusion, in this study we have found that anti-Nogo-A immunotherapy is efficacious up to 25 weeks post-stroke for improving sensorimotor recovery on the skilled forelimb reaching task and is a promising therapeutic strategy for improving disability after stroke, even long after the ischemic injury has taken place, although the exact mechanism by which this improvement occurs is still not known.
CHAPTER SIX
GENERAL DISCUSSION

Summation of Results

In animals with spinal cord injury, anti-Nogo-A immunotherapy has been found to be efficacious when administered up until one-week post-lesion, with treatment potency dropping if administered after two weeks post-lesion (Gozenbach et al., 2011). Given this short treatment window for the use of anti-Nogo-A immunotherapy in spinal cord injury, a previous study from our laboratory set out to determine whether anti-Nogo-A immunotherapy was efficacious over a similar or longer time frame when applied in the chronic phase after ischemic stroke. We found that in animals with mild to moderate stroke deficits (15% stroke size), treatment with anti-Nogo-A immunotherapy up to 9 weeks post-stroke was able to induce functional sensorimotor functional recovery in adult rats after stroke (Tsai et al., 2010). Interestingly, the effect size and time course of treatment-induced recovery was similar to that of animals treated immediately or up to 1 week post-stroke, implying no loss in treatment effect potency. We therefore set out to further examine the critical time window for the application of anti-Nogo-A immunotherapy in the chronic phases after stroke. In addition to the critical time window for applying such a neuro-restorative therapy, we were also interested in studying whether the initial severity of stroke deficit mitigates the efficacy of anti-Nogo-A
immunotherapy. Therefore, we investigated the efficacy of anti-Nogo-A immunotherapy for improving moderate to very severe stroke deficits when applied either at 9 weeks (~ 2 months) and 25 weeks (~ 6 months) post-stroke in adult rats.

Previous studies have shown that a limited amount of spontaneous recovery occurs in rats as well as humans post-stroke. Therefore, we evaluated the time-course of spontaneous recovery of skilled forelimb performance after stroke for the first 8 and 24 weeks prior to the application of anti-Nogo-A immunotherapy. Interestingly we found two important parallels in motor recovery post-stroke in adult rats that correspond very well to human post-stroke recovery. Firstly, limited but statistically significant post-stroke recovery in skilled reaching performance was seen overall in animals during the first month post-stroke. Statistically significant recovery post stroke was seen until post-stroke week 2, however minimal improvement was seen up to 4 weeks post-stroke with a stable plateau in recovery seen thereafter. The timeframe in recovery seen in adult rats post-stroke is similar to the timeframe seen in humans. Typically, the majority of spontaneous recovery seen in humans post-stroke is seen within three months post-stroke. However, some estimates show that the majority (80%) of significant motor recovery takes place within 4.5 week post-stroke (Jorgensen et al., 1995a; Jorgensen et al., 1995b) and corresponds well to the time-frame of spontaneous recovery we see in adult rats post-stroke in this study. Secondly, we found that initial deficit severity after stroke modulates the rate and magnitude of spontaneous recovery seen after stroke in adult rats. This relationship between initial deficit severity after stroke and recovery potential has also
been well demonstrated in human stroke recovery (Jorgensen et al., 1995a; 1995b; 1995c) and underscores the similarity between rat and human post-stroke recovery profiles. Animals with initial mild to moderate deficits at 1 week post-stroke generally showed both little deficit and little improvement over time. Effectively, animals in these two deficit categories show very mild deficits and the need for treatment or rehabilitation is low. Conversely, animals with severe deficits at 1 week post-stroke, show statistically significant recovery over time up to post-stroke week 3, however the magnitude of recovery is quite low. This population of animals has the greatest need for a neuro-restorative therapy and most likely will show the greatest benefit. Animals with very severe deficits at post-stroke week 1 show no improvement over time and may be very difficult to rehabilitate with any form of treatment.

Based on the spontaneous recovery profiles seen after stroke in adult rats, we decided to treat animals with anti-Nogo-A immunotherapy at two delayed chronic time points, 9 (subchronic) and 25 (chronic) weeks post-stroke. The stroke deficits seen at post-stroke week 9 and 25 are thought to be stable and the potential for spontaneous recovery of function minimal. The cohort of animals treated with anti-Nogo-A immunotherapy at 9 or 25 weeks post-stroke included animals with moderate, severe, and very severe deficits. Two separate approaches to analyze the recovery data were used for these results. An intention-to-treat analysis was performed that included all deficit severity animals (moderate, severe, very severe) as well as any protocol deviations. A
per-protocol analysis was performed including only animals with moderate and severe
deficits as well as no protocol deviations.

The experimental data from these experiments supports the existence of an
extended therapeutic time window for the use of anti-Nogo-A immunotherapy to improve
sensorimotor functional recovery after stroke in the chronic phase. We demonstrate the
effectiveness of anti-Nogo-A immunotherapy when initiated 9 and 25 weeks post-stroke
in the adult rat to significantly improve sensorimotor performance on the skilled reaching
task using both an intention-to-treat and per-protocol paradigm. Based on these results, it
is likely that anti-Nogo-A immunotherapy is effective chronically after stroke and long
after the lesion-induced neuroplasticity-promoting environment in the brain has
diminished (Biernaske et al., 2004). However, given the fact that animals with very
severe deficits prior to treatment do not improve with anti-Nogo-A immunotherapy and
that the recovery rates and recovery plateau (based on PP analysis) are slower and lower
than that seen in previous studies at similar time points, it is likely that anti-Nogo-A
immunotherapy may have differential efficacy rates based on initial stroke deficit
severity prior to treatment.

Multiple research studies show that inhibiting protein Nogo-A leads to axonal and
dendritic reorganization that is associated with functional recovery (Seymour et al., 2005;
Papadopoulos et al., 2006; Tsai et al., 2011). Therefore, we evaluated dendritic plasticity
in the perilesional and contralesional forelimb motor cortex. We did not find any changes
in complexity across layer V pyramidal neurons in either the perilesional or
contralesional caudal forelimb motor cortex or layer II/III neurons in the contralesional caudal forelimb motor cortex. These results underscore the difficulty relating neuroanatomical structural plasticity with recovery in function and potentially imply other mechanisms or brain areas are responsible for the functional recovery seen in these experiments.
Translation of Anti-Nogo-A Immunotherapy to the Clinic

Over the last two decades, there has been a paradigm shift regarding the ability of the adult CNS to undergo spontaneous cortical reorganization as well as the ability to induce substantial neuronal plasticity through neuro-restorative therapies. With the discovery and cloning of the neurite outgrowth inhibitory myelin associated protein, Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), a new era of investigation geared towards improving function following various CNS lesions has taken place. Since then, the inhibition of Nogo-A has been shown to promote functional recovery following various brain injuries such as stroke, spinal cord injury and traumatic brain injury in animal studies.

Proof of concept experiments using anti-Nogo-A immunotherapy to improve functional recovery after stroke has been extensively studied in pre-clinical studies and been shown to be effective. Studies primarily from the Kartje laboratory have shown that inhibiting protein Nogo-A using function inhibiting monoclonal antibodies after ischemic middle cerebral artery occlusion leads to substantial sensorimotor functional recovery when applied immediately (Papadopoulos et al., 2002), 24 hours (Weissner et al., 2003), 1 week (Seymour et al., 2005), 9 weeks (Tsai et al., 2011; this dissertation), and 25 weeks post-stroke (this dissertation). Furthermore, significant improvement using this therapy was seen in the Weissner study that used a rat model that is spontaneously hypertensive, showing the efficacy of this therapy in a model with hypertension as a comorbid factor. Additionally, we have shown that this therapy is efficacious in the aged
rat, showing only a slowing of recovery, but ultimately the same magnitude of recovery (Markus et al., 2005). Finally, other methods of inhibiting the protein Nogo-A signaling pathway have also been shown to be significant at inducing sensorimotor recovery after stroke, i.e. targeting the Nogo-A related receptor, NgR (Lee et al., 2004) as well as using NEP1-40 to competitively antagonize the NgR receptor (Fang et al., 2010). All these findings support the use of blocking protein Nogo-A in order to improve functional recovery after stroke.

General limitations to the translation of this therapy to the clinic is the necessity of either intrathecal or intracerebroventricular infusion of the antibody. Although an intravenous route of application would improve the feasibility of this therapy, intrathecal and intracerebroventricular drug application routes are already in possible in clinical practice and therefore can be used. Examples of drugs delivered intracerebroventricularly include the anti-spasmodic drug baclofen used for dystonia (Rocque et al., 2012), chemotherapeutic agents such as methotrexate for brain neoplasms (Gabay et al., 2012), and anti-fungals such as amphotericin B for fungal brain infections (Tsung et al., 2010). However, development of a less invasive route of delivery would be of great clinical importance, especially if clinical trials for the use of this therapy show positive results for spinal cord injury and pave the way for this therapy to be tested in clinical trials for other central nervous system injuries and disorders.

Currently, anti-Nogo-A immunotherapy is being evaluated in a phase I human clinical trial in patients with spinal cord injury and a phase II trial is currently being
planned (Abel et al., 2011). A phase I clinical trial is meant to evaluate the safety of a new treatment and identify side effects. In the case of anti-Nogo-A immunotherapy, since Nogo-a plays a role in stabilizing mature neuronal circuits in the human adult brain, it is possible that increasing neuroplasticity in the brain could have other effects than to restore impaired functions. There is a potential risk of destabilizing important circuits involved in long term memory and a potential risk of psychiatric symptoms (Mirnova et al., 2013). Although, no side effects of the treatment have been reported in animal or the phase I clinical trial (Abel et al., 2011), it will be important to monitor cognitive function during anti-Nogo-A immunotherapy in humans. Other potential side effects of too much neuroplasticity in the brain could include the development of seizures and pain syndromes.

The results from our laboratory over the years and specifically this project can be used to better design a human clinical trial for the evaluation of anti-Nogo-A immunotherapy in human stroke. Firstly, our studies have shown that anti-Nogo-A immunotherapy can be used in chronic stroke, and therefore many more stroke survivors could be enrolled in a clinical trial. Secondly, we have shown that anti-Nogo-A immunotherapy is effective in chronic stroke in adult rats with moderate and severe deficits after stroke. Therefore, it is possible that this therapy could be efficacious in moderate and severe human stroke, thereby also providing a larger group of patients eligible for testing. Finally, an important caveat regarding patient enrollment in an anti-Nogo-A immunotherapy study is the fact that patients will need to have some residual
function in their upper extremity since we have shown that adult rats with very severe deficits where no forelimb grasping is possible do not improve after anti-Nogo-A immunotherapy.
Future Directions

It is of interest to further characterize the efficacy of anti-Nogo-A immunotherapy across acute and chronic time points of administration and how treatment effects are modulated by initial deficit severity, stroke size, and stroke location. Results from this type of analysis would have the ability to further inform clinical trial design for testing anti-Nogo-A immunotherapy in humans after ischemic stroke. Therefore, future analyses will incorporate data from all experiments from the Kartje laboratory utilizing anti-Nogo-A immunotherapy, including those from this dissertation, in a non-linear regression model using stroke lesion size and location as co-variate parameters. This way, differences in recovery rate and magnitude can be evaluated directly to see what factors modulate anti-Nogo-A immunotherapy efficacy (age, lesion size, lesion location, initial deficit severity, treatment delay time).

Furthermore, additional experiments can be conducted to evaluate which other neural correlates of recovery may be playing a role in the improved recovery seen in the experiments in this dissertation. Additional brain areas associated with spontaneous recovery after stroke in humans and non-human primates involve the premotor and supplementary areas (Benowitz et al., 2010). An area in the rat brain that is important for forelimb reaching and corresponds to either a premotor or supplementary motor function is called the rostral forelimb area (also known as the forelimb area of Neafsey; Neafsey et al. 1986). Therefore, dendritic plasticity could be evaluated in the ipsilesional/contrallesional rostral forelimb area. Furthermore, other brain areas involved
in motor function could be evaluated such as the ipsilesional/contralesional dorsolateral striatum and ipsilesional/contralesional cerebellum. Additionally, axonal sprouting into the denervated red nucleus was already shown to be correlated with sensorimotor recovery displayed by rats with mild to moderate stroke deficits treated at 9 weeks post-stroke with anti-Nogo-A immunotherapy (Tsai et al., 2010). It is possible that axonal plasticity is playing a larger role than dendritic plasticity in recovery after anti-Nogo-A immunotherapy and axonal sprouting from the undamaged corticospinal tract could be evaluated at the level of the red nucleus, basal pontine nuclei and spinal cord. Additionally, axonal sprouting within the ipsilesional dorsolateral striatum, or callosal sprouting in the perilesional cortex or ipsilesional rostral forelimb motor cortex could be evaluated.

Further experiments could look to see how to rehabilitate the animals that did not respond to therapy, the very severe deficit animals. It is possible that additional methods to improve functional recovery need to be used in addition to anti-Nogo-A immunotherapy. Methods such as intensive skilled reaching rehabilitation or environmental enrichment have been shown to improve functional recovery after stroke in adult rats. Therefore, a combinatorial approach could be taken with these very severe animals to see if a regimen of anti-Nogo-A immunotherapy, intensive skilled reach training, and environmental enrichment could lead to improved skilled forelimb reaching performance. Additionally, increased dosage and length of treatment using anti-Nogo-A antibodies could also be effective.
REFERENCES


VITA

Katherine Marie Podraza was born on March 13th, 1984 in Chicago, IL to Bozena Grodkowska Podraza and Jozef Podraza. At a young age, Katherine’s parents moved to Phoenix, Arizona. Katherine has one older sister named Bernadette Rabiej. Currently, both Katherine’s parents and sister and brother-in-law live in Phoenix, Arizona.

Katherine attended Centennial High School in Peoria, Arizona and graduated as the Valedictorian. For college, she attended Pepperdine University in Malibu, California and majored in Physiological Psychology while following the pre-medical track. After graduating with honors from Pepperdine University, she spent one year working as a clinical trial research assistant at University of California, San Diego in the laboratory of Dr. Adam Fleisher. During this time she met her future husband, Nitin Bangera, who at the time was completing his PhD in biomedical engineering at UCSD.

Katherine was accepted into the MD/PhD program at Loyola Stritch School of Medicine and matriculated in the Fall of 2007. After completion of the first two years of medical school, she joined the laboratory of Dr. Kartje where she spent four years completing her dissertation experiments. During this time, Katherine and Nitin were married on May 19th, 2012 in San Diego, California. Katherine will be graduating with her MD, PhD degree in May of 2015 and will be pursuing Neurology as a specialty.