Characterization of the Effect of Anti-Nogo-A Antibody Treatment on Sensorimotor Recovery After Traumatic Brain Injury

Stephen Nawara

Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

CHARACTERIZATION OF THE EFFECT OF ANTI-NOGO-A ANTIBODY TREATMENT ON SENSORIMOTOR RECOVERY AFTER TRAUMATIC BRAIN INJURY

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

PROGRAM IN MOLECULAR PHARMACOLOGY
AND THERAPEUTICS

BY

STEPHEN G NAWARA

CHICAGO, IL

AUGUST 2015
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LIST OF ABBREVIATIONS

11c7 A monoclonal antibody directed towards AA 623-640 of rat Nogo-A
3T3 3-day transfer, inoculum 3 x 10^5 cells (mouse fibroblast)
AA Amino Acid
AAALAC Association for Assessment and Accreditation of Laboratory Animal Care
Ab Antibody
CCI Controlled Cortical Impact
cDNA complementary DNA; copy DNA
CNS Central Nervous System
cm centimeter
CT Computed Tomography
Da Daltons; kDa -kiloDaltons
DNA Deoxyribo-Nucleic Acid
dpi dots per inch
EC50 half-maximal (50%) Effective Concentration
Fab Fragment antigen-binding
FWHM Full-Width-Half-Maximum
GTP Guanosine-5’-triphosphate
HDI Highest Density Interval
IACUC Institutional Animal Care and Use Committees
ID Identification
iid independent and identically distributed
IgG1 Immunoglobulin G subclass 1
<table>
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<tr>
<td>IN-1</td>
<td>monoclonal Ab towards a 250 kDa protein fraction later identified as Nogo</td>
</tr>
<tr>
<td>JAGS</td>
<td>Just Another Gibbs Sampler</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LINGO-1</td>
<td>Leucine rich repeat and Ig domain containing 1</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM domain kinase 1</td>
</tr>
<tr>
<td>mAB</td>
<td>monoclonal Antibody</td>
</tr>
<tr>
<td>MCMC</td>
<td>Markov Chain Monte Carlo</td>
</tr>
<tr>
<td>m/sec</td>
<td>meters per second</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>msec</td>
<td>millisecond</td>
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<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<tr>
<td>NEP1-40</td>
<td>Nogo Extracellular Peptide AA 1-40</td>
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<tr>
<td>Nobs</td>
<td>Number of Observations</td>
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<td>Nogo</td>
<td>Neurite Outgrowth Inhibitor; Reticulon-4; Nogo-A/B/C-isoforms of Nogo</td>
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<td>NI-35/250</td>
<td>Neurite Inhibitor of MW 35/250; later identified as Nogo</td>
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<td>NgR1</td>
<td>Nogo Receptor 1; Nogo-66 Receptor</td>
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<tr>
<td>p75NTR</td>
<td>Low-Affinity Nerve Growth Factor Receptor; p75 Neurotrophin Receptor</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC12</td>
<td>Rat Pheochromocytoma cell line #12</td>
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<tr>
<td>PirB</td>
<td>Paired Immunoglobulin-like Receptor B</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
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<tr>
<td>Rac-1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
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<tr>
<td>S1PR2</td>
<td>Sphingosine-1-Phosphate Receptor Number 2</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal Cord Injury</td>
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<tr>
<td>SDS/PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Taj/TROY</td>
<td>Tumor necrosis factor receptor superfamily, member 19</td>
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<td>TBI</td>
<td>Traumatic Brain Injury</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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<td>UV</td>
<td>Ultra-Violet</td>
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CHAPTER 1

ABSTRACT

Estimates indicate that over 5 million Americans are currently living with Traumatic Brain Injury (TBI) related disability. Much effort has been put into enhancing functional recovery with physical therapy and other rehabilitative approaches. These strategies are thought to function by facilitating the brain’s innate capacity for neuroregeneration and neuroplasticity. However, the success of these strategies may be limited due the presence of growth- and plasticity- inhibiting factors expressed in the adult brain.

The presence of the membrane protein Nogo-A has been associated with reduced neurite outgrowth in vitro, identifying it as one such molecule. In addition, rodents treated with antibodies raised against Nogo-A have been reported to show a greater degree of axonal growth and motor performance after a variety of injuries to the spinal cord and brain. Here, the effect of anti-Nogo-A immunotherapy was studied using the controlled cortical impact (CCI) model of TBI.

Rats received CCIs to the sensorimotor cortex opposite the preferred forelimb. Beginning one week later, anti-Nogo-A antibody (11c7) was administered to the lateral cerebral ventricle for two weeks. Recovery of these animals was assessed using the skilled reaching task and compared to controls from one day post-injury until eight weeks.
later.

The anti-Nogo-A treated animals recovered an average of 90% baseline skill, while controls recovered only 60%, suggesting that the treatment may be beneficial. However, this conclusion depends on the assumption that performance on the reaching task is a measure of capability. If so, after a period of re-learning the skill, the individual rat should enter a plateau phase that reflects maximal performance. During this phase, the distribution of day-to-day success rate for each individual should follow a specific probability distribution (binomial). However, in our study the scores during the plateau phase were often much more consistent than predicted, a strong indication that capability was not measured. Further, neither the final performance nor profile of recovery were uniform within each group. When fit with a sigmoidal curve, the distribution of the plateau skill levels appeared multimodal.

It is possible that performance was determined by some neurological characteristic of the rats, perhaps related to the brain injury. However, total lesion size and cortical lesion location were not well correlated with performance and could not explain the greater degree of recovery observed for anti-Nogo-A treated animals.

Another possibility is that performance was determined by neuroplastic processes, previously proposed to be influenced by anti-Nogo-A antibody. To this end, the brains of the same rats were stained using the Golgi-Cox procedure. The degree of dendritic branching and soma area was measured for pyramidal neurons from the contra-lesional cortex. However, these measurements also did not correlate with performance on the reaching task nor differ between groups.
While any number of unmeasured neurological aspects could be responsible for differences in performance that were not examined in this study, it is also possible that there are none. The low day-to-day variance and multimodality indicated that there were at least two strategies that could be used by the rats during the reaching task. Therefore, a simple stochastic model of two-strategy habit learning is proposed, as follows:

The model required only four parameters shared amongst all rats: 1) probability strategy A is successful; 2) probability strategy B is successful, 3) initial probability of choosing strategy A; 4) rate of learning strategy A. Despite the low number of parameters, this model is capable of generating recovery profiles similar to those observed for each rat. Further work is required to determine the best set of parameters to consistently capture the low plateau variance and other aspects the data. However, this result does show that differences in plateau performance need not be related to any underlying neurological difference.

In conclusion, anti-Nogo-A immunotherapy administered one week following TBI was associated with average recovery of 90% baseline skill (controls: 60%) on the forelimb reaching task. However, no correlation between reaching score and lesion size/location, or number of dendritic branches was found. Further study is needed to determine the mechanism of recovery and a better approach to behavioral modeling of recovery profiles.
CHAPTER 2

REVIEW OF LITERATURE

Traumatic Brain Injury

In the last decade, it has become increasingly common for Traumatic Brain Injury (TBI) to be considered a “silent epidemic”. Efforts at characterizing the epidemiology of TBI have been plagued by diagnostic ambiguity and confounding by shared symptoms from other causes. Further, many people with minor TBIs likely do not attempt to seek medical help. With this in mind, the current estimate of around 12 million people living with TBI related disability in the United States and European Union alone is likely an underestimate (Roozenbeek et al., 2013).

A recent definition of TBI is “an alteration in brain function or other evidence of brain pathology caused by an external force” (Menon et al., 2010). Injury due to TBI is often considered to take place in two phases. First is the primary injury, during which a focal ablation/contusion to brain occurs and/or diffuse biomechanical damage disrupts axonal tracts. These processes are commonly associated further with vascular damage and thus haemorrhagic and ischemic damage. Secondary injury follows, which is characterized by an inflammatory response accompanied by oxidative stress and excitotoxic phenomenon (Werner and Englehard, 2007). This second phase is thought to continue for months to years following the primary insult and has been targeted (thus
far unsuccessfully) by “neuroprotective” strategies aimed at suppressing inflammatory responses, scavenging free radicals, and/or reducing brain energy consumption (Faden and Stoica, 2007).

Clinicians report there is little evidence that progress has been made during the last 25-35 years in reducing overall mortality from TBI (Stein et al., 2010) or improving functional outcomes after TBI (Rosenfeld et al., 2012; Roozenbeek et al., 2013). For example, Roozenbeek et al. (2013) write:

“The substantial decrease in mortality between 1970 and 1990 was attributed to the introduction of CT scanners and advances in intensive care, which led to improvements both in detection of TBI and in patient care. The static mortality after 1990 is surprising... Similar conclusions were drawn in a recently published meta-analysis of observational studies that took place between 1980 and 2011... Neither a clear reduction in mortality nor a decrease in the rate of unfavourable outcome over time was observed.”

Despite much effort, no treatments showing promise in preclinical animal trials have been successfully translated into therapies suitable for clinical use (Maas et al., 2012). The above holds true for neuroprotective agents as well as rehabilitative approaches. Similar to the neuroprotection literature, the evidence for efficacy of current strategies aimed at improving either cognitive (Koehler et al., 2011) or physical (Bland et al., 2011) function after TBI have been questioned. The failure to develop an unequivocally useful strategy has been commonly attributed to factors such as wide variability in injury properties in patients and inapplicability of rodent results to human pathology, amongst others (Mass et al., 2012).

**Animal Models of Traumatic Brain Injury**

A number of strategies have been developed to model TBI in animals (for review
The controlled cortical impact (CCI) model used for the current study utilizes a pneumatic piston to impact the cortex through a craniotomy. It was developed in response to the need for reproducible injuries (Lighthall, 1988). The primary advantage over earlier strategies such as the weight drop model (Feeney et al., 1981), which involves dropping a weight from a pre-specified height onto the head (either with or without craniotomy) of the animal, is the relatively greater controlled and reproducible nature of the injury event. A third common model is termed fluid percussion injury which is implemented by placing a reservoir of fluid over the cortex and striking an attached piston that creates pressure waves over the dura mater (Xiong et al., 2013). This is meant to mimic closed head injury but, like the weight drop model, is considered less consistent than the CCI technique.

**Neuroplasticity Research**

One plausible strategy for improving functional outcome after TBI is to increase the ability of the remaining undamaged tissue to compensate for that which has been lost via processes termed neuronal regeneration and plasticity. This could occur due to new growth of damaged neuronal tracts (axonal regeneration), formation of new axonal branches from undamaged neurons (axonal sprouting), alterations in the relative strengths of preexisting neuronal connections (synaptic plasticity), and growth of new dendritic branches and spines to accommodate new axonal projections (dendritic plasticity).

The ability of neurons of the central nervous system to alter their structure has been hypothesized since at least the time of Galen when he claimed (referring to the brain) “a softer substance is always more easily modified than a harder” (Bennet and
Hacker, 2002). In the late 1800s, Hughlings Jackson’s work with hemiplegic patients led him to state “nervous arrangements near to those destroyed, having closely similar duties, come to serve, not as well, but, according to the degree of gravity of the lesion, next and next as well as those destroyed” (Jackson, 1888). In contrast with these claims of clinical practitioners, the neurohistologist Ramón y Cajal wrote in 1913 “once the development was ended, the founts of growth and regenerations of the axons and dendrites dried up irrevocably” (Stahnisch et al., 2002). Despite accompanying this claim with a warning that it was a conjecture and later expressing optimism for “restorative plasticity” after injury, the concept that the adult central nervous system was static and that no regeneration could occur became dogma into the 1950s (Freeman, 1952).

Much of the work during the first two-thirds of the 20th century on this matter focused on spinal cord transections and attempts to determine the mechanism for the lack of regeneration despite common observance of partial functional recovery (Liu and Chambers, 1958). A common explanation for lack of regeneration at the time was that the peripheral nervous system contained Schwann cells while the CNS did not, implying that the Schwann cells must be required for neural growth (Sugar and Gerard, 1940). Alternatively, it was proposed there could be some innate property of the CNS neurons or their environment that prevented plasticity (Sugar and Gerard, 1940). Research of this phenomenon remained highly conflicting and controversial for many years due to numerous confounds and technical issues. The beginning of a resolution to these questions was found only after the development of horseradish peroxidase staining (Weisblat et al., 1978). This method allowed tracing of CNS axons across a PNS graft
to the lesioned spinal cord, leading to the conclusion that “Schwann cells are more conducive to axonal regeneration than central neuroglial cells” (Richardson et al., 1980). This suggested that adult CNS neurons were innately able to grow and that some aspect of the CNS environment was responsible for the lack of regeneration.

Around this time studies of cortical plasticity were also being performed. At the time, the possibility of “functional remodelling” after an injury was accepted. However, the mechanisms proposed did not allow for long distance structural changes (Jenkins et al., 1990). This changed as new evidence was published such as that from the “Silver Spring Monkey” experiments that suggested a greater degree of cortical reorganization could occur than could be explained by preexisting neural circuitry (Pons et al., 1991).

**Mechanism of Action of Nogo-A**

Nogo-A is thought to have two main inhibitory regions (Figure 1) termed Nogo-Δ20 (Oertle et al., 2003a) and Nogo-66 (GrandPré et al., 2000). Both are said to sequentially modulate the activity of RhoA (Niederöst et al., 2002) and Rho Kinase (Niederöst et al., 2002). Nogo-66 is then thought to further affect LIMK (Hsieh et al., 2006) and Cofilin (Hsieh et al., 2006) signalling leading to destabilization of actin filaments and inhibition of neurite outgrowth and growth cone collapse (Hsieh et al., 2006).

A receptor for Nogo-Δ20 is reported to be Sphingosine-1-Phosphate Receptor 2 (S1PR2) (Kempf et al., 2014), while Nogo-66 may signal via receptor complexes variably consisting of: NgR1 (Fournier et al. 2001), p75NTR (Wang et al., 2002a; Wong et al., 2002), LINGO-1 (Mi et al., 2004), TAJ/TROY (Park et al., 2005; Shao et al. 2005), and
Figure 1. Schematic of Human Nogo-A. 

A. Nogo-A (Reticulon 4a) is a 1,192 AA protein with two primary regions of interest. In at least one conformation the N-terminus (Exons 1-3) appears to be extracellular (Oertle et al., 2003a). Recombinant peptides derived from the regions labeled Nogo-Δ20 and Nogo-66 are reported to inhibit neurite outgrowth and induce growth cone collapse. The Nogo-Δ20 and Nogo-Δ2 fragments also inhibit 3T3 fibroblast spreading (Oertle et al., 2003a). Two other main isoforms exist that share a common 188 AA C-terminus. Nogo-B lacks the regions coded by Exon 2 and Exon 3. Nogo-C lacks the regions of Exons 1-3, it is the product of an alternative promoter encoding an 11 AA N-terminus. Thus Exon 3 and Nogo-Δ20 are unique to the Nogo-A isoform. 

B. The amino acid sequence of human Nogo-A (NCBI Reference Sequence: NP_065393.1) is shown. Colors correspond to those of the schematic. Peptide P472(NYESIKHEPENPPYEEA) is the target of the 11c7 antibody (Oertle et al., 2003a). KRKAE is an ER-retention signal (GrandPré et al., 2000). KELRR mutants did not affect neurite outgrowth. (Li. et al., 2008). AA: Amino Acid, TM: Transmembrane Domain.
However, the above reviews do not examine that the effects appear to depend upon factors such as cell type (tissue, species, age) (Fournier et al., 2001), dosage, recombinant Nogo-A construct (tags, expression system) (Fournier et al., 2001), form of the construct (substrate vs. soluble) (Fournier et al., 2001), and duration of the experiment (Niederöst et al. 2002). Attempting to summarize evidence regarding the activity of Nogo-A without respect for these factors is somewhat misleading.

Neither neurite outgrowth nor growth cone collapse appear to have been demonstrated under the same conditions that RhoA-GTP levels were measured. If true, this places a major limitation on the confidence we can place in the above narrative (Nogo-A activates RhoA/etc. leading to inhibition of neurite outgrowth). While there is indirect evidence that treatment with the RhoA inhibitor C3 transferase or the Rho Kinase inhibitor Y27632 can attenuate the Nogo-A effect, these treatments also increase neurite outgrowth (Niederöst et al., 2002, Fournier et al., 2003) and fibroblast spreading (Schmandke et al., 2013) under the control conditions. The doses of Y27632 used (≥10 μM) should also be sufficient to affect a number of other kinases (Ishizaki et al., 2000). However, one factor is particularly interesting: the role of dimerization and aggregation of Nogo-A constructs.

**Similarity Between Nogo-A and Amyloids**

Nogo-A proteins and peptide fragments derived from them appear to be natively unfolded in aqueous solution. This is evidenced by far-UV circular dichroism (Li and
Song, 2007; Zander et al., 2007; Li et al., 2004a, Vasudevan et al., 2010, Li et al., 2008) and unusual migration on SDS-PAGE gels (GrandPré et al., 2000; Oertle et al., 2003a; Dodd et al., 2005; Zander et al., 2007; Li et al., 2004a; Li and Song, 2007; Fiedler et al., 2002; Schweigreiter et al., 2004; Joset et al., 2010).

Consistent with their unfolded state, size exclusion chromatography experiments indicate that N- and C-terminal Nogo-A fragments tend to form aggregate and/or oligomeric structures (Zander et al., 2007; Huebner et al., 2011). The high insolubility of recombinant Nogo-66 constructs is also well known (GrandPré et al., 2000; Oertle et al., 2003a; Atwal et al., 2008; Li et al., 2004a; Li et al., 2006).

The amyloid forming proteins alpha-Synuclein (Cookson, 2009; Moussa et al., 2004) and amyloid-beta (Kawooya et al., 2003; Ni et al., 2011) as well as others (Uversky, 2008) are reported to share these properties. In particular, soluble amyloid-beta is also reported to increase RhoA-GTP, decrease Rac1-GTP, and inhibit neurite outgrowth (Petratos et al., 2008). This peptide is also reported to stimulate RhoA and regulate the cytoskeleton of 3T3 fibroblasts (Pianu et al., 2014). Others have reported it can induce growth cone collapse (Kuboyama et al., 2015) and cause p75NTR-dependant activation of RhoA and Rho Kinase (Saadipour et al., 2013). Substrate-bound amyloid-beta is also reported to decrease cell adhesion and neurite outgrowth (Postuma et al., 2000).

Additionally, amyloid beta has been reported to interact with the Nogo-66 receptors p75NTR (Wang et al., 2011b), PirB (Kim et al., 2013) and NgR1 (Park et al., 2006), while amyloid-beta precursor protein has also been said to interact with Lingo-1 (Laat et al., 2015). In addition, amyloid beta has been reported to induce cofilin
dephosphorylation (Davis et al., 2011) and affect sphingosine-1-phosphate signalling (Kaneider et al., 2004).

It is thought that the ability to form amyloid structures is a generic property of polypeptide chains, independent of amino acid sequence (MacPhee and Dobson, 2000; Knowles et al., 2014). Since the activities of amyloid-beta and Nogo-A appear to overlap in essentially every case that has been reported, the physical properties of the Nogo-A constructs should be investigated. It is quite possible the experimental conditions are causing them to form generic amyloid structures or other aggregates, something that may or may not be relevant in vivo.

Conditions that encourage aggregation are required for, or enhance, the in vitro effects of both N- and C-terminal fragments of Nogo-A (Fournier et al., 2001; Niederöst et al. 2002; Oertle et al. 2003a). Further, at least the inhibition of fibroblast spreading induced by Nogo-A substrates correlates very closely to the molecular weight of the different fragments (see Appendix A). This also suggests some kind of sequence-independent mechanism.

Overview of Nogo-A Research

Early Nogo-A Research (IN-1 Antigen Era)

As mentioned above, by the 1980s there was increasing acceptance that the adult mammalian CNS was at the very least not innately static. It was at this time that Dr. Martin Schwab began a series of experiments aimed at elucidating the role of the CNS environment in axonal regeneration. First, it was reported that failure of axonal
regeneration was not due to a lack of soluble neurotrophic factors (Schwab and Thoenen, 1985), but instead to the presence of oligodendrocytes or CNS myelin (Schwab and Caroni, 1988). Two protein fractions were isolated from myelin with axonal growth inhibitory properties in vitro (Caroni and Schwab, 1988b), and a monoclonal antibody (named IN-1) was raised against these fractions that neutralized this inhibitory activity (Caroni and Schwab, 1988a). Further reports supported the idea that contact inhibition was the responsible mechanism (Bandtlow et al., 1990).

These cell culture studies were soon followed by intra-cerebroventricular implantation of IN-1 antibody secreting hybridoma cells into rats that had received spinal cord transections. Long distance sprouting of axons at the lesion site was reported (Schnell and Schwab, 1990), thus issuing in the era of Nogo-A (originally referred to as NI-35/250; IN-1 antigen) research.

The next decade witnessed further reports that this protein played a role as an axon guidance molecule (Schwab and Schnell, 1991), limiting in vivo axonal regeneration (Cadelli and Schwab, 1991; Bartsch et al., 1995; Varga et al., 1995; Raineteau et al., 1999), limiting functional recovery after chronic injury (von Meyenburg et al., 1998), and suppressing growth-related gene expression of contacted neurons (Zagrebelsky et al., 1998).

Other studies reported that in vivo distribution of the protein during development and injury correlated with reduced plasticity (Stichel et al., 1995; Stocker-Buschina et al., 1996). It was also observed that embryonic neurons were less responsive (Bandtlow and Löschinger, 1997), and human myelin was reported to be as inhibitory as myelin
from the rat (Spillmann et al., 1997). Further, Amberger et al. (1997) proposed that oligodendrocyte precursor cells could neutralize inhibitory CNS myelin via a metalloprotease.

The primary investigator of the current work, Dr. Wendy Kartje, joined this research network in 1998 leading a study reporting increased functional recovery and corticofugal plasticity after pyramidotomy and neutralization of the IN-1 antigen (Z’Graggen et al., 1998). These experiments were the first to incorporate the skilled reaching task into anti-Nogo-A research, which Dr. Kartje had learned from her time in the lab of Anthony Castro who had earlier developed the task (Castro, 1972). This same year she also reported that IN-1 treatment is associated with increased innervation of transplants into damaged sensorimotor cortex (Schulz et al., 1998). Further reports that IN-1 increased corticofugal (Wenk et al., 1999) and corticostriatal plasticity (Kartje et al., 1999) followed the next year.

In 1998, the purification of “bovine neural growth inhibitor” allowed the first cDNA cloning of this inhibitory protein (Spillmann et al., 1998). This year also saw the first use of the name “Nogo” for the protein in a Society for Neuroscience conference abstract (Chen et al., 1998).

Later Nogo-A Research (Nogo Era)

The year 2000 was a “breakout year” for Nogo-A research. The IN-1 antibody was humanized (Brösamle et al., 2000), along with simultaneous publication of cloning and identification of Nogo-A as a member of the Reticulon family of proteins by the Schwab group (Chen et al., 2000) and two others (GrandPré et al., 2000; Prinjah et al.,
At this point a comprehensive account of Nogo research becomes unwieldy. As shown by Figure 2, while around 25 papers on NI-35/250/Nogo-A had been published during the previous two decades, at least 14 were published in 2000, 14 in 2001, followed by an increase to 44 in 2002. Over 900 papers have been published in total as of October 2013.

During this time, further research characterized the Nogo-A protein by mapping its inhibitory regions, of which 3 were identified (Oertle et al., 2003a). Analysis of the evolution of the Nogo gene identified homologues in species as distant as yeast and found evidence that the most inhibitory region arose ~350 million years ago during the appearance of the first land vertebrates (Oertle et al., 2003c). Study of the genomic structure of the Nogo gene identified a total of ten variants, the three most common of which are Nogo-A, Nogo-B, and Nogo-C (Oertle et al., 2003b).

Expression of Nogo-A was detected in both oligodendrocytes and multiple populations of neurons in adult mouse (Wang et al., 2002b), rat (Huber et al., 2002), and human (Buss et al., 2005). Nogo-A expression in rat oligodendrocytes was reported to first occur during myelination (Postnatal days 5-9) (Huber et al., 2002). A number of other studies are described in the following sections.

Nogo-A Research Network

Two major subsets have arisen within the Nogo-A research network, the first centered around Dr. Schwab in Zurich, Switzerland, the second around Dr. Strittmatter at Yale in New Haven (see Figure 2). Network analysis indicated that at least one of these two investigators is linked to the majority of others via co-publication (not shown).
Figure 2. Nogo-A Research Network. A Pubmed search was made, and results were manually filtered for false positives. The location of the publishing authors, year of publication, and word frequencies of the abstracts were then extracted. The above shows the results for 2013. Nogo-A research consists of over 900 publications performed in over 180 cities, with over 60 journal articles published on the topic each year since 2005. A large proportion of the research has taken place in Zurich, Switzerland and New Haven, Connecticut, USA which corresponds to the location of labs headed by Martin Schwab and Steve Strittmatter, respectively. Word frequency analysis indicates that top topics for 2013 include the Nogo receptor (NgR1), regeneration, and expression patterns of Nogo and related proteins.

PubMed Search Terms: “Nogo-a OR NogoA OR “Nogo A” OR Nogo OR RTN4 OR “NI-250” OR “NI-35/250” NOT GO/Nogo NOT go-Nogo”
Enhanced recovery has been reported by collaborators of the Schwab network after use of neutralizing antibodies in a number of CNS injury models, such as spinal cord injury (Merkler et al., 2001; Fouad et al., 2004; Liebscher 2005), pyramidotomy (Blöchlinger et al., 2001; Raineteau et al., 2002; Bareyre et al., 2002;), and stroke (Papadopoulos et al., 2002; Emerick and Kartje, 2004; Wiessner, et al., 2003; Markus et al., 2005; Papadopoulos et al., 2006; Tsai et al., 2007; Tsai 2011).

Meanwhile, the collaborators of the Strittmatter network has focused on the Nogo-66 receptor (NgR1) as a drug target, which they identified (Fournier et al., 2001). Strategies have included neutralization of Nogo-A inhibitory activity with 1) a solubilized form of the receptor (Fournier et al., 2002), 2) a NgR1 antagonist in the form of a peptide fragment of Nogo-66 they named NEP1-40 (GrandPré et al., 2002), and 3) a monoclonal antibody directed at NgR1 (Li et al., 2004b).

**Nogo-A and Spinal Cord Injury**

The majority of the preclinical trials have been performed in the context of spinal cord injury (SCI). While a substantial literature exists supporting the role of Nogo-A in preventing recovery after SCI (Kwon et al., 2011), the evidence for the effectiveness of targeting Nogo-A after SCI is mixed. In studies of Nogo-A knockout (KO) mice some have reported lack of regeneration and/or functional recovery (Zheng et al., 2003; Zheng et al., 2005; Lee et al., 2009; Lee et al., 2010a; Lee et al. 2010b) and suggested the existence of artifactual labeling of axons (Steward et al., 2007). Others have reported CST regeneration in the KO mice (Kim et al., 2003; Simonen et al. 2003; Cafferty et al. 2010).
Mice have also been generated to lack NgR1 which is reported to result in increased axonal growth and functional recovery (Kim et al., 2004; Harel et al., 2010; Wang et al., 2011a). Inhibition of Nogo-66 signalling with a Ngr1 antagonist (GrandPré et al., 2002; Li and Strittmatter, 2003), soluble NgR1 (Li et al., 2004b; Ji et al., 2005; Li et al., 2005; Wang et al., 2006; Wang et al., 2011a), or inhibition of ROCK (Fournier et al., 2003) has been reported successful. However, Steward et al. (2008) attempted a direct replication of Li and Strittmatter (2003) and reported lack of consistent functional recovery.

Anti-Nogo-A treatment has been reported to improve functional recovery (von Myenburg et al., 1998; Merkler et al.; 2001; Liebscher et al., 2005; Maier et al., 2009; Gonzenbach et al., 2010; Gonzenbach et al., 2012; Zhao et al., 2013) and increase axonal growth (Schnell and Schwab, 1990; Cadelli and Schwab, 1991; von Myenburg et al., 1998; Brosamle et al., 2000; Liebscher et al., 2005; Müllner et al., 2008; Maier et al., 2009; Gonzenbach et al., 2012; Schnell et al., 2011; Zhao et al., 2013) in rats. However, Oudega et al. (2000) reported that anti-Nogo-A treatment did not enhance regeneration of rat sensory axons.

Axonal growth after anti-Nogo-A has also been reported in the Marmoset (Fouad et al., 2004), macaque (Freund et al., 2006; Freund et al., 2007) and primate (Freund et al. 2009). However, Ho and Tessier-Levigne (2006) questioned the results of Freund et al. (2006) and others have not observed promising axonal growth in the macaque (Wannier-Morino et al., 2008; Beaud et al., 2012; Hoogwoud et al., 2013). Further, Beaud et al. (2008) reported no effect on cell body shrinkage of axotomized cortico-spinal neurons in
Phase I clinical trials coordinated by Novartis using the anti-Nogo-A approach of a human antibody in traumatic SCI were initiated in 2006 and reported no side effects (Zorner and Schwab, 2010). Phase II efficacy trials are underway but results have not yet been reported.

**Nogo-A and Traumatic Brain Injury**

There have been a number of studies characterizing the role of Nogo-A in animal models of TBI. Enhanced recovery was reported after fluid percussion model of TBI to the parieto-temporal cortex and anti-Nogo-A therapy in the rat (Lenzlinger et al., 2005). However, others have reported impaired functional outcome after the CCI model of TBI to the parieto-temporal cortex (Marklund et al., 2009) in aged Nogo-A/B-/- knockout mice. Marklund had previously reported improved cognitive but not motor recovery after fluid percussion injury and anti-Nogo-A treatment to rats (Marklund et al., 2007). Others reported that vaccination against Nogo-A along with other neurite growth inhibitors is associated with improved function after fluid percussion injury to the rat (Zhang et al., 2008).

Nogo-A expression was observed to be upregulated in the cortex ipsilateral to fluid percussion injury in rats but downregulated in the hilus of the hippocampus using immunostaining (Marklund et al., 2006). However, a similar study reported upregulation of Nogo-A in the hippocampus using western blot data. These increased expression levels were reduced to control levels in rats that underwent exercise (Chytrova et al., 2008). Consistent with the latter, one report concluded that there was upregulation of Nogo-A in
the hippocampus that could be suppressed by treatment with indomethacin after injury in the weight drop model (Chao et al., 2012). Another study reported reduction in neuronal Nogo-A expression but upregulation of Nogo-A in non-neuronal cells in the ipsilesional cortex after CCI to the sensorimotor cortex in rats (Jones et al., 2012).

Studies characterizing the role of Nogo receptors include a report that knockout of a subunit of the Nogo receptor PirB did not enhance recovery after CCI to the mouse motor cortex (Omoto et al., 2010). Another study reported that knockout or antagonism of NgR1 impairs cognitive recovery after CCI to the parieto-temporal cortex in mice (Hånell et al., 2010). However, a more recent report is that enhanced cognitive recovery occurs after CCI to the parietal cortex in NgR1 knockout mice (Tong et al., 2013).

Reports of receptor expression changes after TBI include upregulation of NgR1 in the spinal cord after aspiration lesion to the motor cortex of rhesus monkeys (Nagamoto-Combs et al., 2010), as well as upregulation of Nogo-A and NgR1 in the ipsilesional hemisphere of brains of rats that underwent weight drop model of TBI (Yang et al., 2013). In the latter study the upregulation appeared to be reduced by treatment with a form of vitamin E (alpha-Tocopherol).

When collagen scaffolds were crosslinked to NgR1 and placed into the cortical cavities of rats that received penetrating brain injuries, an increased infiltration of cells and extracellular matrix was observed (Elias and Spector, 2012).

**Nogo-A and Stroke**

Kilic et al. (2010) reported that neutralization of Nogo-A decreased neuronal survival
in the acute phase of stroke, and Guo et al. (2013) reported a Nogo-A fragment reduced oxidative damage. Besides this, all studies published targeting Nogo-A for inhibition in models of stroke have reported promising results (Papadopoulos et al., 2002; Wiessner et al., 2003; Lee et al., 2004; Markus et al., 2005; Papadopoulos et al., 2006; Seymour et al., 2005; Ma et al., 2007; Tsai et al., 2007; Wang et al., 2007; Zhou et al., 2007; Wang et al., 2008; Gillani et al., 2010; Tsai et al., 2011; Wang et al., 2010; Wang et al., 2012).

As both stroke and TBI are cortical injuries, this supports the idea that such a treatment could be useful after TBI. The evidence for improved recovery after targeting of Nogo-A by a variety of methods in a variety of models could be taken as a strong corroboration of the treatment effect.
CHAPTER 3
RESEARCH OBJECTIVES AND RATIONALE

Significance of the Project

There are currently few, if any, effective therapies available for people living with disability due to TBI. Recovery due to physical therapy is limited at best (Roozenbeek et al., 2013). Previous work in animal models of spinal cord injury and stroke suggest that anti-Nogo-A therapy could be beneficial in facilitating the capacity for the adult CNS to compensate for lost tissue via neuroplastic mechanisms.

Main Hypothesis

Intra-cerebro-ventricular infusion of anti-Nogo-A antibody into the lateral ventricles of adult male rats one week after controlled cortical impact to the motor cortex is capable of facilitating improved functional recovery by inducing an environment conducive to neuronal plasticity in the contralesional homotopic cortex.

Specific Aim 1

Assess the effect of anti-Nogo-A therapy on functional recovery as measured with the skilled reaching task after TBI.

- Define functional recovery in the context of the skilled reaching task and determine what assumptions and predictions can be derived from this definition.
-Determine whether recovery profiles can be informatively summarized with an equation that captures both the degree of recovery as well as timecourse of recovery.

**Specific Aim 2**

Develop a method to map the cortical lesion locations of each animal and assess whether this factor influences recovery profile.

**Specific Aim 3**

Assess the contralesional sensorimotor cortex for signs of increased or altered patterns of neuronal plasticity after anti-Nogo-A therapy by determining the degree of dendritic branching and the distribution of neuronal cell body size.
CHAPTER 4

MATERIALS AND METHODS

Animal Welfare and Housing

All animals were housed as pairs in solid-floored plexiglass cages and fed standard rat chow. The light-dark cycle had a period of 12 hours. Anaesthesia was sustained with ~2.5% isoflurane during all surgical procedures while body temperature was monitored and maintained at 37 degrees Celsius with a heating pad. All procedures were approved by the Hines Veteran Affairs Hospital AAALAC accredited IACUC and complied with USDA Animal Welfare Act regulations as well as Public Health Services policy.

General

Forty-seven adult, male Sprague-Dawley rats (Harlan) were used in this study. Handling was begun at 8 weeks of age followed by 2-4 weeks of training at the skilled reaching task. The experiments were run in four batches (cohorts) of 12 rats consisting of mixed treatment groups (see Figure 3 and Figure 4). One rat did not develop baseline reaching skill and was removed from the study. Once baseline reaching skill level was attained (>15 successes per session) for the entire cohort, all rats underwent the Controlled Cortical Impact (CCI) procedure described below. After a one week delay, animals were distributed to anti-Nogo-A, Control Ab, or TBI-only treatment groups using
Figure 3. Experimental Timeline and Overall Workflow. A. Experimental Timeline as described in the methods text. A cartoon of a rat with a subcutaneous pump installed gives an idea of the bulk of the device. Also shown is a rat reaching for a sucrose pellet and a typical example of a controlled cortical impact (CCI) lesion. The coronal section is not from the same brain as the “whole brain” picture.
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Figure 4. List of Animal IDs for Each Cohort. Cohorts consisted of 12 rats each. Animals in the shaded grey cells were not analyzed for this study. Animals in yellow highlighted cells were available for behaviour but not lesion analysis. Animals with one star next to their ID were unavailable for branching analysis, those with two stars for neither soma size nor branching. One exception was Rat 2280 (three stars) whose stain was sufficient for tracing branches but had fewer somas stained than the others. As described in the text, the animals in the first cohort all recovered by four weeks post-TBI and thus the study was terminated early. Assessment post-sacrifice indicated these animals had lesions posterior to the location specified in the protocol. Animal 9024 did not attain baseline reaching skill. When referring to treatment groups throughout this report, the colors Blue, Red, and Green are used to indicate TBI-only, anti-Nogo-A, and Control Ab groups respectively.
a matched-subjects design based upon recovery after one week. A pump was installed (except for the TBI-only group) for infusion of antibody (Ab) into the contralesional lateral cerebral ventricle. Two weeks later the pumps were removed. Sensorimotor testing (Skilled Reaching) was performed throughout this period and for 5 weeks further. After a two week delay the rats were sacrificed and brains collected for modified Golgi-Cox Stain to assess dendritic plasticity. Three-parameter logistic curves were fit to each rat’s skilled reaching timecourse. Golgi-Cox stained sections were then analyzed for contralesional neuroplasticity in terms of both soma size distribution and dendritic branching profile. Correlations between lesion location and reaching outcome were calculated. Histology (lesion and plasticity analysis) could not be performed for all animals due to 1) use for other pilot studies, 2) researcher error in processing the tissue sections, or 3) weak staining. The cause of the latter is unknown.

Researchers were blinded while testing rats on all behavioural tasks and plasticity assessment as well as when “correcting” artifacts (see Figure 8) during the lesion analysis process. However, many of the later stages of analysis were exploratory and blinding was inconsistent. It was not possible to avoid becoming familiar with the appearance of different animal’s data while determining what analyses were of interest.

Skilled Reaching

During the “handling” phase (see Figure 3), a handful of small round sucrose pellets (45 mg; Bilaney Consultants, Frenchtown, NJ) were placed in each animal’s home cage to habituate them to the food. To perform training/testing, animals were placed in a transparent Plexiglas chamber (30 x 36 x 30 cm) with a rectangular opening
(1.5 x 3 cm) in one wall, with a Plexiglas shelf attached outside and underneath the opening. To encourage reaching, animals were food restricted to 90% of their normal body weight. Training was performed in 2 sessions per day (morning/afternoon), and consisted of first habituating the animal to the chamber and allowing them to attain pellets from the platform with their tongues (15 minutes per session). Once attention was paid to the pellets they were moved out of range of the tongues and the animals would begin to attempt reaching. At this point, the pellets were placed one after the other onto the shelf with forceps at the animals preferred reaching location. Animals reached through the opening to obtain pellets placed on the shelf until a total of twenty attempts or 5 minutes elapsed. All animals developed a preferred forelimb. Training was complete when the animal attained >15/20 successes three sessions in a row. All animals except one (who was subsequently dropped from the study, Figure 4) attained this baseline skill level within 4 weeks. The results of these last three training sessions were averaged to give a baseline value for each animal.

The number of successful reaches was defined as the number of pellets grasped with the appropriate limb and placed into the mouth on the first attempt. Attempts with the ipsilesional forelimb were not counted. Attempts with the contralesional forelimb that hit the wall of the chamber were counted as misses. Attempts when the animal reached out, missed the pellet, and then attained the pellet without retracting the forelimb into the chamber (“double-pumps”) were counted as one miss and one success. The reaching movement is very quick (<1 second), therefore assessing the double-pump live was difficult and may not have been completely consistent. Each animal was tested one day
after TBI and then multiple times (3-5) a week for 8 weeks (one week pre-treatment, two weeks during treatment, and five weeks post-treatment). Videos were taken at baseline, one week post-TBI (before osmotic pump implant), and at the end of the study. Videorecording was performed in a different room under brighter light than everyday testing and by a different investigator (although one they were familiar with). Nearly all animals performed worse during the taping sessions, indicating a strong environmental influence on reaching performance. The first cohort of 12 rats all recovered baseline skill level by four weeks post-TBI regardless of treatment group and were sacrificed at that point (see Figure 4). This was deemed to be due to lesions posterior to the forelimb area, although no careful analysis was performed to verify this.

**Procedure for Producing Traumatic Brain Injury**

Throughout the procedure anaesthesia was sustained with ~2.5% isoflurane, body temperature maintained at 37 degrees Celsius with a heating pad, and the rat head was held in place using ear bars. A 4 mm diameter circular craniotomy was performed over the region of the forelimb sensorimotor cortex contralateral to the preferred reaching forelimb and the skull piece removed. This corresponds to a position centered on 0.5 mm anterior and 4 mm lateral to bregma. The cortical impact was delivered by a controlled cortical impact injury device. The device consists of a small bore, double acting, pneumatic piston cylinder with a 40 mm stroke mounted on a stereotaxic micromanipulator. The pneumatic piston cylinder was angled 22º away from vertical to maximize the chance that the flat impactor tip (cylinder with 3 mm diameter) was perpendicular to the surface of the brain. The impactor tip was set to penetrate the
brain at 2.5 m/sec at a depth of 2.0 mm below the cortical surface (dorsal to subcortical structures) for 250 msec. Following the lesion, the skull fragment was replaced and scalp incision sutured closed.

**Drug Administration Via Osmotic pump**

Osmotic pumps were installed one week after TBI. Throughout the procedure, anesthesia was sustained with ~2.5% isoflurane, body temperature maintained at 37 degrees Celsius with a heating pad, and the rat head was held in place using ear bars. The anti-Nogo-A antibody 11c7 or control Ab (IgG1 anti-Wheat Auxin; both gifts from Novartis) was infused for 2 weeks into the contralesional lateral ventricle using an Alzet osmotic pump (model 2ML). The pumps have dimensions of 5.1 cm in length, 1.4 cm in diameter, and mass of 5.1 grams. This is small enough to be placed subcutaneously in the midscapular area. The device is regulated by a flow moderator with a pumping rate of 5.0 µl/hr (± 0.75 µl/hr). This corresponds to a dosing rate of 12 µg/hr (85 nmol/hr) for a total dose of ≈4 mg of antibody. The cannula (0.36 mm outer diameter) was placed so as to maximize the chance of piercing the contralesional lateral ventricle at coordinates: lateral 1.3 mm; caudal 0.8; and dorsoventral 3.8, relative to bregma. Due to excess solution remaining in the pumps during pilot testing (possibly due to debris in the lesioned hemisphere resulting in clogged cannulas), the contralesional hemisphere was cannulated. It was deemed unlikely that the damage caused would have significant direct impact on reaching recovery due to the caudal location. TBI-only animals did not receive a pump. No assessments of drug distribution or biological (e.g. serum, cerebrospinal fluid) concentrations were performed. Also see Figure 5.
**Figure 5. Properties of the anti-Nogo-A Antibody.** Schematics of human Nogo-A protein is shown. Green, purple and blue colors indicate N-terminal regions encoded by different exons. The C-terminal region is the one flanked by grey segments which is encoded by 6 different exons and highly conserved, yellow regions indicate transmembrane domains (TM). Black filled segments show regions reported as inhibitory to 3T3 cell spreading in cell culture studies. (Oertle et al., 2003a). The orientation of the N-terminal tail of Nogo-A in membranes is somewhat controversial and it appears to be able to take on multiple orientations (Oertle et al., 2003a). The model proposed for Nogo-A in oligodendrocyte plasma membranes is shown here. The N-terminal tail is not drawn to scale and is shown with arbitrary secondary structure as this is currently unknown. The Antibody binding site is as indicated, possibly sterically hindering contact of the Nogo-A protein with exploring axons (the triangle with curved arrow depict a neuron with extending axon). To account for degredation/adsorption the total amount of antibody is 1 mg in excess of that which will be administered according to the nominal pumping rate. Pharmacodynamic data from US patent 7,785,593 B2: “Antibody (11C7) Anti-Nogo-A and its Pharmaceutical Use”. Distribution data from Weinmann et al. (2006).
Golgi-Cox Stain

Animals were sacrificed approximately 10 weeks after TBI under overdose with intraperitoneal pentobarbital followed by five minutes in a chamber of 5% isoflurane. After cardiac perfusion with 0.9% heparinized saline, the brains were removed and placed whole in Golgi-Cox (Potassium Dichromate, Potassium Chromate, and Mercuric Chloride) solution for 14 days followed by at least 2 days in 30% sucrose.

An account of the staining process can be found in Gibb and Kolb (1998). Materials are shown in Figure 6 and Figure 7. The brains were blocked from as rostral as possible to caudal to the posterior extent of the lesion using a new razor blade each time. The two halves of the brain were then adhered to the vibratome tray with a small “dab” of Krazy Glue (~1 ml), and subsequently submerged in 6% sucrose, thus allowing them to be sectioned into 200 μm coronal sections using a vibratome and placed on slides.Slides had been dipped in 2% gelatin solution and left to dry overnight at 37 degrees Celsius. After a slide was populated, pressure was applied with moist bibulous paper and it was left to “settle” in a humid container (a toolbox lined with wet paper towels) for at least 2 days. Following this they were placed upright in trays and carefully submerged in the sequence of 300 ml trays of the solutions named in steps 3-15 above. To maintain the dehydration of the 100% ethanol it was stored with molecular sieve. Steps where the “# of Times Used” = 2 (Figure 6) indicates that the solution was reused for a second batch of slides before being replaced. Finally, the slides were coverslipped using permount as a mounting agent. Steps 3-16 were performed in a low humidity environment while avoiding any direct light. Slides were then stored in the dark face up for at least 2 weeks.
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Figure 6. Sacrifice and Golgi-Cox Processing Supplies.
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Figure 7. Sacrifice and Golgi-Cox Processing Supplies (continued). Details on materials used as described in the methods.
to allow the permount to set before analysis began. Exposure to light was kept limited during analysis. Once processed and coverslipped, these sections were assessed for lesion and neuronal plasticity of layers IV/V/VI pyramidal cells from the contralesional motor cortex as described below.

**Lesion Assessment**

Each slide was first scanned to a high resolution (1200 dpi) uncompressed “.tiff” image and imported to Adobe Photoshop. The lesion was assessed both as total lesion size and as a function of cortical location. The amount of lesion at each pixel measured (dimensions 500 microns medial-lateral and 200 microns rostral-caudal) was defined as the measured area of the contralateral intact hemisphere minus the area of the damaged hemisphere at that location. The first appearance of the genu of the corpus callosum was taken as 2.4 mm + Bregma. The measurement of the lesion size could have been affected by processing artifacts ([Figure 8](#)), or secondary injury processes resulting in, for example, atrophy of the damaged hemisphere or hypertrophy of the contralesional hemisphere. Post-sacrifice damage was distinguished from lesion via knowledge of the impacted hemisphere and inspecting the area surrounding the cavity for the presence of scar tissue. The location of the lesion along the ventral-dorsal axis was not assessed, rather the number of pixels found along this axis at each position rostral-caudal and medial-lateral was used as the area for each hemisphere at that location. See [Figure 8](#) for more details.

**Quantification of Dendritic Branching**

For inclusion, a neuron needed to have the attributes of a pyramidal neuron (approximately pyramidal soma shape, prominent apical dendrite extending towards
Figure 8. Method for Assessing Lesion Size and Location. (A) A custom semi-automated procedure was developed to assess each individual rat’s lesion. After brain sections were processed, each slide was scanned to a high resolution (1200 dpi) uncompressed “.tiff” image and imported to Adobe Photoshop. Each hemisphere was then cut from the slide image, rotated, and positioned onto a template file. It was common for sections to be damaged dorsally due to rongeur during harvesting and ventrally due to vibratome damage (distinguished from CCI lesion by the lack of scar tissue). Thus each section was manually painted over in an effort to “correct” for these processing artifacts. Once this was complete, an automated procedure assessed the number of pixels every 0.5 mm medial-lateral for each hemisphere. The number of pixels was scaled to an area measurement by a scanned ruler (providing a px/mm conversion factor). Local “lesion size” was then calculated by taking the difference between the intact and damaged hemispheres at each location. This allowed creation of lesion maps with resolution 0.2 mm Rostral-Caudal X 0.5 mm Medial-Lateral as shown. The Rostral-Caudal location of each section was calculated using the first appearance of the Genu of the Corpus Callosum as +2.4 Bregma. For the rat brain used as an example above (B) this corresponded to section #24, thus the section # 23 used as an example is located at 2.4 mm - (23-24)*0.2 mm = 2.6 mm + Bregma. The thick ovals show the planned location of the lesions as described in methods, the surrounding thinner-lined oval shows the craniotomy. The dashed line going from bottom-left to upper-right shows the expected width of the hemispheres according to Paxinos and Watson (1998).
the pia), be contralateral to a lesion, and located in layers IV/V/VI of the motor cortex according to Paxinos and Watson (see Figure 9). Six neurons from the contralesional motor cortex were traced for each animal.

Basilar dendrites were traced using 40x and 63x objectives (Leitz DMRB microscope with the aid of Neurolucida software (MBF Bioscience). Due to the use of a camera and monitor, the total magnification of the system was about 40x greater than indicated by the power of the objective. Basilar dendritic trees were analyzed for total dendritic length and total number of branches of each order. Branch order was determined by considering branches emanating directly from the cell body as first order; once bifurcation occurs two secondary dendrites are formed and so on.

Quantification of Soma Size Distribution

The soma size of additional neurons was assessed. This consisted of establishing an initial region of interest filling the space from +5 to -1 mm rostral-caudal, 1 to 4 mm medial-lateral, and 0.8 to 1.8 mm deep to the pial surface in the contralesional hemisphere and tracing all visible pyramidal neuron cell bodies found in each section analyzed. The procedure was a simple one that consisted of tracing the outside edge of each soma while keeping the edge in focus. Initially sections were chose once every 1 mm rostral-caudal per each animal but additional sections were added later in the region between +3 to +4 mm from bregma.

Materials

Anti-Nogo-A antibody
Figure 9. Region from which Pyramidal Neuron Were Selected. Cartoons of neuron cell bodies are shown as triangles with enclosed white circles (representing nuclei). The processes extending from these triangles indicate axonal tracts. Examples of cortico-cortical, cortico-rubral (red nucleus), and cortico-spinal tracts are shown. The location of the lesion is indicated by red color. Lower-Right Inset. Soma, Basilar Arbor, and oblique apical dendrites are indicated. Axons were not assessed. Branches are color coded by branch order. The apical shafts often extended outside of the section being analyzed and thus were of inconsistent length. For that reason apical dendrite data was deemed unreliable and dropped from analysis.
The anti-Nogo-A antibody used was 11c7, an IgG1–isotype monoclonal antibody developed to recognize an epitope of rat Nogo-A corresponding to amino acids 623-640 (see Figure 5). The antibody has been reported to bind with subnanomolar affinity to the Nogo-A specific inhibitory domain of Exon 3 (Figure 5). The control Ab was also an IgG1 monoclonal antibody directed towards Wheat Auxin. Both antibodies were gifts from Novartis.

Histological and Other Supplies

A complete list of histological supplies can be found in Figure 6 and Figure 7.

Data Presentation and Statistics

Assessment of Total Lesion Size

To assess total lesion size, first each set of brain sections was inspected to determine the first and last sections containing ablated tissue. This could be distinguished from post-sacrifice damage under a microscope by the presence of scar tissue. The total area of the damaged hemisphere between these two sections was then subtracted from the total area of the corresponding sections from the intact hemisphere. This value was then multiplied by the distance between sections (0.2 mm) resulting in a measure of volume.

Group Average Lesion Location and Comparison

The average lesion location for each group was determined as shown in Figure 10. In short, the average difference was calculated for each group at each pixel. This was then used to generate a map of the average lesion. To determine whether any regions were differentially damaged between groups, a t-test was performed at each
Figure 10. Calculation of Average Lesion Location. Individual lesions were assessed as described in Figure 8. To calculate average lesion location, the difference between intact and damaged hemispheres at each pixel, for each rat, was calculated by treatment group. The figure shows an example calculation for the pixel corresponding to +3.8 mm from bregma and 1.5 mm lateral to midline. The corresponding location is denoted by the black box in each lesion map. This was then smoothed to give Figure 23. The thick ovals show the planned location of the lesions as described in methods, the surrounding thinner-lined oval shows the craniotomy. The dashed line going from bottom-left to upper-right shows the expected width of the hemispheres according to Paxinos and Watson (1998).
Regions consisting of adjacent pixels with p-values $<0.05$ were deemed a region of interest.

Curve-Fitting to Reaching Recovery

The interpretation of the three main parameters of the logistic (sigmoidal) curve is shown in Figure 11. The Gibbs sampling Markov Chain Monte Carlo (MCMC) method was used to fit a three parameter sigmoidal curve to the timecourse of each animal’s recovery using a the JAGS model found in Appendix B and depicted in Figure 12. The model used uniform prior distributions for each parameter (i.e. plateau, midpoint, and rate), and a gamma (5, 5) distribution was used for the precision (inverse variance). Each animal was assessed independently, no group level priors were used.

Plateau Phase Analysis

The plateau phase was defined to extend from the first timepoint at which the three-parameter logistic curve was $\geq 95\%$ of the plateau score to the final timepoint. As shown in Figure 13, the number of observations ($N_{obs}$; number of sessions), plateau mean, and plateau variance were then calculated from these values. The corresponding binomial distribution is described by $n=20$ trials and probability of success ($p$) equal to the plateau mean divided by the $n=20$ trials. The expected distribution of sample variances was then determined by $10^6$ monte carlo simulations, each generating $N_{obs}$ values from the binomial distribution and calculating the variance of these values. The number of simulated variances less than the measured plateau variance was then divided by the number of simulations to give a lower-tailed $p$-value. See Figure 13 for a graphical description of this process.
The Logistic (Sigmoidal) Curve

Recovery after TBI on the Skilled Reaching Task follows a logistic (sigmoidal) curve:

\[ \text{Reaching Score}(t) = \frac{\text{Plateau}}{1 + e^{\frac{t - \text{Midpoint}}{\text{Rate}}}} \]

where, \( t \) = Sessions/Days Post-TBI

The shape of the curve is determined by three parameters. Plateau, Midpoint, and Rate. By testing different combinations of parameters a best-fit curve can be found:

The Logistic (Sigmoidal) Curve

The function used has three parameters (Plateau, Midpoint, and Rate), which determine the asymptote, time to half plateau, and time elapsed between ~34% and ~68% of the plateau level being reached, respectively. The method used was Gibb’s Sampling (JAGS 3.2 via Rjags). Actual data is shown as the points, while the black curve for each panel graphs the equation shown. The deviation of each fit from the actual data can be seen as the grey line. Deviation from fit was modelled as a normal distribution when fitting, thus the error term minimized was the root mean square error. Right panels each have the corresponding parameter set equal to the best fit divided by two to demonstrate the importance of each to the shape of the curve. See text for details.
Figure 12. JAGS Model. Estimates for each parameter (Plateau, Midpoint, and Rate) were sampled from uniform distributions. Precision (inverse variance) were sampled from a gamma (5,5) distribution. Code is available in Appendix B.
Figure 13. Determination of Plateau Variance. Rat 282 was determined to have spent Nobs=34 sessions in the plateau phase with mean=10.38 and variance=2.49. This corresponded to a lower-tailed p-value of p=0.006, indicating underdispersion relative to the expected binomial distribution. A. A three-parameter logistic curve was fit as described in Figure 11 and Figure 12. The plateau phase was considered initiated when the fit value was \( \geq 95\% \) of the estimated plateau. This timepoint is marked by a vertical dashed line while the included scores (Nobs=34) are depicted as red points. B. Histogram of plateau phase scores. The mean and variance of the scores were calculated from the red points in panel A. The corresponding binomial distribution is shown as the black curve using n=20 trials per session and probability of success p=mean/n=10.38/20=0.519. The theoretical mean and variance for this distribution are shown on the top-right. C. The expected distribution of sample variance was determined via \( 10^6 \) monte carlo simulations. It can be seen that the mean of these simulated variances (vertical black line) corresponds well with the theoretical expected variance shown in panel B. The vertical red line shows the measured plateau variance for this rat which was less than 99.4\% (p=0.006) of the simulated variances. D. The relationship between plateau mean and plateau variance is shown for Nobs=34, see Figure 14 for further description. The blue point corresponds to the current data.
Correlation Maps

Lesion versus skilled reaching correlation maps were generated by calculating Pearson’s R between the local lesion size at each pixel for each animal’s reaching parameter. Only pixels where data was available for >19 rats were used.

Attributions

This work was performed by Stephen G. Nawara, Cathy M. Papadopoulos, Shih-Yen Tsai, Gwendolyn L. Kartje, Vicky A. Husak, Lauren Goworowski, Westley A. Huisinga, Jenny Biesiadecki, and Catherine Taylor. Funding was provided by the Department of Veterans Affairs CDA-2 to C.M.P. (Project #B7495-W), NINDS 40960 as well as the Loyola University Chicago Neuroscience Institute and Department of Molecular Pharmacology and Therapeutics. Additional materials/equipment were made available by G.L.K. (microscope, lab/office space, consumables), while V.A.H. ensured the safety and cleanliness of the laboratory environment.

Surgical procedures (CCI, osmotic pump install/retrieval) were performed by S-Y.T. He was assisted in this for all four cohorts by C.M.P., and the latter three also by S.G.N. Animal sacrifice and brain retrieval was done by S.G.N. and C.M.P. Reaching data was collected by C.M.P. for all four cohorts and assisted by S.G.N. for the latter three. Golgi-Cox staining was done by S.G.N. Processing of slide images for lesion analysis was done by S.G.N., W.A.H., J.B., and C.T. Quantification of dendritic branching and soma size was done by S.G.N. and L.G. All data analysis procedures were devised and performed by S.G.N. This document and all figures were created by S.G.N. W.A.H assisted with artwork and formatting.
CHAPTER 5

THEORY

Skilled Reaching as a Measure of Capability

Performance on the skilled reaching task (see Methods) has been used as a measure of “motor function”, or “functional recovery” after various types of brain injury (e.g., Castro 1972; Papadopolous et al., 2002). However, “function” appears ill-defined. In some sense performance on any arbitrary task (or clinical score) can be defined as “function.” In a clinical setting, this may be sufficient because the task chosen can be the one of interest. In these cases the mechanism by which a treatment affects function may be informative, but is not crucial to the ultimate goal of improving performance on that task.

This is not helpful in pre-clinical trials such as those using the skilled reaching task since it is wished that the measure of performance generalize to other tasks/species (i.e. human), the ultimate goal is not to assess the success rate of different rats reaching for sugar pellets. For such generalizations to be credible the following must be true:

1) There is/are determinant(s) of performance common to the various tasks by different species.

2) The relative performance by different individuals is independent of task-specific factors such as the approach used and motivation to receive the reward.

A meaningful definition of “motor function” would then be the capability to
attain/maintain a given performance level. Likewise, “functional recovery” refers to the 
capability to perform at a certain level after a brain injury. Thus, if success rate on a task 
is to be a useful measure of “motor function,” it should measure capability.

An attempt to justify interpreting the skilled reaching task in this way is 
made below. A deduced prediction of the “capability interpretation” is followed by 
identification and discussion of the assumptions that seem to be necessary for it to be 
accurate.

Justification for the Capability Interpretation

For the capability interpretation to be strictly valid, the rat should strive to attain 
a pellet on as many trials as possible. If given the opportunity on n=20 trials per day, the 
goal of the rat is to attain 20 pellets every day. It follows that failure to achieve this goal 
must be due to some kind of physiological limitation.

In some extreme scenarios, sub-100% performance can be clearly attributed to 
physiological limitation. If the forelimb is paralyzed the rat will not be able to move it to 
the location of the pellet. However, in these cases 0% success would be expected rather 
than one intermediate between 0-100% percent.

That the rats learn the task shows that it is possible for them to remember and 
repeat approximately the same movement, while the complexity of the act seems to 
preclude anything more than extremely infrequent “random” successes. Once a rat 
“knows” how to successfully attain the pellet, it should be possible for it to repeat the act 
in future trials.
A distinction can be made between motor execution and motor planning (van Beers et al., 2013). An analogous situation is a basketball player shooting free throws. There is not necessarily a single motor plan, or “strategy”, by which a success can be accomplished. In the case of free throws, successful strategies can differ quite drastically: e.g. overhand vs. underhand. More subtle variations such as the position of the elbow underneath rather than to the side of the ball also occur. Such strategies could be distinguished both qualitatively (use of different gestures) and quantitatively (kinematic differences) (Foroud and Whishaw, 2006). Perhaps, due to physiological variability, the execution of a strategy will deviate from the “plan” from trial-to-trial.

It is plausible that some strategies are never, or only very rarely, successful. Further, other strategies may be more or less adaptable to varying external conditions and execution errors, which can be combined under the umbrella term “noise”. Each strategy may be then associated with a robustness to the noise experienced. If this reasoning is correct, failure to attain 100% success would indicate some or all of the following:

1) Environmental variability (e.g., the exact position of the pellet) causes the optimal movement to differ from trial-to-trial. Bright light, talking, etc., may also distract or disorient the rat on some trials.

2) The rat does not “remember” the movement that leads to success. This may occur transiently, perhaps due to some distraction in the environment.

3) Physiological variability. Even if the rat selects/chooses the optimal movement, it cannot always execute the movement to within the tolerance limit required for success. For example, in healthy animals this can be due to normal levels of muscle tremor. Rats that have received brain injuries may additionally receive incorrect or insufficient proprioceptive/sensory information, or fail to execute the appropriate movements in the appropriate sequence (Foroud and Whishaw, 2006).

The above points indicate that a primary determinant of performance on the task is the magnitude of internal/external variability from trial to trial. Therefore, the
limitations on performance appear to be:

1) The degree of variability (“noise”) experienced by the rat from trial to trial.
2) The capability to perform reaching movements robust to this variability.

Prediction of the Capability Interpretation

In that case, the probability of success on any given trial may be described by:

\[ p = \max(0, 1 - \frac{n_o}{R_s}) \]

where,

- \( p \) = probability of success on a given trial
- \( n_o \) = Quantity of noise
- \( R_s \) = Robustness of the strategy (motor plan) used to this noise. If there are \( S \) possible strategies, then \( R_s \) is an element of the set: \( S = \{R_1, R_2, ..., R_S\} \)

If the “noise level” exceeds the robustness of the strategy \( (n_o > R_s) \), then \( p = 0 \). Otherwise, the probability of success is determined by the ratio of the noise level to robustness. If the strategy is very robust to the noise \( (R_s >> n_o) \) there is 100% probability of success. Note that robustness is a property of the strategy used, not a property of the rat. In general, there may be more than one strategy used by different rats (Gholamrezaei and Whishaw, 2009), or the same rat on different trials.

This equation does not attempt to capture the precise relationship between robustness of various strategies and different sources of noise, which may be rather complex. Noise may differ qualitatively. In that case different strategies may be optimal in the face of different “types” of noise. After injury, rats may not have access to the same strategies (i.e. some members of the set \( S \) are now unavailable), and thus must use a compensatory strategy (Whishaw, 2000) which may have less robustness to the noise. In
addition, the level/type of noise may be altered by a brain injury.

However, if a reaching score is to be interpreted as a measure of the rat’s capability, each rat must have eventually adopted a strategy that maximizes $p$. If the rat is not performing at a maximal level it is difficult to understand how the reaching task could be said to measure “motor function,” rather than something else such as motivation or rate of learning. Thus, the ratio of $n_o/R_s$ should be constant: there is a constant probability of success on any given trial.

Previous publications using the skilled reaching task seem to acknowledge this, since rats are not simply tested on a single day (e.g., Papadopolous et al., 2002). Rather, a period of learning/recovery is allowed to elapse, followed by a phase of relatively consistent success rate (i.e. a “plateau” or “asymptote”), and only then are the success rates of different rats compared to assess their relative “motor function” (Fenrich et al., 2015). As mentioned above, if there is no apparent plateau in success rate, it seems inappropriate to use that data as a measure of “motor function”.

Tasks with binary (success/failure) outcomes and constant probability of success are Bernoulli trials (e.g., there is 50% chance of tails on any given flip of a fair coin). The number of successes observed over a series of independent and identically distributed (iid) Bernoulli trials follows a binomial distribution. A binomial distribution is described by the probability of success $p$, number of trials per session $n$, and number of sessions observed $N_{obs}$. For a given $p$ and $n$, on average it is expected that:

$$\text{mean} = np$$

$$\text{variance} = np(1-p)$$
Here, “expected” refers to the mean value of a large number of repetitions, these values are independent of \( N_{obs} \). E.g., if 100,000 people flip fair \((p=0.5)\) coins \( n=20 \) times each day for \( N_{obs}=5 \) days and record the number of tails each day, the mean and variance for each person could be calculated. Although the values would differ from person to person, the mean of the observed variances will be near \( 20 \cdot 0.5(1-0.5)=5 \). This would not change if the experiment was extended to \( N_{obs}=20 \) days, etc.

However, the distribution of samples consisting of \( N_{obs} \) sessions will be spread around these expected values in a characteristic way. If there is a plateau consisting of \( N_{obs} \) sessions, we predict a specific relationship between the mean success rate and the session-to-session variance during this plateau. An analytical description of the relationship between sample mean and sample variance could not be found, but this can be estimated numerically for any combination of \( N_{obs} \), \( n \), and \( p \). Some examples are shown in Figure 14. Sample variance as a function of sample mean is symmetrical around a maximum when \( p=0.5 \) (10/20 successes). Also, smaller \( N_{obs} \) is associated with a larger spread and greater degree of positive skewness.

Deviations from this predicted relationship would indicate that there is something wrong with the above justification for using reaching score as a measure of capability. Specifically, the probability of success on different trials are not independant and identically distributed. The immediately observable consequence would be that session-to-session performance is more or less consistent than expected (termed “underdispersion” and “overdispersion” respectively).
Figure 14. Predicted Relationship Between Mean and Variance During Plateau Phase.
Monte Carlo simulations (1 million each) were performed using binomial distributions with the indicated number of observations (N\text{obs}), \(n=20\) trials, and \(p=\{0, 0.05, 0.1, 0.15, \ldots 1.0\}\). Quantiles of the sample variances were calculated and plotted as polygons filled between q and 1-q (e.g. 5% and 95%). Dotted curves indicate median values. A. The spread between 5% and 95% quantiles decreases as N\text{obs} increases. B-D. Additional quantiles are shown to provide a clearer sense of the distribution shape for the indicated number of observations. Mean-variance pairs far from the expected value are unlikely if the binomial model is accurate, thus would provide evidence against it.
Assumptions of the Capability Interpretation

The above narrative depends on the assumption that the plateau reaching score is solely a measure of capability, i.e., it is the maximal score attainable. This, in turn, depends on a number of other assumptions:

1) The goal is to maximize the probability of a successful reach rather than some other metric such as reward/time or reward/effort. Such goals may correspond in some cases but not others.

2) A rat's probability of success (i.e. \( n_0 / R_s \)) is equal on every trial once plateaued. Violations of assumptions three and four below may, in turn, cause \( n_0 \) to vary from session-to-session. The rat may require a “warm-up” series of trials or become fatigued over the course of the session. The rat may also use different strategies on different trials.

3) Motivation (e.g., due to hunger) plays no role in performance. This would seem to be contradicted by the food deprivation aspect of the skilled reaching protocol (Smith and Metz, 2005).

4) External noise is experienced by all rats equally. Even if true, the effects of these influences may still differ due to anxiety, sensory deficits, etc.

5) Each rat explores the entire set of reaching strategies and after this period of exploration/learning settles upon the optimal strategy available. Instead, the rat may simply try different approaches until one is successful a few times then form a habit (Alaverdashvili et al., 2008).

In practice, at least the last four of these assumptions are only expected to be approximately true. As noted by Whishaw et al. (2008):

“The analysis of “cognitive” factors related to skilled reaching show that many aspects of the reaching act are mediated by higher-level control independent of the actual motoric act... measures of motor performance do not provide an absolute index of motor ability...”

However, as long as capability is the primary determinant of plateau success rate, the capability interpretation would be reasonable. Violations that may cause us to question this can occur at three levels: inter-individual, inter-session, and intra-session. At the inter-individual level such violations are most obviously problematic. They can lead
to egregiously incorrect inferences, especially if due to a treatment effect:

1) A rat attempting to maximize reward/time may prefer to attain e.g. 10 pellets in 40 seconds rather than take its time to get 18 pellets in 80 seconds. If this is the case, comparisons of that score to one of a substantially faster/slower rat or a rat attempting to maximize pellets/session would be uninformative.

2-4) A treatment affecting factors such as motivation, anxiety, or sensitivity to distractions may cause increased or decreased scores in one group of rats, which may then be misunderstood as an effect on “motor function”.

5) A treatment that decreases the rate of habit formation (i.e. learning) may allow the rat to explore a greater number of strategies before settling on the most optimal. This could be interpreted as a deleterious effect on memory rather than a positive effect on motor function.

Violations at the inter-session level cause the probability of success to vary from session-to-session, but they do not pose much risk of being misleading evidence if the magnitude is minor and similar across individuals. However, the resulting presence of overdispersion would provide evidence we are measuring something other than, or in addition to, “motor function.” For example, if a rat is more or less motivated one day to the next a corresponding increase in success rate variability would be expected.

Intra-session violations cause the probability of success to vary from trial-to-trial. In these cases, the expected number of successes instead follows the Poisson-binomial distribution. For \( n=20 \) trials, there can be up to \( n=20 \) different probabilities of success. The consequences of this depend on the exact set of probabilities and whether this is consistent from session-to-session.

If the same pattern of trial-to-trial variability occurs each session, this will result in underdispersion relative to that expected from the binomial model. This can occur if the rat always requires the same warm-up period or tires after the same number of trials. Obviously the success rate now depends upon the number of trials, so it is an unreliable
measure of motor function. Note that all other assumption violations would tend to mask this phenomenon.

In contrast, trial-to-trial violations that are inconsistent from session-to-session will tend to result in overdispersion (counteracting the underdispersion). Due to, e.g., varying degrees of anxiety, a rat may require a different number of practice attempts each session to calibrate itself to the task. The probability of success would then increase at different rates each session. This would be more insidious than the consistent case, since the underdispersion evidence is masked, yet the success rate would still depend on the experimental design (trials per session).

**An Urn Model of Learning the Skilled Reaching Task**

The capability interpretation only attempts to describe the plateau phase of skilled reaching, but it may be extended by modifying the learning function proposed by Thurstone (1930). Above, it was assumed there is some set of strategies available to each rat from which it eventually chooses the one yielding the maximum success rate. Out of this set, some number of the strategies will lead to $p=0$, and others are associated with $0<p\leq1$. The former can be grouped as erroneous strategies ($e$), and the latter labeled as successful strategies ($s_1, s_2, ...$). The simplest non-trivial scenario would be two successful strategies ($s_1, s_2$) each associated with different but constant probability of success ($p_1, p_2$), if selected. These “two” strategies may also be interpreted as subsets of the possible strategies $S$, perhaps corresponding to “goal strategy” and “habit strategy” (Gholamrezaei and Whishaw, 2009). There must also be some probability of selecting each strategy from those available. This can be accomplished by assigning quantities to
s1, s2, and e such that:

\[ p(s1) = \frac{s1}{s1+s2+e} \]

\[ p(s2) = \frac{s2}{s1+s2+e} \]

\[ p(e) = \frac{e}{s1+s2+e} \]

where,

\[ p(s1) = \text{probability of selecting successful strategy } s1 \]

\[ p(s2) = \text{probability of selecting successful strategy } s2 \]

\[ p(e) = \text{probability of selecting an erroneous strategy } e \]

If learning is to occur, the probability of selecting a strategy should increase by some amount \( k \) after a successful execution. It may also be easier to learn one strategy than another, therefore learning rate \( k1 \) is assigned to strategy \( s1 \) and \( k2 \) associated with \( s2 \). As noted by Thurstone (1930), this is like an urn problem. Each type of strategy can be represented by a ball of different color, e.g. errors \( e \) are represented by red balls, strategy \( s1 \) by blue, and strategy \( s2 \) by green (Figure 15).

The probability of drawing a ball (selecting to use a strategy) is determined by the proportion of balls of each color. The probability of success given that a strategy is selected would be analogous to the balls of different color being more or less “slippery”. If we draw a blue ball it would slip out of our grasp with probability \( 1-p1 \), and an error would be recorded. If it does not slip, then a success is recorded and the blue ball is placed back in the urn along with \( k1 \) additional blue balls. Perhaps the arcade “claw game” would make for an even better analogy.

Learning from failure may also occur, in which case an unsuccessful attempt
Figure 15. **Urn Model of Learning the Skilled Reaching Task.** As described in the text, different strategies are analogous to different colored balls. The quantity of balls of each color represents both the number of strategies leading to the same probability of success and the “strength” of the habit to choose that strategy. The ball is replaced after each draw, successful draws from the urn also lead to addition of more balls of the same color on the next draw. “Successful” strategies need not be 100% successful. This can be envisioned as the ball being selected, but having some probability of slipping back into the urn.
would decrease the probability of selecting that strategy by some amount. For simplicity, this model will be limited to only success-based learning. It is not implausible for tasks such as skilled reaching (Leibowitz et al., 2010). The animal may receive little useful feedback when it fails; it does not know which aspect or combinations of aspects of the reaching movement is responsible for the failure. In contrast, a success can provide a great deal of information regarding the sequence and coordination of body orientation, limb motion, and timing of the grasp.

Thus, if strategy \( s1 \) is selected and results in a success on trial \( t \), then \( s1 = s1+k1 \) for trial \( t+1 \) and \( p(s1) \) increases at the expense of \( p(s2) \) and \( p(e) \). Alternatively, if strategy \( s1 \) is selected but does not result in a success on trial \( t \), the situation remains unchanged on trial \( t+1 \). The same goes for strategy \( s2 \). Selection of erroneous strategies \( e \) will have no effect other than to record an error for that trial. The course of learning will then be influenced by seven parameters: the learning rates \( (k1, k2) \) the initial values for \( s1, s2, \) and \( e \) \( (s01, s02, \) and \( e0, \) respectively), and the probabilities of successful execution \( (p1, p2) \). However, the first five parameters are not uniquely identified, only the relative values affect the outcome. So \( e0 \) can be set to a value such as \( e0=100; \) if desired, the urn analogy can be maintained (i.e., avoid fractional \( s1, s2, e \)) by setting this arbitrarily high.

The model can be further simplified with the assumption that both the initial probability of selecting a successful strategy and the ease with which it is learned are inversely proportional to its relative probability of success. The motivation for this is two-fold. First, it should require less effort to learn a “quick-and-dirty” strategy. Second, a greater number of possible strategies should be capable of producing a lower success
rate. Therefore:

\[ s_{02} = s_{01} \frac{p1}{p2} \]
\[ k2 = k1 \frac{p1}{p2} \]

The number of free parameters has now been reduced to four \((s_{01}, k1, p1, p2)\). R code to run this algorithm can be found in Appendix III. To accomplish this a number of assumptions have been made:

1) Constant probability of success for a given strategy.

2) Constant learning rate for a given strategy.

3) There are only two possible successful strategies (or groups of strategies with equivalent effectiveness).

4) No learning from errors.

5) No “forgetting” from trial-to-trial or session-to-session

6) No “consolidation” from session-to-session, e.g. due to sleep.

7) The initial probability of selecting a successful strategy, as well as its learning rate, are inversely proportional to relative effectiveness.

An interesting aspect of this model is that the outcome is historically contingent. The same initial parameters can result in a diversity of learning curves, e.g. depending on how many trials take place before an initial success occurs. Also, in some instances the more effective strategy is not adopted. If the less effective strategy, e.g., \(s2\), happens to be selected and successful on enough trials before the more effective \(s1\) is attempted, it becomes unlikely that \(s1\) will ever become the dominant strategy. It is also possible that a mixture of the two strategies occurs, resulting in an overall probability of success intermediate between \(p1\) and \(p2\). In other cases, the more effective strategy \(s1\) is initially dominant, but a series of successes using \(s2\) can result in that one becoming dominant and thus a decreasing probability of success. Therefore, when fitting this model it is possible
to make one final assumption:

8) All rats are exactly the same (share $s01, k1, p1, p2$).

Insofar as this model will describe reality, any apparent differences in plateau “motor function”, rate of learning, and initial skill are illusory: they are not reflected by any underlying neurological differences. Instead, the different outcomes result from historical accident, i.e., which strategies happened to be attempted in what sequence.
CHAPTER 6
RESULTS

Skilled Reaching

Discussion of Rats Requiring Modified Analysis

The three-parameter (plateau, midpoint, rate) logistic curves were fit to each individual (n=46) reaching timecourse as shown in Figure 16 and Figure 18. These curves appear to provide an informative summary of the data in the majority of cases, but there were some exceptions. These rats were not dropped from the study because the information provided helps in interpreting the results, however it was also not appropriate to analyze their reaching data in the same way as the others.

Cohort 1 rats in all three treatment groups displayed baseline performance levels by ~20 sessions post-TBI. For this reason those experiments were terminated early. Although no formal lesion analysis was performed, visual inspection of the lesions in the removed brains lead to the belief that the TBIs these Cohort 1 animals received were posterior to the targeted region. Two Control Ab treated rats (Cohort 2 #2954 and Cohort 4 #9035) and one TBI-only rat (Cohort 2 #2279) did not demonstrate any appreciable increase in success rate by the end of the study. It is uncertain whether these rats would have recovered if given additional time. The individual timecourses of the “no-recovery” (n=3) and Cohort 1 (n=11) rats are shown in Figure 16 but thier reaching data will be not
Figure 16. Timecourses of Cohort 1 and “No-Recovery” Rats. Each panel shows the result for a different rat. The number of successes (attained pellets) is plotted against the session post-TBI as yellow points connected by a line. The smooth black curves show the best fits of the three-parameter logistic curve equation. The values of these parameters are shown above each plot. A fourth parameter in the model was the variance, which describes the spread of the data around the curve fit assuming a normal distribution. A colored polygon around the estimate is shown at +/- one variance. Color of this polygon indicates treatment group (Red: anti-Nogo-A; Green: Control Ab; Blue: TBI-only). Grey rectangles indicate the sessions during which the anti-Nogo-A and Control Ab rats had pumps installed (corresponding to days 8-22 post-TBI). The thick vertical dotted line indicates the estimated beginning of the plateau phase. This was taken to be the session at which the curve fit was ≥95% of the plateau score, if this did not occur the line is shown at the right of the plot.
be included in the comparison of treatment groups or fit of the learning model.

Group and Cohort Comparison

From Figure 17A we can see that, on average, anti-Nogo-A > Control Ab > TBI-only in terms of plateau, midpoint, and rate on the skilled reaching task. Most notably, the anti-Nogo-A treated animals plateaued at ~90% of baseline performance compared to only ~60% in the case of both control groups. If the “no-recovery” rats from the two control groups had eventually begun improving, the lower-bounds on the midpoints would be ~40 sessions. If this occurred, at least that apparent difference would be diminished. On the other hand, the Cohort 1 anti-Nogo-A rats had later midpoints as well. A tendency towards higher plateau scores is clearly present in the anti-Nogo-A animals, but one TBI-only animal also had a plateau score of ~16 and three of the anti-Nogo-A rats plateaued at 11-13 pellets. The treatment is neither necessary nor sufficient to elicit the high plateau.

Plateau and midpoint for all three groups may be distributed multimodally. There appears to be plateau modes at 9.5, 12.5, and 15.5 successes/session. The putative middle mode is near the mean of the other two, possibly suggesting two different reaching strategies associated with ~50% and ~80% success rates that were used on different trials by the rats with plateaus ~65% success rate. Midpoint modes appear at ~4, 8, 15, 24 sessions post-TBI, each mode is ~3 sessions more distant than the last.

Besides the full recovery of the cohort 1 rats, there is additional evidence for cohort effects. The two anti-Nogo-A cohort 3 rats performed worse on average than the other two cohorts. The TBI-only rats from the same cohort also differed from the others
Skilled Reaching Timecourse

Baseline

# of Pellets Attained

Plateau

Antinogo (n=11)
ControlAb (n=8)
TBI Only (n=13)

Midpoint (Sessions Post−TBI)

Cohort #

Rate Constant

Range of 5−95% Quantiles for 5/20/35 Observed Sessions

Post−TBI Plateau Variance
Assuming Binomial Distribution

Frequency

Lower−Tailed P−value
Figure 17. Cohort 2-4 Results. A. The individual curve fits from Figure 18 are plotted together in the top-left panel, the other three panels show the distributions of the plateau, midpoint, and rate by cohort and treatment group. The mean and 95% HDI of each parameter estimate are shown as a yellow point flanked by a line. Thick black bars indicate the mean of these means for each treatment group. The beanplot helps show the distribution of these means. Boxplots are also shown for each cohort of each group. B. The mean and variance of each rat’s plateau phase reaching scores were calculated and plotted against one another. The shaded background shows the area between 5% and 95% quantiles expected if the indicated number of observations (5, 20, or 35) were sampled from a binomial distribution, dashed lines indicate the medians (the same plot is shown in Figure 14A). The number of plateau phase observations differed amongst rats so the quantiles are only depicted to give a sense of the expected range. Mean-variance pairs that fell below the 5% quantile for that rat’s number of plateau phase observations are highlighted with a yellow border. C. For each rat, a probability of success \( p \) was calculated from the plateau mean divided \( n=20 \) trials. One million replications were then generated from a binomial distribution having \( n=20 \) trials and the same \( N_{obs} \) and \( p \) as each rat. The variance of each of these simulations was calculated and compared to that observed for the rat. The p-value (different from the probability of success mentioned above) corresponds to the percent of simulation results less than the observed value. Low p-values indicate underdispersion, high p-values indicate overdispersion. The histogram has bin size of 0.01, thus 9 rats had p-values less than 0.01.
by having low rate constants. It is also interesting that all three rats with midpoints >20 were from cohort 2. Two of the “no-recovery” rats were also from this cohort.

Plateau Phase Analysis

The plateau phase was defined to consist of the session at which the curve-fit was ≥95% of the plateau score until the end of the study. These timepoints are indicated by the vertical dashed lines in Figure 18. The mean and variance of these data were calculated for each animal and plotted in Figure 17B. When the variances were compared to those expected from the corresponding binomial distributions, underdispersion was very common. Out of n=32 rats, 18 had variances <95% of simulation results, 9 of these were <99% and 3 of those <99.9%. A histogram of the corresponding p-values is shown in Figure 17C.

Results of the Learning Model

It was found that reasonable output was generated using: $k1=7.51$, $s01=0.517$, $p1=0.815$, $p2=0.532$. The two derived parameters were then: $k2=11.078$ and $s02=0.792$. As mentioned earlier, $e0=100$. The code used can be found in Appendix C. The model is stochastic, it does not return the same output each time even when the same parameters are used. It therefore proved difficult to choose the parameters algorithmically. The values above were arrived at largely by manual tweaking so they may not be optimal. The best way to “fit” this model to capture all relevant aspects of the data is left to future work.
Antinogo Cohort 2: 2277
Plateau= 15.34 Midpoint= 25.01
Rate= 4.12 Variance= 2.77

Antinogo Cohort 2: 2280
Plateau= 11.79 Midpoint= 15.7
Rate= 3.32 Variance= 1.55

Antinogo Cohort 2: 2953
Plateau= 12.93 Midpoint= 17.8
Rate= 1.38 Variance= 4.47

Antinogo Cohort 3: 284
Plateau= 12.42 Midpoint= 8.99
Rate= 6.62 Variance= 1.33

Antinogo Cohort 4: 9025
Plateau= 16.31 Midpoint= 8.89
Rate= 1.38 Variance= 3.97

Antinogo Cohort 4: 9028
Plateau= 14.24 Midpoint= 8.14
Rate= 2.05 Variance= 1.87

Antinogo Cohort 2: 2278
Plateau= 9.66 Midpoint= 10.04
Rate= 0.78 Variance= 4.47

ControlAb Cohort 4: 9034
Plateau= 14.41 Midpoint= 6.34
Rate= 1.38 Variance= 3.97

ControlAb Cohort 3: 2777
Plateau= 13.73 Midpoint= 14.5
Rate= 2.3 Variance= 4.7
Figure 18. Individual Timecourses. See the caption of Figure 16 for explanation.
For this analysis, sets of \( n=32 \) simulated “individuals” were generated using the parameter values mentioned above. Each simulated individual was then assigned to a real rat. At each timepoint, the curve-fits shown in Figure 18 are described by a normal distribution with mean equal to the estimate (black line) and standard deviation as indicated by the colored polygons. Each real-simulated pair is then associated with a vector of log-likelihoods (one for each timepoint), the sum of which describes the similarity of the pair. The assignments were made using the hungarian algorithm so that the total log-likelihood was maximized.

The model was assessed in two ways. First, in Figure 19 we check to see how close the model can come to generating results like the real data. To do this the best result of 10,000 simulations was used, so it is not necessarily representative of the output. However, it does show that the model can generate results very similar to that observed in the data. To emphasize, only four parameters are neccesary to allow generation of this diverse set of outcomes. When comparing the results of the 32 different rats, we tend to assume that major differences reflect underlying differences of some neurological characteristic. Here we see that this is not necessarily true.

The main deficiency, at least for this set of parameters, appears to be too little diversity in the rate constants. The patterns of plateau scores and midpoints, however, were reproduced very well.

The individual plots upon which Figure 19 is based are shown in Figure 20. These are in the order corresponding to the best match with the real data from Cohorts 2-4 shown in Figure 18. One visible difference in these plots from the real data is that
Figure 19. Best Simulation Results. See the caption of Figure 17 for explanation of the plots. The model is able to generate results very similar to those observed in the real data, including the presence of underdispersion.
simAntinogo Cohort 2: 1
Plateau= 15.34 Midpoint= 7.56
Rate= 2.6 Variance= 2.3

simAntinogo Cohort 2: 2
Plateau= 15.07 Midpoint= 2.14
Rate= 1.87 Variance= 1.92

simAntinogo Cohort 2: 3
Plateau= 19.7 Midpoint= 13.68
Rate= 2.48 Variance= 1.3

simAntinogo Cohort 2: 4
Plateau= 14.85 Midpoint= 24.96
Rate= 1.21 Variance= 6.71

simAntinogo Cohort 2: 5
Plateau= 15.74 Midpoint= 1.77
Rate= 2.78 Variance= 1.66

simAntinogo Cohort 2: 6
Plateau= 12.44 Midpoint= 17.57
Rate= 1.68 Variance= 1.4

simAntinogo Cohort 3: 7
Plateau= 11.28 Midpoint= 14.94
Rate= 2.13 Variance= 6.54

simAntinogo Cohort 3: 8
Plateau= 10.65 Midpoint= 8.65
Rate= 1.63 Variance= 1.26

simAntinogo Cohort 3: 9
Plateau= 14.75 Midpoint= 15.51
Rate= 1.65 Variance= 1.23

simAntinogo Cohort 4: 10
Plateau= 15.63 Midpoint= 13.79
Rate= 3.27 Variance= 1.94

simAntinogo Cohort 4: 11
Plateau= 15.69 Midpoint= 5.23
Rate= 2.1 Variance= 4.19

simControlAb Cohort 2: 12
Plateau= 13.9 Midpoint= 4.63
Rate= 1.27 Variance= 1.7

simControlAb Cohort 3: 13
Plateau= 11.94 Midpoint= 8.23
Rate= 2.7 Variance= 1.53

simControlAb Cohort 3: 14
Plateau= 9.52 Midpoint= 21.76
Rate= 1.65 Variance= 0.32

simControlAb Cohort 3: 15
Plateau= 9.88 Midpoint= 17.64
Rate= 2.39 Variance= 3.95

simControlAb Cohort 4: 16
Plateau= 13.69 Midpoint= 14.94
Rate= 1.16 Variance= 1.45

simControlAb Cohort 4: 17
Plateau= 12.67 Midpoint= 9.14
Rate= 2.59 Variance= 1.35

simControlAb Cohort 4: 18
Plateau= 15.09 Midpoint= 2.81
Rate= 3.16 Variance= 1.48
Figure 20. Individual Simulation Results. See the caption of Figure 16 for explanation. Each panel of a simulated outcome corresponds to a panel of real data in Figure 18.
Figure 21. Additional Simulation Results. Nine sets of 32 simulated individuals using the same parameters mentioned in the text and used for Figure 19 and Figure 20. The three-parameter curves were fit to each set of results. The colors indicate the group of the assigned rat.
the simulations that end up with late midpoints do not have any previous successes. This differs from e.g., Control Ab #2957, and anti-Nogo-A #2956.

Nine additional simulations are presented in Figure 21 to provide a sense of the types of results that occur. The entire range of results observed in the real data may be observed here, although perhaps not in the same proportions. Intermediate plateaus between values corresponding to \( p_1 \) and \( p_2 \) are present, but most often the simulated results clusters around the scores corresponding to those two probabilities. The bottom-right panel even shows a “no-recovery”, suggesting that those rats may have begun learning at a later timepoint if the study were extended.

Lesion Analysis

The maps of each animal’s lesion are shown in Figure 22. It can be seen that the location of the lesion and its extent varied quite a bit. For example compare the lesions of the two TBI-only animals with ID 2279 and 287 (Figure 22). The lesion for the former covered the rostral forelimb area while the latter was solely of the caudal forelimb area with little to no overlap.

Only partial lesion data was available for some animals due to damage to the tissue during processing, so not all regions are available for comparison for each animal. This was somewhat more common for the anti-Nogo-A and control Ab groups, possibly because the tissue was more fragile due to the additional damage from the cannula that distributed the antibody solution. Damage to the intact hemisphere during processing can also lead to “negative” lesion size in some areas, for example see the lateral edge for anti-Nogo treated animal number 2280 in Figure 22. It was common for there to be a “lesion”
Figure 22. Individual Lesion Maps. The lesion of each rat was analyzed as described in methods (Figure 8). For easy comparison, each panel depicts a hemisphere of the rat corresponding to the same position in the figure showing the individual reaching curves (Figure 18). The front of the brain is shown to the left and lateral is towards the top. The treatment group, cohort, ID, damaged hemisphere (R: Right, L: Left), and total measured lesion volume are shown above each map. Where lesion data was missing an empty panel is shown. Additionally the maps for the three “no-recovery” rats are provided at the end. The final panel shows the color scale used. The thick ovals show the planned location of the lesions as described in methods, the surrounding thinner-lined oval shows the craniotomy. The dashed line going from bottom-left to upper-right shows the expected width of the hemispheres according to Paxinos and Watson (1998).
measured at the lateral edge of the hemisphere, possibly corresponding to atrophy of the damaged hemisphere, although this could be some artifact of the lesion analysis process. However, the widths corresponded well with those shown in the atlas (Paxinos and Watson, 1998). The expected location and size of the lesions is also shown, it is clear that this does not correspond to the data. The lesions appear more medial, anterior, and narrower than expected. Perhaps the narrowness is due to deformation of the remaining tissue into the cavity, this could also explain the apparent lateral “lesion”. The impactor was meant to be perpendicular to the cortical surface, in which case it should have pushed towards the midline. However, if the tissue was pushed down-and-out, the upper part could sink into the cavity as the lower bulges outward. In this fashion the lesion would appear narrower in these 2-D maps, the depth at the lateral edge would decrease, but the width of the hemisphere could be maintained.

The average lesion at each pixel was calculated and smoothed to give Figure 23. Student’s t-tests were performed at each pixel and a cluster of pixels with relatively low p-values were found when comparing the TBI-only and Control Ab group in the rostral forelimb area, indicating that the control Ab groups had smaller lesions on average to this area.

Correlation Between Lesion and Reaching Outcome

Total lesion volume and correlation between the lesion and reaching outcome was investigated as shown in Figure 24. From Figure 24A it appears first that the lesions tended to be larger in the TBI-only group, and second that many of the smaller lesions were measured for rats that received damage to the right hemisphere (yellow circles).
Group Average Lesion Location

Intact - Damaged Hemisphere Area (mm^2/pixel)

-1 -0.5 0 0.5 1
Increasing Lesion Size

A  TBI only (n=10)
Distance (mm) +/- Bregma
+6 +4 +2 0 -2 -4 -6 -8

B  Anti-Nogo-A (n=10)
Distance (mm) +/- Bregma
+6 +4 +2 0 -2 -4 -6 -8

C  Control Ab (n=9)
Distance (mm) +/- Bregma
+6 +4 +2 0 -2 -4 -6 -8

Forelimb Area (Neafsey 1986)

Region of interest

Difference between TBI-only and Control Ab lesions p=0.03
Figure 23. **Average Lesion Size and Location.** Average lesion is indicated by colors on the right side of the color key at the top. Narrowing of damaged hemispheres was observed for all groups. A-C. Lesion Size was calculated as the difference between the area of all analyzed lesions from each treatment group. Grayscale lines trace out contiguous areas with >0.5mm^2/pixel differences for each animal in that group. Note the prominent difference in area at the lateral edge. This may represent hypertrophy of the unlesioned hemisphere, atrophy of the lesioned hemisphere or deformation due to the cavity. The TBI-only group may have had greater lesion sizes in the region of the rostral forelimb area than the Control Ab group. The region is outlined in panels A and C, p=0.03.
Figure 24. Correlation Between Lesion and Reaching Outcomes. A. The measured total lesion volume is shown for each rat. Rats that received lesions to the left hemisphere (L) are shown by a yellow circle, the right hemisphere (R) is indicated by a yellow square. Boxplots are shown for each cohort of each treatment group. The beanplots show the distribution for all animals in each treatment group. Thick horizontal bars shown the group means. No lesion analysis was performed for TBI-only animals from the fourth cohort. TBI-only rats
tended to have larger lesions, as did rats that received damage to the right hemisphere relative to the left. **B-D.** Total lesion volume is plotted against the three reaching parameters. The vertical lines associated with each point indicate the 95% for the estimate of that parameter. There is no clear relationship between total lesion volume and any of the reaching parameters. Colors indicate treatment group (Red: anti-Nogo-A, Green: Control Ab, Blue: TBI-only. Shape indicates the damaged hemisphere as in **panel A. ****E-F.** Maps of Pearson’s R calculated at each pixel to determine correlation between lesion location and reaching outcome. Only pixels where data was available for >19 rats are included. The dotted lines are as described in **Figure 22.** A negative correlation between plateau and more anterior damage is suggested. This would correspond to the rostral forelimb area (**Figure 23**). However, this is primarily due to the large lesion in the area of TBI-only animal 2279 who did not recover on the skilled reaching task. This animal may have recovered had the study been extended and the correlation would disappear. No strong relationships appear between lesion location and midpoint or rate.
However, the aspects of the lesions that were measured do not appear to correlate well with performance on the reaching task (Figure 24B-D). Figure 24E at first seems promising, but it is mostly due to the large anterior lesion and low score of a single rat (TBI-only 2279). The plateau is not reliable for this rat because it did not begin improving by the end of the study. While it is possible this is due to the size and location of the lesion, the presence of rats that did not begin to recover until ~20 sessions post-TBI (Figure 17 and Figure 18) suggests the low “plateau” could be an artifact of the experimental design. Figure 21 also shows that the urn model could generate such data. In the case of the model, the simulated rat would eventually improve if allowed more sessions.

Golgi-Cox Analysis

As mentioned in Methods, only the basilar arbor was assessed. No obvious group differences were present in terms of total dendritic length, total number of branches, or soma area (Figure 25A-C). No correlation between these and reaching outcome was detected (not shown). The distribution of soma area appears to be either log-normal or bimodal with the most common area being around 230 μm² and a long tail with a few somas larger than 500 μm (Figure 25C). These two distributions would suggest positive feedbacks during the growth process or different subtypes, respectively. The somas measured from anti-Nogo-A treated animals 281 and 284 tended to be larger on average (Figure 25C).

There was great intra-individual diversity on all measured parameters, and possibly also inter-individual differences. For example, compare the branch length of
Figure 25. Neuronal Properties. A-C. Beanplots show the distribution for each group, each column contains the individual data points (yellow circles) and a boxplot calculated from these points. The animal id is shown in alternating fashion at the top and bottom of each plot. A. Total length of the dendritic arbor. B. Total number of branches. C. Soma area. D. Soma area plotted vs. total number of branches. E-F. Soma area plotted against location rostral-caudal, medial lateral, and depth from the surface of the brain. In the case of rostral-caudal location, it was recorded with resolution of 0.2 mm. Random jitter has been added to the points in the plot of +/- 0.1 mm for easier visualization.
control Ab animals 2957 and 9035 (Figure 25B). Similarly, the pattern of branching varied greatly from neuron to neuron even from the same rat, but the average number of branches per branch order were rather similar from rat to rat. There was one control Ab animal with exceptionally few branches higher than 3rd order (Figure 26: rat ID 2957).

Of the neurons that had both soma area and branch data available, there may be two subtypes (Figure 25D). Neurons with soma area >300 square microns had >40 total branches with only one exception. On the other hand, the neurons with smaller somas displayed the entire range of total branches. This suggests there is a lower bound on the number of branches for the neurons with larger somas.

When soma area was plotted against location (Figure 25E-G), only depth appeared to play a role. In this case, the entire range of soma sizes was found at 1000-1200 microns from the pial surface. A gradually decreasing upper bound on soma size is then visible both shallower and deeper to this location. The group averages by branch order were compared to previous results from the literature. This can be found in Figure 29.
**Figure 26. Individual Branching Profiles.** The width of the filled polygons indicates +/- 1 sd from the mean for that animal. The mean is shown by the dashed line. Branch profiles of each neuron are shown as the yellow points connected by thin lines. Color of the polygons indicates treatment group (Red: anti-Nogo-A, Green: Control Ab, Blue: TBI-only)
CHAPTER 7
DISCUSSION

In summary, the anti-Nogo-A treated rats were the best performers on the skilled reaching task by the end of the study but this had no strong association with any of the measured aspects of lesion or contralesional neurons. The reaching results are consistent with the improved recovery reported after a number of other types of CNS lesion as described in subsections “Nogo and Traumatic Brain Injury” and “Nogo-A and Stroke” of Review of Literature.

Skilled Reaching

These results show rats that received anti-Nogo-A treatment more often had high plateau scores. However a number of lines of evidence indicate this may be for a reason other than improved “motor function”. First, the unanimous recovery of the cohort 1 rats was attributed to a systematic difference in lesion location. If this is true, perhaps some rats had “better” TBIs than others and many happened to end up assigned to the anti-Nogo-A group. The parameters of the lesion that are relevant to skilled reaching, if any, must be identified and observed to be similar across groups before it is possible to confidently attribute a difference to the treatment. Although these results show full recovery is possible with relatively large lesions, it is implausible that the lesion has no effect.
Second, underdispersion relative to the binomial model was very common. Two types of plausible explanations for this would be either a “warm-up” or fatigue effect (see Figure 27). Rats with higher scores would then be ones that habituate faster, tire slower, or show delayed loss of motivation. In any case, the “treatment effect” will be dependent upon the number of trials per session. While the first two proposed explanations could be interpreted as influencing “motor function” in some sense, the reaching score of a fixed set of trials is clearly not an appropriate way to measure or distinguish between them.

Little information is available in the literature regarding day-to-day variability on the skilled reaching task. Fenrich et al. (2015) compare an automated version of the reaching task with the usual manual method. In figure 7G of that paper, they present a plot of the mean difference in success rates between sessions during the plateau phase. This average difference was less for the rats who were tested in the automated group, which they interpret as increased consistency. However, the rats in the automated group were allowed many more attempts per session.

When data like theirs was generated using the binomial model (Figure 14), the rats in the automated group were about twice as variable as predicted, while those in the manual group were near the expected value. If anything, overdispersion as in the automated group of Fenrich et al. (2015) would be expected due to various day-to-day differences in environment. Most studies report only group averages, which appears to hide the information that may conflict with the assumed interpretation of the results.

One concern is that the rats did receive a few practice pellets each session before the first trial. The duration of practice was not recorded so it is possible that some rats
Within-Session Patterns That May Lead to Underdispersion

Trial #

Probability of Success

0.0 0.2 0.4 0.6 0.8 1.0

5 10 15 20

Figure 27. Possible Explanations for the Observed Underdispersion. A. Three possible patterns of trial-to-trial success probability are shown that would lead to similar plateau mean and variance. Each session of reaching consisted of 20 trials, if a rat followed any of these patterns consistently from session to session during the plateau phase it would lead to a plateau mean of ~9.5 pellets and plateau variance of ~ 1 pellet. Colors correspond to the histograms shown in panels B-D and horizontal dashed line shows overall mean. B. Histogram of the number of successes observed from 10^6 simulations of twenty-trial sessions using the “warm-up” curve (yellow; panel A). In this case, the performance of the rat would improve throughout the session. The solid black line shows the expected number of successes according to the binomial distribution corresponding with p=9.5/20=0.475. The dashed black line shows the results expected from the corresponding Poisson binomial distribution. C. As in panel B except now fatigue is modeled by the corresponding decay. D. If a rat uses a mixture of two strategies in any order it will also result in underdispersion as long as the number of times each is used is relatively constant from session to session. In this example, the rat was equally likely to choose either strategy one with Pr(Success)=0.921, or strategy two with Pr(Success)=0.0285. E. All three patterns are examples of Poisson binomial distributions with mean and variance calculated as shown. The binomial distribution is the special case of the Poisson binomial with constant Pr(Success) (i.e., \( p_1 = p_2 = \ldots = p_n \)) from trial to trial.
had greater/lesser number of practice trials than others. For example, if a rat was more “frantic”, it may have been allowed more practice at the beginning of the session. In this way, a treatment that increased general level of activity could appear to increase “motor function”. Such an effect has been ascribed to knockout of Nogo-A in rats (Petrasek et al., 2014).

Third, the urn model is able to generate results covering the entire diversity of those observed from one set of parameters. In the bottom-right panel of Figure 21 there are apparent plateau scores ranging from 0-16. Although this is consistent with the idea that simple habit formation explains the data, the take-away is not so much that the model is literally/approximately true as it is that simple processes can result in a wide range of outcomes.

The process actually occurring in the brains of these rats is undoubtedly more complex and different at baseline across individuals. If the simple model can produce this diversity with no underlying difference in parameters, the more complex reality can do so with little/no measurable neurological differences. Further characterization of the model is needed to determine the effects of altering different parameters (e.g., increase learning rate) and how likely it is for such a large proportion of high-performers to be assigned to the same treatment group.

As mentioned in Methods, rats were not randomly assigned to treatment group. Rather they were matched based on performance after one week. There may be some form of selection bias that occurred. From Figure 18 it is evident that many of the worst performers before day 7 were assigned to the anti-Nogo-A group. It may be that rats that
recover early are simply those that end up learning the easier but less effective strategy. These possibilities can be explored using the urn model or extensions of it.

There have also been reports that rats forced to use the impaired forelimb early after sensori-motor cortex stroke were found to have larger lesion size and increased level of functional deficit compared to controls (Kozlowski 1996; Humm 1998; Bland 2000). If high performance early on is similar to forced use, perhaps a similar effect can occur due to good performance on the skilled reaching task soon after lesion which later limits the level of recovery.

**Lesion Analysis**

The current results provided some limited evidence that the amount of damage to the rostral forelimb area is relevant to performance on the reaching task, but this was mostly due to a single animal that may have recovered later. The location of the lesions in this study was not consistent across animals, which raises concerns regarding the comparability of behaviour by the different groups. Since it remains unknown what aspects of the lesion determine reaching performance, it is not possible to say how large an effect this may have had. While it is possible that the stroke lesions used in previous trials of anti-Nogo-A antibody are more consistent, Tsai et al. (2007) reported standard deviations in total lesion size of up to ~15%, which suggests otherwise.

The methods developed for analysing lesion location from histological sections tissue were not perfect as artifacts due to post-sacrifice tissue damage added a significant amount of noise. Yet this method seems to be superior to only measuring the total lesion size since the large amount of “lesion” attributed to the lateral edge of the damaged
hemisphere must clearly arise from a process different than the direct ablation of tissue. If this phenomenon is common in previous studies it would cast doubt on the utility of comparing total lesion size, which may be in part artifactual or due to atrophy. Assessing the lesions via small animal fMRI may allow a higher signal to noise ratio.

It may also be helpful to develop a biophysical model of the lesion, incorporating the forces involved, properties of rat brain tissue, etc. Then it would be possible to compare the observed lesions to those predicted and make claims about secondary injury processes. For example, if the unsmoothed average lesion for the TBI-only rats in Figure 10 is inspected rather than the smoothed in Figure 23, the measured “lesion” appears to surround the targeted area. It is almost as if the tissue is deforming in such a way to fill the cavity. This would render comparison between any localized function in healthy tissue difficult to compare to the damaged without an informed guess as to where the areas have migrated.

Golgi-Cox Analysis

Results reported previously differ much across publications (Figure 28). Some studies report branching profiles for control animals that are more similar to those of the anti-Nogo-A treated group than they are to the control groups in the previous report of Papadopoulos et al. (2006) (Figure 29). Indeed, the results from the current study for all groups appear to be more similar to the previous anti-Nogo-A treated animals.

A close inspection of the results shown in Figure 29 leads to the realization that there are two primary differences between the branch order profiles. First, there is high variability in the number of high order (>4) branches. This could likely be attributed
Figure 28. Varying Branching Profiles from Previous Studies. Seven previous publications were identified that reported branch order profiles for the basilar branches of pyramidal neurons in the intact cortex after unilateral damage. Each result is shown along with key information about the study. See Figure 29 for selected results plotted on the same axis.
Figure 28. Varying Branching Profiles from Previous Studies. Continued. These three reports plotted number of branch points rather than number of branches. These values were converted for comparison to the others in Figure 29.

Study: Jones 1992  
Animal: 4.5-6 month old male Long Evans  
Injury: Electrolytic Lesion  
(3-4.5 mm lateral, 1.5 to -0.5 mm from bregma, 0-1.7 mm deep)  
Treatment: None  
Sacrifice Time: 18 or 120 days Post-Lesion  
Stain: Golgi-Cox  
Neuron Location: 0.1-2.2 mm +Bregma, the region just lateral to the dorsal ‘peak’ of the corpus callosum and 4.0 mm lateral to midline.

Study: Jones 1994  
Animal: 4.5-7 month old male Long Evans  
Injury: Electrolytic Lesion  
(3-4.5 mm lateral, 1.5 to -0.5 mm from bregma, 0-1.7 mm deep)  
Treatment: Cast (Forelimb Immobilization)  
Sacrifice Time: 45 days Post-Lesion  
Stain: Golgi-Cox  
Neuron Location: 0 to 1.8 mm +Bregma, the region just lateral to the medial agranular area, bordering the dorsal peak of the corpus callosum, and approximately 4.0 mm lateral to midline.

Study: Kozlowski 1997  
Animal: Male Long Evans (400-650 g)  
Injury: Electrolytic Lesion  
(3-4.5 mm lateral, 1.5 to -0.5 mm from bregma, 0-1.7 mm deep)  
Treatment: Ethanol Diet 18-45 Days Post-Lesion  
Sacrifice Time: 45 days Post-Lesion  
Stain: Golgi-Cox  
Neuron Location: -0.7 to 1.8 mm +Bregma
No Lesion

Lesion

Current: TBI Only
Pruskey1996: Lesion
Forgie1996: Electrolytic Lesion
Forgie1996: Aspiration Lesion
Voorhies2002: Electrolytic
Voorhies2002: Aspiration (18d)
Voorhies2002: Aspiration (30d)
Hurtado2007: MCAO+SAL
Papadopolous2006: Stroke Only
Kozlowski1998: Lesion+Vehicle
Jones1992: Day 18
Jones1994: Lesion−No Cast
Kozlowski1997: Lesion−Vehicle
Figure 29. Comparison of Branching Profiles from Previous Studies. Values were extracted from the plots shown in Figure 28 by manually estimating each data point with the help of an overlying grid. The profiles varied substantially across studies even when conditions were similar, primarily for branch orders >3. A number of aspects are notable:

1) The results of all three groups in the current study were very similar to those reported for unlesioned animals by Voorhies 2002, Forgie 1996, and Clarke 2009. They were also similar to the animals that received lesions in Forgie 1996 and 30 days after aspiration lesion from Voorhies 2002. The Clarke 2009 lesion groups were very similar to the sham group from that study so they have been omitted for clarity, note these profiles were also similar to the current results.

2) The Papadopolous 2006 lesion-control groups were most similar to the results of Hurtado 2007. Notably these two studies were the only ones that used middle cerebral artery occlusion to cause the lesion, but the unlesioned animals from Papadopolous 2006 shared a similar profile distinguished by relatively few 3rd order branches. The anti-Nogo-A treated animals from Papadopolous 2006 were most similar to those reported after electrolytic lesion by Voorhies 2002.

3) The animals of the Forgie 1996 study were sacrificed 18 days after lesion. Yet, the neurons of animals sacrificed 30 days after aspiration lesion in Voorhies 2002 were more similar to the results of Forgie 1996 than those sacrificed 18 days after lesion.

4) The only study to use a staining method other than Golgi-Cox was Pruskey 1996, who reported a larger number of 1st order branches than seen in the other studies. Because this study traced neurons labelled by injection of tracer into the spinal cord it likely represents the average profile closest to that of only cortico-spinal neurons.
to different “thresholds of inclusion” of the researchers tracing the neurons. It is often difficult to distinguish branches of the neuron being traced from crossing branches that arise from other neurons as the distance from the soma increases (personal observation).

An interesting difference is whether there are more or less 3rd order branches than 2nd order, i.e., whether over 50% of 2nd order branches for a given neuron bifurcate. Studies in the mouse have identified two types of cortical pyramidal neurons found in a ratio ~3:1 (Groh et al., 2010). Further, there is a report (Shin et al., 2006) of two types of pyramidal neurons in the rat that differed in Nogo-A expression and soma size. The non-Nogo-A expressing neurons all had soma areas <300 μm², which is similar to that found for the neurons with a greater number of branches in this study. Let us refer to neurons with a greater number of order 3 than order 2 branches as type I, and the others type II.

Of the results shown in Figure 29, the treated group in Papadopoulos et al. (2006) consisted mostly of type I neurons while the controls were type II neurons. A further two papers (Forgie et al., 1996; Clarke et al., 2009) reported results like type I neurons. Both groups of Hurtado et al. (2007) appeared to consist of type I neurons, while the other studies report more intermediate results. As mentioned above, the results for all three groups in the current study are more consistent with the previous (Papadopoulos et al., 2006) anti-Nogo-A branching profile than either of the controls from that study.

One difference between studies is that the current control groups were tested on the skilled reaching task, while no behaviour was performed as part of the earlier work. It is possible that the reaching task may have functioned as a enriched environment. There are also a number of alternative explanations for the larger number of branches
observed by Papadopoulos et al. (2006) other than dendritic plasticity due to anti-Nogo-A treatment, which will be discussed below.

Although the data is not reported in enough detail to say, it appears likely that the current and previous studies are reporting on a mixture of two types of pyramidal neurons. The relative percentage of neurons of each type that are being traced could be influenced by a number of factors. If the stain is very robust, one type of neuron may be less likely to be obscured by other nearby neurons and blood vessels. Conversely, if the stain is weak one type or the other may be preferentially stained. In the absence of precise inclusion criteria, the choice of which neurons to trace, in terms of appearance and location, may be adjusted by the researcher in an attempt to achieve an equal number of neurons for each animal. Given the small number of animals and neurons traced per animal in these studies (usually <10 in both cases), this could easily lead to inadvertent selection bias that is not balanced across groups. The problem of selection bias may be exacerbated if researchers stop the study when a significance difference is detected and/or continue adding neurons/animals to the study in search of a significant difference.

As the mechanism by which neurons are stained by the Golgi procedure is unknown, experimental manipulations involving injection of material (e.g., antibody) into the cortex may affect the relative proportions of each type of neuron that is stained. The antibody may be preferentially interacting with the type I neurons since Shin et al. (2006) suggested that only pyramidal neurons with large soma size (>300 μm^2) express Nogo-A. It is possible that this could influence the chance of the neuron being filled with the Golgi material, although no mechanism for this is known.
An increased number of branches could also be due to real anatomical changes other than sprouting. For example, a treatment could give the appearance of increasing dendritic branching by selectively damaging the type II neurons. This could manifest as either cell loss or degeneration to a point that would cause a researcher to avoid choosing them for tracing. In the case of the anti-Nogo-A antibody, it has been reported that treatment administered before stroke in mice is associated with decreased neuronal survival in the damaged hemisphere, although not in the intact hemisphere (Kilic et al., 2010).

Longitudinal studies using two-photon microscopy to observe the same neurons over time in mice have not reported observing any large scale changes in dendritic branches (Mostany et al., 2011; Johnston et al., 2013, Schubert et al., 2013). While these studies did not include anti-Nogo-A treatment and were performed in mice, the total lack of observed sprouting of branches casts doubt on the plausibility of a treatment doubling the number of 3rd order branches, total branches, and total dendritic length as was observed by Papadopoulos et al. (2006). Zemmar et al. (2014) includes a table reporting on branching of measured using two-photon in vivo imaging. This data included anti-Nogo-A treated rats, but was not longitudinal. In this case the treated animals had ~50% the number of branch segments of controls. The table is not discussed in the text of that paper so it is not clear how they chose to interpret those results in light of Papadoplous et al. (2006).

That said, none of these studies have been directly replicated and it is possible species differences exist or the researcher’s performing the two-photon studies simply
missed these effects or that some technical factor is responsible. For example, one could speculate that the fluorescent proteins used to allow imaging of the neurons may not be trafficked to newly sprouted branches in the adult mouse. Another possibility is that the expression of exogenous protein is somehow inhibiting branch sprouting.

**Future Directions**

There are a few possibilities regarding the possible role of putative neuroplasticity enhancing treatments such as anti-Nogo-A. First, the benefits of any such treatment would be limited by the maximal capacity of the remaining tissue. Information regarding this could possibly be gathered by purposely varying lesion location, perhaps also mapping the forelimb area of each animal beforehand, and determining the maximal degree of recovery. Second, there are a few means by which such a treatment could enhance functional recovery. Perhaps the treatment somehow directs the neurological changes in a way that leads to realization of a state approaching the optimum. Alternatively, it could increase the set of possible neurological states and associated behavioural strategies that are explored before stabilization allowing greater chance for the animal to hit on a globally optimum strategy.

An effect directed towards a specific neurological structure due to a compound infused into the lateral ventricle seems unlikely. However, this could occur if there is a specific bottleneck to achieving an optimal neurological state that is targeted by the antibody. As an example, there have been a number of studies reporting increased number of axons crossing from the intact motor cortex to the red nucleus ipsilateral to motor cortex lesions after anti-Nogo-A treatment. Presumably this would allow increased
communication between the intact cortex and contralesional spinal cord. These papers report relatively consistent results of ~4-5 % of total stained fibers (i.e. axons) crossing the midline (Wenk et al., 1999; Papadopoulos et al., 2002; Seymour et al., 2005; Tsai et al., 2011). However, these studies do not report on the relationship between the crossing fibers and functional outcome.

There is a study in which rats instead received amphetamine and environmental enrichment that reported a similar level of crossing fibers and included a comparison of these versus week 8 reaching score (Papadopoulos et al., 2009). The overall correlation was rather weak and determined primarily by the presence of three animals with an exceptionally large number of crossing fibers and high reaching scores, but no animals with any low-fiber-count yet high reaching score. This data would be consistent with the idea that a large number of these axons is sufficient to enable optimal recovery but not necessary for it to occur in the case of e.g., limited amount of damage to structures important to reaching skill. Also note that this study indicates that increased sprouting across the red nucleus is not an anti-Nogo-A specific effect. If amphetamine is equally effective this would be the cheaper treatment option although side effects (e.g. addiction) would need to be considered. Regardless, cortico-efferent axonal plasticity is definitely something to be assessed if possible. In this study it was not due to the use of Golgi-Cox stain.

The second possibility is that enhanced neuroplasticity increases the number of strategies that are explored, thus providing greater opportunity to find a global optimum. This could occur in a number of ways. Each would act on the rate at which the animal
converges on a stable reaching strategy and hence could indirectly affect plateau skill level. One mechanism is by increasing the reward threshold necessary for the animal to accept modifications to its strategy, for example, by ignoring successes that require a relatively large amount of time or effort between decision to attain the pellet and the reward. Another possibility is that these treatments interfere with memory of previous strategies. Thus, the animal would less precisely repeat the previous approach after some delay between reaching sessions. Alternatively, neuroplasticity enhancement could introduce noise into the system, thus increasing the degree of strategy modifications for a given level of reward.

Evidence in favour or against these possibilities could be gained experimentally based on observation of behaviour alone. If there is a specific tissue-directed effect of the anti-Nogo-A antibody, there should be some pattern of motion that develops which is characteristic or prototypical of animals that receive the treatment. If the animals have a higher threshold for accepting modifications, then they should be less likely to repeat the strategy used for successful reaches if they involve more complicated movements. If the treatment is interfering with memory of previous successful strategies, the variability of the behaviour should increase from day to day or after pauses in testing such as over the weekend. If the treatment is increasing the noise levels, there should be variability from trial to trial even when the animal has attained a high level of performance.

The literature on dendritic plasticity after cortical injury is currently in conflict with the more recent in-vivo imaging reports. It seems clear that observing the same neuron over time to assess changes is superior to comparing samples of neurons from
different groups at the end of the study. In the latter case it is not possible to distinguish between seemingly opposite phenomena such as inducing branching and selectively damaging neurons with fewer branches.

On the other hand, Golgi-stained tissue has the potential advantage of allowing the assessment of a large number of neurons from the same animal at the researcher’s leisure. Use of the tissue in this manner appears to be uncommon, but if attempts were made to trace nearly all stained neurons along with their coordinates it may be a good way to search for regions that best correlate with functional outcome or may be affected by anti-Nogo-A. However, the problem of whether this is due to a beneficial or harmful effect would remain. It seems unwise to continue using rat tissue for this purpose unless that issue can be resolved.

Further, the method of analysis used here and elsewhere (counting branches, etc.) may not be ideal. Cuntz et al. (2010) have proposed a model for dendritic growth based on “laws of neuronal architecture” first proposed by Ramón y Cajal. The model can “grow” a realistic diversity of neurons based on the idea that the neuron attempts to minimize both the material used and time elapsed for a signal to reach the soma from anywhere in the arbor. Morphologically these costs correspond to the total dendritic length and the length of the path from each point to the soma. The single parameter of this model is a “balancing factor” that determines the relative importance of these two costs. Preliminary exploration of this model showed it was able to generate data similar to that presented here.
Conclusion

This project found that rats receiving TBI and anti-Nogo-A immunotherapy one week later had a higher plateau score when compared to those that received TBI and control immunotherapy or TBI alone. However, the success of the urn model suggests it is possible to measure stochastic noise and observe results similar to those presented here albeit the different performance of the treatment group remains unexplained.

It was also found that success rate alone does not allow distinguishing between different explanations for individual and group differences. Skilled reaching data should be collected and analyzed at the individual trial-to-trial level. Kinematic data may also be necessary, along with some assessment of the most plausible confounds (e.g., motivation, activity level, anxiety). Of course, the number of explanations for the existence of a mere difference between two groups of rats is prohibitively large. The set consistent with data showing one average is higher than another is only slightly less so. The methods available to assess the confounds may also suffer from their own deficiencies, *ad nauseam*.

The best way to provide positive evidence for a given explanation is a precise *a priori* prediction consistent with new evidence. Promisingly, the exceedingly simple urn model of learning was able to “fit” >1000 datapoints with only four parameters. A more complete exploration of this model, methods of fitting it to data, and identification of possible behavioural/neurological correlates to its parameters remain be performed.

In the context of brain injury, one can imagine that the s01/s02 parameters are related in some way to initial deficit due to the lesion, k1/k2 to neuroplasticity, and p1/p2 to variability in execution of different motor strategies. If the effect of a treatment can be
related to these parameters, precise predictions could be made regarding the distribution of outcomes, lower/upper bounds on time to attain a certain performance level, form of a relationship between plateau score and kinematics, etc. More work is required on this front.

More detailed information regarding the behaviour would seem to be required to propose anything specific. Group averages, even the individual timecourse of success scores, are too abstract to precisely relate to any but the most drastic neurological effects a treatment may have. Similar arguments have been made regarding the ability to distinguish recovery vs. compensation using skilled reaching (Alaverdashvili and Whishaw, 2013).
APPENDIX A:

INHIBITORY ACTIVITY OF NOGO-A FRAGMENTS CORRELATE WITH MOLECULAR WEIGHT
As shown in Figure 2, Strittmatter and Schwab are the two most prolific sources of information regarding Nogo-A. They co-authored a recent review (Schwab and Strittmatter, 2014) which describes the most up-to-date ideas regarding the signalling pathways via which Nogo-A is thought to play its role:

“The sphingosine-1-phosphate receptor 2 (S1PR2) has now been identified as a specific binding partner and signal transducer for Nogo-A-d-20 in neurons and Nogo-A responsive non-neuronal cells by a yeast 2-hybrid screen, co-immunoprecipitation, binding studies and in situ co-localization experiments [12]. Nogo-A binding to this 7-transmembrane domain, G-protein coupled receptor activated the G-protein G-13, the rhoGEF LARG and rho A; all these steps are required for Nogo-A-d-20 induced inhibition of neurite growth or fibroblast spreading.”

Nogo-A-d-20 is a peptide fragment including the region targeted by the 11c7 Ab used in the current study, see Figure 5. As shown in that figure, it is one of three “active regions” of Nogo-A. The effects of this peptide have been primarily tested in the form of a substrate (the physical properties of this substrate do not appear to have been described) rather than in solution. Reference 12 is to Kempf et al. (2014). In figure S3 of this paper there is a gradual increase in inhibition of 3T3 fibroblast spreading (~0, 40%, 70%, and 90%) as the dose of Nogo-A-d-20 is increased from 0 to 20 to 40 to 80 pmol/cm^2 adsorbed to tissue culture dishes. First, this is a quite steep dose response curve, characteristic of a non-specific mechanism of action (Shoichet, 2006).

Second, one pmol/cm^2 corresponds to 6,022 molecules per square micron. If one amino acid is approximated by a 1 nm^2 square, ~1,000,000 amino acids could fit in one square micron. If all the peptide adsorbed, each molecule standing straight up, then the minimum amount of the surface covered by the peptide can be calculated. The doses of Nogo-A-d-20 in these experiments correspond to 12.44%, 24.88% and 49.6%. It is rather
implausible that such a large percent of the cell surface is devoted to Nogo-A receptors, so the presence of a dose response is curious.

If all the peptide adsorbs, the effect must be due to formation of aggregates or even layers and thus the in vitro results have questionable biological relevance. Likewise, if only some percentage of the added peptide actually adsorbs, these experiments lack appropriate controls. They must show that a different peptide that adsorbs to a similar degree does not have an inhibitory effect. Both scenarios suggest that the inhibitory effect of Nogo-A-d-20 is due to non-specific physiochemical characteristics. This possibility was investigated by Oertle et al. (2003a), who concluded:

“Nonspecific physicochemical properties (acidity of the fragments, structural effects attributable to proline and serine residues) are not responsible for this effect.”

In that paper they compared the inhibitory characteristics of a number of Nogo-A fragments to determine the “inhibitory regions”. Of the fragments in figure 2 of Oertle et al. (2003a), it was possible to estimate the half-maximal effective concentration (EC50) for 33 of them. The result of plotting this against molecular weight is shown in our Figure 30. These results indicate Oertle et al. (2003a) are incorrect.

According to Erickson (2009), the density of most proteins is similar. Therefore, the minimum volume (nm³) occupied can be calculated from the molecular weight (in g/mol; Daltons): \( V = 1.212e^{-3} \times MW \). As a preliminary analysis, the inverse of these values (i.e., 1/volume), multiplied by a few different constants, were then plotted against EC50 (Figure 30). Inhibitory activity appears to correspond to ~500-3000 times the inverse of the minimum volume occupied by each different peptide fragment (the constants...
Figure 30. 3T3 Fibroblast Spreading Inhibitory Activity of Nogo-A Fragments Correlates with Molecular Weight. **A.** This is a plot of data from Oertle et al. (2003a). The height of each bar in figure 2 of that paper was estimated and the doses were converted from μg to pmol. From this, EC50 values were estimated via linear interpolation of the dose-response curves. These values were then compared to the molecular weight of the associated peptide. The pattern of results suggest a simple inverse relationship between molecular weight and inhibitory activity, possibly related to the volume occupied by the peptides (i.e., the surface area of interaction with the culture dishes). Nogo-A-d-20 is claimed as one of the most inhibitory region of Nogo-A, this result is shown in red. It does have a somewhat lower EC50 for its size, but is consistent with the overall pattern. Also, the data from Kempf et al. (2014) indicates an EC50 > 20 pmol/μm² for this peptide. No attempt was made to assess the uncertainty due to sampling error (the bar height represented mean values) or estimation error (from only 4 dosages) of the EC50 values. **B.** The same data shown as a log-log plot.
are determined by the units; if EC50 was shown in nmol/cm² this would be ~0.5x-3x, the center line corresponding to a 1:1 relationship). One could imagine that the volume occupied by a peptide is related to the surface area of its interaction with a substrate. Larger volume would then be associated with a greater number of bonds and thus stronger adhesive forces. Of course, other properties of the peptides are likely to also play a role. As noted by Goebel-Stengel et al. (2011), discussing loss of peptide to containers:

“at this time it is impossible to predict which surface will give the best recovery for any given peptide. Possible indicators such as net charge, regional charge distribution, regional or net hydrophobicity, or chain lengths do not consistently predict the correct container surface that would yield optimal peptide recovery.”

It is surprising that this relationship is visible at all, since we would expect to need to measure the amount adsorbed to find evidence for such an effect. For that reason it is compelling evidence that at least some of the in vitro effects of Nogo-A are due to its non-specific physical properties. The experiments used to elucidate the signalling pathways activated by this protein have failed to control for the amount of peptide that is adsorbed to the surface. No instances of measuring the actual amount of Nogo-A, Nogo-fragments, or control peptides present was found. This is quite surprising because the issue was identified with regards to PNS and CNS myelin (referred to as “antigen” or “substrate” below) as early as 1988:

“Values obtained with different antigens cannot be compared quantitatively, as adsorption to the wells varied among different antigens.” (Caroni and Schwab, 1988a)

“Differences in the extent of spreading were most reproducibly observed after short periods (1 h) of substrate contact. Differences tended to diminish or even to vanish after prolonged exposure to the substrate (10 h). Possible interpretations of this diminution include protease digestion of substrate, matrix production by the fibroblasts, and possibly also progressive detachment of dish-adsorbed substrate.” (Caroni and Schwab, 1988b)
It is also notable that, at least in some cell types, RhoA is said to be regulated by many of the same things it is claimed to cause to happen downstream of Nogo-A. For example, the assay used by Kempf et al. (2014) to determine RhoA activity was first used by Ren et al. (1999) with the title: “Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton”. That data indicates any substance that non-specifically interferes with cell adhesion can cause higher levels of activated RhoA in 3T3 cells, because RhoA levels are lower in adhered cells.

Interestingly, in the very first paper proposing the presence of CNS “inhibitory substrates” Schwab and Theonen (1985) write:

“The major differences between optic and sciatic nerves are the different types of glial cells and the exclusive presence of basement membranes in the peripheral nerves. In fact, the preferential localization of the axons within our sciatic nerve explants points to an important role of specific substrates... axons grew mostly in contact with the surface of living Schwann cells and the Schwann cell side of the basal lamina. This association with basal laminae was particularly striking in the dead sciatic nerve explants, where axons often squeezed themselves between the myelin, the Schwann cell debris, and the overlaying basal lamina...

...Major constituents of basement membranes are collagen type IV, laminin, and fibronectin... Particularly outstanding is laminin...”

It was recognized that a major difference between CNS and PNS tissue is the lack of favorable substrate (basal lamina) in the former, of which they identify laminin as an especially important component. They dismiss this explanation for their results with the following rationale:

“...In the case of our optic nerve explants, immunofluorescence experiments with an antiserum against laminin showed a pronounced staining of astrocytes... Nevertheless, this laminin production in the nerve explants or at lesion sites in vivo does not seem to be capable of or sufficient to support axonal regeneration. Therefore, besides the possible lack of substrates favoring neurite growth in the differentiated CNS, inhibitory substrate molecules should be considered.”
This evidence against basal lamina as the important factor is rather weak. Intermittent laminin expression by astrocytes is not comparable to the presence of a layer of basal lamina, which forms a “path” along which axons can grow. An alternative explanation for the inhibitory effect of Nogo-A substrate on 3T3 cells, PC12 cells, etc. is becoming clear.

In all cases, there is some favorable substrate present for that particular cell line, be it plastic, poly-lysine, laminin, etc. When Nogo-A is added, it sticks to the favorable substrate, thus preventing the cells from interacting with it. An analogous situation would be comparing planting a seed in fertile dirt compared to fertile dirt that has been covered by a layer of gravel.

Adding anti-Nogo-A antibody to the situation could selectively solubilize that adsorbed protein, similar to sending someone to specifically pick up the gravel and leaving behind the dirt. Unless the quantity adsorbed in these experiments is much less than that added, this is the likely means by which the antibody counteracts the inhibitory effect in vitro. It does not make much sense that a layer of antibody bound to Nogo-A would be just as preferable a substrate as the one that has been optimized for each different cell line. Careful choice of substrate would not be necessary were that the case.

It seems that the following information is required to distinguish between the two explanations:

1) The quantity and spatial distribution of inhibitory and control peptides adsorbed to the underlying substrate. Also, how this changes over time for different amounts of added peptide.

2) All the above performed with antibody present. Antibodies should be tested that are directed towards the inhibitory peptide, control peptide, and some other peptide that is not present.
3) How points 1 and 2 are altered in the presence of the cells.

Armed with this information, it should be possible to disprove the proposed adsorption mechanism of Nogo-A substrate. Even if that explanation is incorrect, one will still need to be devised for the phenomenon in Figure 30; i.e., how can it be explained as due to an interaction between the peptides and a receptor? Perhaps it is due to a correlation between size and proper folding? Despite the questionable applicability of information arrived at via the \textit{in vitro} experiments, it is still possible that strategies targeting Nogo-A \textit{in vivo} are effective.
APPENDIX B:

JAGS MODEL
model {
    for (r in 1 : Ndata) {
        mu[r] <- Plateau[ subj[r] ]/(1+exp(( Midpoint[ subj[r] ] - t[r])/Rate[ subj[r] ]))
    }
    for (s in 1 : Nsubj) {
        Plateau[s] ~ dunif(0, 20)
        Midpoint[s] ~ dunif(0, 39)
        Rate[s] ~ dunif(0, 39)
        Precision[s] ~ dgamma(5, 5)
    }
    Variance<-1/Precision
}

APPENDIX C:

R CODE FOR URN LEARNING MODEL
UrnModel<-function(Ntrials=780, e0=100, Nsim=32,
    k1=7.231, k2=11.078, s01=0.517, s02=0.792,
    p1=0.815, p2=0.532)
{
    DailyScores=matrix(nrow=Ntrials/20, ncol=Nsim)
    for(sim in 1:Nsim){
        s1=s01; s2=s02; e=e0
        out=matrix(0,nrow=Ntrials)
        samp1<-sample(0:1, Ntrials, prob=c(1-p1,p1), replace=T)
        samp2<-sample(0:1, Ntrials, prob=c(1-p2,p2), replace=T)
        for(i in 1:Ntrials){
            total<-s1+s2+e
            samp0<-sample(0:2,1,prob=c(e/total,s1/total,s2/total))
            if(samp0==1){
                if(samp1[i]==1){out[i]=1; s1=s1+k1}
            }
            if(samp0==2){
                if(samp2[i]==1){out[i]=1; s2=s2+k2}
            }
        }
        DailyScores[,sim]<-unname(tapply(out,(seq_along(out)-1)%%20, sum))
    }
    return(DailyScores)
}
REFERENCES


regeneration in the CNS. Neuron 15, 1375–1381.


Kaneider, N.C., Lindner, J., Feistritzer, C., Sturn, D.H., Mosheimer, B.A., Djanani, A.M.,


of cerebral circulation 42, 186–190. doi:10.1161/STROKEAHA.110.590083.


VITA

Stephen was born in Chicago, IL on March 13th 1986 to Bruce and Dorothea Nawara. He was awarded a Bachelor of Science in Psychology: Natural Science by Loyola University Chicago on May 10th 2008 and accepted as a PhD student by the Loyola University Chicago Department of Molecular Pharmacology and Therapeutics (then called Department of Pharmacology and Experimental Therapeutics) in fall of 2008. There he joined the lab of Dr. Gwendolyn Kartje which specializes in preclinical trials using rodent models of brain damage such as stroke and traumatic brain injury (TBI) where he performed this work.