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Anti-Nogo-A Immunotherapy Facilitation of Environmental Enrichment's Effects on Recovery From Stroke in the Aged Rat

Sarah Jane Hein
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LOYOLA UNIVERSITY CHICAGO

ANTI-NOGO-A IMMUNOTHERAPY FACILITATION OF ENVIRONMENTAL
ENRICHMENT’S EFFECTS ON RECOVERY FROM STROKE IN THE
AGED RAT

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN NEUROSCIENCE

BY
SARAH J. HEIN
CHICAGO, IL
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<thead>
<tr>
<th>Ab</th>
<th>Antibody</th>
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<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled Cortical Impact</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CSPG</td>
<td>Chondroitin Sulphate Proteoglycans</td>
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<tr>
<td>EE</td>
<td>Environmental Enrichment</td>
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<tr>
<td>ErbR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>FA</td>
<td>Focused Activity</td>
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<td>GAP-43</td>
<td>Growth Associated Protein-43</td>
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<td>hABM-SCs</td>
<td>Human Adult Bone Marrow-Derived Somatic Cells</td>
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<td>icv</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MAG</td>
<td>Myelin Associated Glycoprotein</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle Cerebral Artery</td>
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<tr>
<td>OMgp</td>
<td>Oligodendrocyte Glycoprotein</td>
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<tr>
<td>Acronym</td>
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<tr>
<td>NgR</td>
<td>Nogo Receptor</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<tr>
<td>PirB</td>
<td>Paired Immunoglobulin-like Receptor</td>
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<tr>
<td>RAGs</td>
<td>Regeneration Associated Genes</td>
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<td>RTN</td>
<td>Reticulon</td>
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<td>TBI</td>
<td>Traumatic Brain Injury</td>
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<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Stroke and traumatic brain injury are devastating disorders that often lead to permanent neurologic deficits, with few available treatment options once injury has occurred. In previous work, Anti-Nogo-A immunotherapy has been shown to neutralize the neurite inhibitory protein, Nogo-A, and to promote neuronal plasticity and improve functional recovery after stroke in the rat. Furthermore, other reports have demonstrated that environmental enrichment improves functional recovery in rodents following stroke. Our previous work demonstrated that anti-Nogo-A immunotherapy also helps promote cortical plasticity in the rodent. In the current project, we examined two methods to promote recovery after brain injury, a stroke model (MCAO) and a traumatic brain injury model (TBI). This work also studied whether environmental enrichment (enriched housing and focused activity) paired with Anti-Nogo-A immunotherapy shortened the recovery time and increased the neuronal plasticity of aged rats who had received focal ischemic stroke, produced by middle cerebral artery occlusion. Additional work examined whether Anti-Nogo-A immunotherapy alone could lead to a shorter recovery time and increased plasticity in animals that sustained traumatic brain injury. The skilled forelimb reaching task and the skilled ladder rung walking test were used to assess sensorimotor recovery. Dendritic plasticity was studied using the Golgi-Cox staining method. Our results show that dendritic plasticity was increased in the middle cerebral artery occlusion animals but decreased in the traumatic brain injury animals compared to
age-specific naïve animals. However, there was no significant difference in performance on behavioral tasks in the rats receiving Anti-Nogo-A or control antibody in both the MCAO and TBI experiments.
CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Literature Review

The Global Problem of Stroke:

Worldwide, stroke is a devastating neurological condition that often leads to permanent disability or death in adults. The World Health Association estimated in 1999 that stroke led to 5.54 million deaths world-wide, mostly occurring in less developed countries (The World Health Report 1998, 1998; The World Health Report 2000, 2000). The end point of stroke is, of course, not always death but may be a crippling disability that leads to an enormous socioeconomic burden. The approximate costs for each stroke patient range from $59,800-230,000 in their lifetime after stroke (The World Health Report 1998, 1998; The World Health Report 2000, 2000). Stroke mortality trends may have decreased in industrialized nations recently, although this is controversial (Gillum, 1997). Nonetheless, stroke is a deadly killer and disabler that we must work to silence.

Stroke:

There are two types of stroke: ischemic stroke and hemorrhagic stroke. Ischemic stroke occurs when blood flow to an area of the brain is reduced. When this occurs, the
affected area of the brain is damaged and can no longer function properly. Ischemic strokes account for 67.3-80.5% of all strokes (Feigin, Lawes, Bennett, & Anderson, 2003). Some of the known major risk factors of stroke are: high blood pressure, smoking, diabetes, high cholesterol, heart disease and arterial fibrillation. People with known histories of heart disease and diabetes, especially men, African Americans and people over the age of 55 are at most risk for stroke (Feigin et al., 2003; Gillum, 1997; Gillum & Semos, 1997; Murray & Lopez, 1997; Sarti, Rastenye, Cepaitis, & Tuomilehto, 2000).

**Traumatic Brain Injury:**

Traumatic brain injury (TBI) is an acquired injury to the brain that occurs because of sudden trauma. Symptoms in this type of injury range from mild to severe according to amount of brain damage. TBI patients may also experience trauma to the rest of the body as well because of the nature of the accident. Extensive trauma proves to be more difficult to treat because of the likely involvement of various other organ systems in the body. Not unlike ischemic stroke, TBI is also very difficult to study and treat in the aged population because of the multifaceted effects it has on the nervous system. It can lead to disability as well as death, especially in the aged population.
Global Problem of Traumatic Brain Injury:

The National Institutes of Health (NIH) identified adults older than 75 years of age to be in the high risk group for acquiring traumatic brain injury (*Traumatic Brain Injury: Hope Through Research*, 2002). The Centers for Disease Control and Prevention (CDC) conducted a survey across nine states in the United States looking at the hospitalization rate for elderly patients with traumatic brain injury. In this work, the hospitalization rate was 105 patients per 100,000 for those 65-74 years of age, while the rate was 287 per 100,000 for those patients over 75 years of age (*Rates of Hospitalization Related to Traumatic Brain Injury-- Nine States, 2003,* 2007). Another survey conducted by the CDC determined that falls were the highest contributor to TBI in the elderly population and the next was motor vehicle accidents (*Rates of Hospitalization Related to Traumatic Brain Injury-- Nine States, 2003,* 2006). It was found that 79% of TBI victims over the age of 65 suffered from co morbid health condition(s) (Cuadrado, Egido, Gonzalez-Gutierrez, & Varela-De-Seijas, 1999). These data suggest that TBI is indeed a deadly condition within the aged population and great strides are needed to help advance recovery in these patients.

Age and Brain Injury:

As the elderly population is rapidly growing, disorders associated with age such as stroke and traumatic brain injury are on the rise (Popa-Wagner, Carmichael, Kokaia,
Kessler, & Walker, 2007; Uomoto, 2008). Accordingly, clinical research is being developed to further understand the basic mechanisms involved in stroke as well as preventive measures and therapies to ease the burden of the disease on the aged population. Studying stroke and TBI in the aged population is especially challenging because these adults often suffer from other diseases as well. Moreover, old age and the related changes that occur in the brain contribute to the poor recovery of the brain after stroke and TBI (Hukkelhoven et al., 2003; Rosen, Dinapoli, Nagamine, & Crocco, 2005). The difficulty understanding the age-related mechanisms underlying stroke and TBI leads to challenges in designing effective therapies to treat elderly patients.

*Treatment Options—Stroke:*

Due to the challenging nature of stroke, treatment options are quite limited. The current accepted treatment used to reduce the severity of stroke is tissue plasminogen activator (Brouns & De Deyn, 2009; del Zoppo, 2000; Cheatwood, Emerick, & Kartje, 2008). This treatment is only effective in a short-time window of three hours after the stroke has occurred. Numerous clinical drug trials (over 150) have studied a variety of other treatments for stroke, involving ion channel blockers, anti-inflammatory drugs, glutamate antagonists, and free-radical scavengers (Cheng, Al-Khoury, & Zivin, 2004; Faden & Stoica, 2007; Ginsberg, 2007; Savitz, 2007; Savitz & Fisher, 2007; Young, Ali, Duretete, & Vivien, 2007). These clinical trials have all proven to be highly
disappointing. Because of the failed attempts to develop effective neuroprotective agents, patient care after stroke is generally geared toward various rehabilitation techniques that will aid the patient to live close to normal life as possible.

*Treatment Options: Traumatic Brain Injury:*

Treatment options for TBI are also scarce, with no clearly proven treatments available, and this reflects the very short time window where treatments could work to alleviate the injury and promote greater recovery. Little can be done to reverse the injury in TBI patients while medical personnel work to stabilize the patient from further damage. Sometimes, brain surgery is needed to treat contusions or hematomas (*Traumatic Brain Injury: Hope Through Research*, 2002). Rehabilitation currently is the most promising treatment for TBI. The NIH recommends that patients should be fitted with individualized programs to help rehabilitate them according to their strengths and weaknesses (*Traumatic Brain Injury: Hope Through Research*, 2002). Accordingly, scientists are working to develop treatments to solve the complex problem of understanding and treating traumatic brain injury.

*Nervous System and Injury:*

Because the nervous system is extremely complex, the effects and progression of brain injury are multifaceted. After injury, degenerative processes in the peripheral and
central nervous systems are similar in some ways, but inherent promoters of growth and environmental stimuli differ between the two systems (Fenrich, 2003; Liu, Cafferty, Budel, & Strittmatter, 2006). As a striking difference, the central nervous system (CNS) has a very limited capacity for recovery after injury compared to the peripheral nervous system, which is more regenerative and allows for more recovery.

In 1927, Santiago Ramon y Cajal noted that the CNS had a non-permissive nature, meaning various factors in the brain do not allow for new growth to happen after injury (Liu et al., 2006). In landmark studies, Aguayo and Richardson used a peripheral nerve graft to create a permissive environment that promoted axonal regeneration of damaged CNS neurons (Aguayo, David, & Bray, 1981; Richardson, McGuinness, & Aguayo, 1982). Later experiments discovered inhibitors in the myelin that collapsed growth cones and prevented neurite outgrowth in vitro (C. Bandtlow, Zachleder, & Schwab, 1990; Savio & Schwab, 1989) and in vivo (Schnell & Schwab, 1990). Other in vivo work found that regenerating neurons ceased activity when they encountered astrogliosis areas (Davies, Goucher, Doller, & Silver, 1999). Such experiments showed that inhibitory molecules are a good target for therapies to create a more permissive CNS environment and thus more recovery after injury.

Plasticity and regeneration are structural processes that may occur in the CNS and PNS to lead to recovery after injury. Regeneration in this context refers to regrowth of the
damaged axons after damage, and, even though this progression may occur, it does not mean full functional recovery. Plasticity, on the other hand, typically refers to the compensatory measures, such as remodeling, of the undamaged neurons in response to an injury (Kartje & Schwab, 2006).

Regeneration mainly occurs in the PNS, but may occur, although rarely, in the CNS. In order for regeneration to happen, the damaged neuron must somehow be rescued and restored. The process of Wallerian degeneration separates the damaged axon from the neuron, as well as primes the neuron for the possibility of a successful axonal regeneration (Fenrich, 2003). In the initial stages of axonal injury, calcium influx triggers axonal proteases that then help break down the axolemma and axoplasm (E. B. George, Glass, & Griffin, 1995; Schlaepfer & Bunge, 1973; Stoll, Griffin, Li, & Trapp, 1989; Stoll, Jander, & Myers, 2002). Next, proliferating Schwann cells use various mediators such as regeneration associated genes (RAGs), growth associated protein-43 (GAP-43), neurotropic factors, Schwann cell proliferative factor, neuregulin and erb receptors (Boyd & Gordon, 2003; Cohan, 1992; Fu & Gordon, 1997; Hall, 1999; LeBlanc & Poduslo, 1990) in its efforts to form bands of Bünger from the myelin debris (Fenrich, 2003). The bands of Bünger serve as a guide for axon regeneration from the proximal distal stump (Hirata & Kawabuchi, 2002; Liu et al., 2006; Stoll et al., 1989; Stoll et al., 2002). Macrophages are essential in the degeneration process, since they phagocytize the
damaged myelin (Bruck, 1997; Hirata & Kawabuchi, 2002). In the CNS and the PNS, axotomized neurons undergo chromatolysis (morphological changes occurring at the cell soma) to prime the area for regeneration to occur (Kreutzberg, 1996). These neurons also express proteins that aid the interaction between the Schwann cells and neurons to ensure regrowth.

CNS axons undergo the degeneration process described above but at a slower pace due to the limited phagocytic ability of resident microglia (R. George & Griffin, 1994; Rapalino et al., 1998). The presence of growth inhibitors such as Nogo-A decreases growth after injury (C. E. Bandtlow & Schwab, 2000; R. George & Griffin, 1994; Rapalino et al., 1998; Stoll & Muller, 1999). The oligodendrocytes do not dedifferentiate into growth supportive cells like the Schwann cells and therefore are not able to help aid in new growth (Fenrich, 2003). The regrowth of injured axons seen in the PNS is rarely seen in the CNS.

Obstacles to Regrowth in Central Nervous System:

There are many factors that provide obstacles in the path of neurite growth. Some molecular components that act as barriers to new growth include: chondroitin sulphate proteoglycans (CSPG), tenascin, semaphorins-3A (Davies et al., 1999; Letourneau, Condic, & Snow, 1994; Pasterkamp, Ruitenber, & Verhaagen, 1999), neurocan versican, phosphacan and NG2 (Liu et al., 2006). More specifically, Nogo-A, myelin-
associated glycoprotein (MAG) and oligodendrocyte glycoprotein (OMgp) are inhibitors located in the myelin of axons in the central nervous system (Schwab 2004). Neurite growth is hindered by this very potent growth inhibitor—Nogo-A. This inhibitor leads to growth cone collapse in neurites and therefore prevents regrowth in the central nervous system (C. E. Bandtlow & Schwab, 2000). Axonal regeneration was much improved in experiments with mice that do not express Nogo-A compared to wild-type controls (Kim, Li, GrandPre, Qiu, & Strittmatter, 2003; Simonen et al., 2003). The non-permissive nature of Nogo-A is also found in cell migration of fibroblasts as well (Buchli & Schwab, 2005; Caroni & Schwab, 1988).

**Nogo**

The *nogo* gene was initially discovered when investigators fractionated adult CNS myelin and found membrane- associated proteins, identified as NI-35/250, according to molecular weights determined by SDS-PAGE (Caroni & Schwab, 1988). Later, a short peptide sequence was characterized as a homolog of NI-35/250, named bovine bNI220 (Spillmann, Bandtlow, Lottspeich, Keller, & Schwab, 1998) and which possessed potent inhibitory characteristics. Further research determined that application of monoclonal antibody IN-1, specific to the amino region of Nogo-A, after spinal cord injury in rats promoted the regeneration of corticospinal tract axons (Bregman et al., 1995; Brosamle, Huber, Fiedler, Skerra, & Schwab, 2000; Schnell & Schwab, 1990). It was further
determined that IN-1 could neutralize the amino-terminal domain of Nogo-A (Fiedler, Horn, Bandtlow, Schwab, & Skerra, 2002) and thus prevent growth cone collapse in the CNS myelin (C. E. Bandtlow, Schmidt, Hassinger, Schwab, & Kater, 1993).

The \textit{nogo/RTN-4} gene produces three isoforms: Nogo-A, Nogo-B and Nogo-C. Nogo-A, found in oligodendrocytes, myelin, and neuronal populations, is a high molecular weight (200 kDa) protein that is involved in inhibiting neurite growth (Chen et al., 2000; GrandPre, Nakamura, Vartanian, & Strittmatter, 2000; Prinjha et al., 2000). Nogo-B (55 kDa), a splice form of Nogo-A, is expressed ubiquitously in many tissues and functions in vascular remodeling (Acevedo et al., 2004), apoptosis (Li et al., 2001), and modulation of vascular smooth muscles and migration of endothelial cells (Acevedo et al., 2004; Oertle & Schwab, 2003). Nogo-C (25 kDa) comes from the \textit{nogo} gene using an alternate promoter. The function of Nogo-C is not very well known, but it has been found to delay peripheral nerve regeneration in experiments \textit{in vivo} (Kim, Bonilla, Qiu, & Strittmatter, 2003). Nogo-C is expressed in differentiated muscle fibers, as well as Purkinje cells in the brain (Acevedo et al., 2004; Dupuis et al., 2002; Huber, Weinmann, Brosamle, Oertle, & Schwab, 2002; Hunt, Coffin, Prinjha, Campbell, & Anderson, 2003; Li et al., 2001; Tozaki, Kawasaki, Takagi, & Hirata, 2002).

All three Nogo isoforms are reticulon proteins, specifically in the reticulon-4 (RTN-4) family. A fairly short amino (N)-terminal sequence is common to many
reticulon proteins (Schwab, 2004). The reticulon proteins are evolutionarily very old. Nogo-A’s long N-terminal sequence developed later in evolution as a result of a fusion of old homology domains and new sequences, which thus led to a novel function (Oertle & Schwab, 2003; Oertle et al., 2003). The Nogo proteins, like most reticulon proteins, are located in the endoplasmic reticulum (ER) and are highly involved in ER processes, such as developing the shape of the ER (Caroni & Schwab, 1988; Chen et al., 2000; Dodd et al., 2005; GrandPre et al., 2000; Prinjha et al., 2000; Voeltz, Prinz, Shibata, Rist, & Rapoport, 2006). In reticulon proteins and more specifically Nogo-A, -B, and -C, there are 2 large hydrophobic domains near the C-terminus (Chen et al., 2000; GrandPre et al., 2000). Between the two domains lies the 66 amino acid loop, which the Nogo Receptor (NgR) subunit binds to (Fournier, GrandPre, & Strittmatter, 2001). This domain must be displayed on the extracellular face of the oligodendrocyte in order for the receptor on the surface of neurons or fibroblasts to be activated (Dodd et al., 2005).

**Nogo Receptor and Downstream Effects:**

NgR is a glycosyl phosphatidyl inositol (GPI) linked leucine-rich repeat glycoprotein (Barton et al., 2003; Fournier et al., 2001; He et al., 2003). There are currently three known isoforms of this receptor: NgR1, NgR2, and NgR3. NgR1 and 2 are involved in the growth inhibitory processes of the CNS (Fry, Ho, & David, 2007). NgR1 binds selectively to the OMP, MAG, and the Nogo-66 portion of Nogo-A, while
NgR2 only binds to MAG (Filbin, 2003; Venkatesh et al., 2005). NgR is a glycoprotein expressed in many neuronal populations. Due to expression of the Nogo-A protein on the cell surface and lack of an intracellular domain, the co-receptors p75 (Wang, Kim, Sivasankaran, Segal, & He, 2002; Wong et al., 2002), TROY (Park et al., 2005; Shao et al., 2005), and LINGO-1 (Mi et al., 2004) are required in order for intracellular signaling (Fournier et al., 2001).

TROY and p75, members of the tumor necrosis factor family, aid in signal transduction across the cell membrane, while LINGO-1 is an adaptor protein (Gonzenbach & Schwab, 2008). When the receptor complex is stimulated at the surface, a small intracellular GTPase, RhoA becomes activated and leads to actin depolymerization and growth cone collapse (Zhang, Zhang, Zhang, & Qin, 2008). Growth cone collapse occurs through the actions of proteins such as collapsin-1 and semaphoring III (D). These proteins collapse the lamellipodial and filopodial structures of growth cones of neurons and therefore inhibit axonal outgrowth. Another set of proteins, rho, and rac1 are modulators of this process (Jin & Strittmatter, 1997).

Recent evidence has suggested that the myelin inhibitory growth molecules on different populations of neurons may use different receptor systems (Giger et al., 2008). Paired immunoglobulin-like receptor B (PirB) is a high affinity receptor for MAG, OMgp and Nogo-A. This receptor can bind to the Nogo-66 region and thus lead to neurite
outgrowth (Atwal et al., 2008). In *in vivo and in vitro* experiments where NgR was genetically blocked and PirB was blocked by antibodies Atwal et al., (2008) showed that these two receptors may collaborate in the demanding process of growth cone collapse.

Nogo-A also has an Amino-Nogo region, which does not bind to NgR, and also leads to neurite outgrowth (Oertle et al., 2003). This region, independently from NgR, has been shown to prevent fibroblast activity and axonal regrowth *in vitro* (Fournier et al., 2001; Oertle et al., 2003). IN-1 has been shown to bind specifically to the amino region of Nogo-A and neutralize the protein (Fiedler et al., 2002). This region can also bind to integrins and block their downstream signal transduction (Hu & Strittmatter, 2008).

*Blockade of Nogo:*

Blockade of the Nogo-A receptor/protein complex and downstream intracellular signaling may lead to better recovery from injury in the CNS. One such method that has been developed is raising antibodies against Nogo-A, that then block its ability to inhibit neurite growth. This has been heavily studied in animal models of cortical injury, such as traumatic brain injury (TBI) and stroke. The inhibitory effect of IN-1 on Nogo-A is supported by *in vitro* assays (Caroni & Schwab, 1988) as well as spinal cord injury studies *in vivo* (Bregman et al., 1995; Schnell & Schwab, 1990; Thallmair et al., 1998; Z’Graggen, Metz, Kartje, Thallmair, & Schwab, 1998).
Nogo-A Immunotherapy:

Ischemic stroke in the sensorimotor cortex leads to loss of the ability for rats to perform functional tasks such as the skilled forelimb reaching task (Cheatwood et al., 2008; Papadopoulos et al., 2002). This is a very precise task that requires the animal to learn fine digit control and controlled arm movements. The corticospinal tract which arises from layer V pyramidal cells in the sensorimotor cortex is involved in this task, and, when it is damaged, the animal loses its ability to successfully complete the task (Whishaw, 2000). Anti-Nogo-A therapy has been used in experiments to try to rehabilitate rats undergoing middle cerebral artery occlusion (MCAO), an animal model of stroke. This therapy has been used to promote forelimb functional recovery after stroke. Investigations have discovered that the contralesional cortex plays an important role in sensorimotor recovery after injury (Cramer et al., 1997; Cuadrado et al., 1999). Intracerebral administration of the IN-1 antibody leads to improved performance at the skilled forelimb reaching task (Papadopoulos et al., 2002). Further investigations showed that the antibody therapy could be administered a week after MCAO and still promote functional recovery (Seymour et al., 2005). This is an important observation, because an effective treatment one week after ischemic insult extends the previous very short (3 hour) treatment period to a larger window of opportunity to be able to treat stroke.
Anti-Nogo-A immunotherapy has also been found to improve forelimb motor function when administered by the intrathecal route one week after MCAO (Tsai et al., 2007). A more clinically relevant model involving the neutralization of Nogo-A after MCAO in the aged rat found that the use of an anti-Nogo-A antibody (7B12) aided in functional recovery in the aged animal, although significant functional recovery was not seen until 9 weeks after stroke (Markus et al., 2005).

Anti-Nogo-A therapy promotes forelimb function in rats after a cerebral cortical lesion (Emerick & Kartje, 2004). This recovery correlated with the observed remodeling of motor cortical pathways. After administration of anti-Nogo-A mAb, new fibers have been seen to project from the contralesional brain regions to regions associated with the injured hemisphere (Cheatwood et al., 2008). The presence of IN-1 mAb in the lesioned dorsal corticospinal tract has led to regenerative sprouting in the damaged axons (Schwab, 2004). Animals treated with the IN-1 mAb a week after strokes were found to have significantly higher corticorubral fiber ratios as compared with their control counterparts (Seymour et al., 2005). The effective benefits of anti-Nogo-A treatments on cortical pathways and cortical plasticity after cortical aspiration lesions have been shown in multiple studies. Remodeling occurs in the corticorubral and corticopontine fibers in the adult brain after IN-1 treatment in rats receiving unilateral cortical lesions (Wenk, Thallmair, Kartje, & Schwab, 1999). Functional recovery in animals treated with IN-1
after unilateral pyramidal tract lesions was associated with new growth of corticopontine and corticorubral fibers which crossed the midline (Z’Graggen et al., 1998). IN-1 treatment led to structural plasticity after lesions in corticospinal tracts of animals, specifically new growth in intact and lesioned axons in the cervical spinal cord, red nucleus and pons (Thallmair et al., 1998).

Experiments using Nogo-A/B deficient aged mice found that functional outcome was impaired after cortical injury (Marklund et al., 2009). This suggests that Nogo-A may play a role in the development of the brain and the genetic deletion of Nogo-A may exacerbate injury. Marklund’s group also reported an improvement in cognitive function after treatment with anti-Nogo-A therapy after brain injury (Marklund et al., 2009).

Further immunotherapy treatment involved non-human primates subjected to a unilateral cervical lesion to the spinal cord. Using the modified Brinkman board task (a motor function task) the monkeys that received anti-Nogo-A therapy displayed significantly better performance than their control Ab counterparts. An increase in corticospinal axonal sprouting was seen in the area of the lesion in the animals receiving anti-Nogo-A therapy. Therefore, the enhanced sprouting after injury played a role in functional recovery after stroke (Freund et al., 2009).
**Dendritic Plasticity:**

Noticeable shortening of the basal dendrites of prelimbic pyramidal neurons in layers V/VI have been seen in animals after traumatic brain injury (Hoskison et al., 2009). Recovery after cortical damage may be due to the dendritic plasticity in the dendrites of neurons (Biernaskie, Chernenko, & Corbett, 2004; Jones & Schallert, 1994). Treatment of rats with IN-1 after stroke resulted in an increase in dendritic plasticity, specifically in layer V pyramidal neurons of the forelimb motor cortex of the contralesional cortex (Papadopoulos et al., 2006). This increase in dendritic and axonal plasticity may represent a compensatory measure by the brain in its attempt to regain functional use of the limbs.

**Environmental Enrichment:**

Environmental enrichment has long been studied in the laboratory for its effects on various diseases and to better understand its mechanisms. More specifically, it has been found that rodents living in environmental enrichment conditions experience considerable changes in the brain at the cellular and molecular levels (Bennett, Rosenzweig, Diamond, Morimoto, & Hebert, 1974; Rosenzweig & Bennett, 1996). Environmental enrichment allows animals to be housed in conditions that allow for social interactions as well as sensory and motor stimulation (Mora, Segovia, & del Arco, 2007; Rosenzweig & Bennett, 1996; van Praag, Kempermann, & Gage, 2000). There are
several categories of enrichment for laboratory rodents: structure and substrate, manipulanda, novel foods, social contact, and other types of enrichment which stimulate senses other than touch and taste (Hutchinson, Avery, & Vandewoude, 2005).

Environmental Enrichment and Brain Plasticity:

Plasticity of the brain can be impacted by enriched environmental conditions, as shown in numerous studies. Although, there is no published standard of ideal environmental enrichment conditions, a general idea of what is appropriate has developed. Three experimental housing conditions are mentioned consistently across the literature—standard conditions, environmental conditions, and impoverished conditions. Standard conditions refer to typical laboratory rodent environment, in which 2-6 rats are housed per cage, without any play objects. The enriched environment housing has larger groups of animals, as well as larger cages filled with objects for stimulation and play. Lastly, the impoverished condition is when animals are singly housed in small cages without any objects (Will, Galani, Kelche, & Rosenzweig, 2004).

In 1874, Charles Darwin noted that wild rabbits had increased brain sizes, compared to their domestic counterparts (Mohammed et al., 2002). In the 1960s, the first controlled experiments that subjected animals to environmental enrichment were conducted (Diamond, 2001). In the wild-type rodent, enriched environment was shown to enhance dendritic arborization, alter spine morphology, increase neurogenesis in the hippocampus,
increase neurotrophin levels, induces changes in NMDA and AMPA expression, as well as enhance learning and memory. In rodents with ischemic stroke, environmental enrichment enhances functional motor recovery, attenuates learning/memory deficits, increases dendritic arborization, increases levels of neurotropic factors, normalizes deficits seen in cell proliferation in subventricular zone and in glucocorticoid and mineralcorticoid receptors, as well as others (Nithianantharajah & Hannan, 2006). It also has been found that aged animals also reap the beneficial effects from the enriched environment. Environmental enrichment was found to lead to better functional motor recovery in the skilled forelimb reaching task in rats after cerebral ischemia (Knieling, Metz, Antonow-Schlorke, & Witte, 2009). Taking all these observations together, I hypothesize that placing aged animals in enriched environment after ischemic stroke, and giving them Nogo-A immunotherapy will lead to better functional recovery on the behavioral, cellular and molecular levels.
CHAPTER 2
HYPOTHESIS AND SPECIFIC AIMS

_Hypothesis:_ Anti-Nogo-A immunotherapy facilitates environmental enrichment mediated functional recovery and neuronal plasticity in aged rats receiving focal ischemic stroke.

_Specific Aim 1:_ Determine if Anti-Nogo-A immunotherapy added to environmental enrichment will improve performance in the skilled forelimb reaching task or ladder rung walking test in aged (20 months) Fisher 344 rats after stroke.

_Specific Aim 2:_ Determine if Anti-Nogo-A immunotherapy added to environmental enrichment will increase dendritic plasticity in the contra-lesional forelimb area of the motor cortex in aged (20 months) Fisher 344 rats after stroke.
CHAPTER 3
EXPERIMENTAL DESIGN AND METHODS

A. Animals

a. Control Animals

Two aged (24 months) naïve Fisher 344 rats were designated as normal aged controls in this study. They were trained on the skilled forelimb reaching task and the skilled ladder rung walking test. They did not receive any surgeries or any other treatments. They were sacrificed after 4 weeks and their brains were processed by the Golgi-Cox method to determine dendritic morphology.

b. MCAO Experiments

Ten aged male Fisher 344 rats at 18 months upon arrival were used in this study. They received ad libitum water and standard rat food and were maintained on a 12 hour light-dark cycle. Because of the fragile nature and mortality of the aged rats, we completed the experiment with two rats in the control group receiving control antibody and four rats receiving anti-Nogo-A antibody (11c7) in the experimental group. Four animals (23 months of age; two control antibody and two 11c7 antibody animals) were perfused for Golgi analysis. Figure 1 shows the experimental design for the MCAO experiments.
c. **TBI Experiments**

The TBI experiments were designed and conducted by Ian Vaagenes. Twenty aged (18 months) male Fisher 344 rats were used in this set of experiments. The animals received *ad libitum* water and standard rat food and were maintained on a 12 hour light-dark cycle. Because of high mortality, only four animals in the control IgG antibody group and five animals in the anti-Nogo-A antibody group survived. Six animals (three in the control antibody group and three in the 11c7 antibody group) were sacrificed at 24 months of age for Golgi analysis. Figure 2 shows the experimental design for the TBI experiments.

B. **Housing**

a. **MCAO Experiments**

In the training period before the animals received the stroke surgery and treatments, animals were housed in standard lab cages, two per cage.

After the stroke surgery and surgeries for the antibody pumps, all surviving animals were placed in large plexiglass cages (81.5 cm X 61 cm X 45 cm) outfitted with various objects for exploration (Figure 3). These objects were distributed in the cage on a weekly rotation (Week A and Week B). Week A objects were: Bundle of Twig Nibblers, Nature Ball and Bag ‘o’ Chews. Week B objects were: Nylabone, Build ‘n’ Bites, and a Bag of Chew Toys. Toys were
placed in novel areas every week to encourage exploration. Also due to the fragile nature of the animals and weight loss seen in the animals, fruit loops, and mashed rat food were introduced to the cage as well. These food items were only supplied when the colony seemed sickly. We provided the food to promote stable weights in the animals, but the food could also be seen as a form of environmental enrichment because it added a novel stimulus to their surroundings.

b.  **TBI Experiments**

TBI animals were housed in standard lab cages, two per cage throughout the duration of the study.

C.  **Behavioral Tasks**

a.  **Skilled Forelimb Reaching Task**

Upon arrival, all animals were trained everyday on this task, which measures fine digit motor control and coordination of the forelimbs. Animals were trained in a clear plexiglass box (30 X 36 X 30 cm) with a rectangular window (1.5 X 3 cm) open at the base of the floor (Markus et al., 2005; Papadopoulos et al., 2002; Seymour et al., 2005; Tsai et al., 2007) (Figure 4A). To promote forelimb reaching, small sucrose pellets (45 mg; Bilaney Consultants, NJ) were placed on a platform outside of the window of the box.

When the initial training period began, food restriction was initiated and the animal’s weight was not allowed to drop below 95% of their baseline body
Animals were trained in this task for 6-7 weeks before receiving MCAO or TBI. Animals were required to reach a baseline (of 15/20 pellets in less than five minutes) to be considered for this study. Animals were tested in the task five days after MCAO or TBI surgeries, as well as five days after the antibody pump was inserted and again five days after it was removed. After all surgeries, animals were tested everyday (Monday-Friday) for the duration of the study. The TBI animals were tested for 13 weeks following surgery, while the MCAO animals were tested for 9 weeks after surgery. Animals were video taped once a week for analysis.

b. Skilled Ladder Rung Walking Test

Animals were familiarized on the ladder rung test, twice a week, two weeks before MCAO or TBI. This test consists of an animal walking across a narrow 1 meter long Plexiglass frame with bars placed every 2.5 cm as described in previous work (Emerick & Kartje, 2004; Metz & Whishaw, 2002)(Figure 4B). The animals were encouraged to walk across by placing a tray of sucrose pellets on the opposite end of the walk-way. The animals tended to walk toward the tray of sucrose pellets. This activity is not forced.
This test is designed to measure foot slips and this test evaluates sensory and motor function. The test sessions included three runs on the apparatuses and were video-taped for analysis. Before the MCAO or TBI surgeries, animals were video-taped in a baseline test session. After the MCAO or TBI surgeries, animals were video-taped once a week for the duration of the experiments. The TBI animals were tested for 13 weeks after injury and the MCAO animals were tested for 9 weeks.

D. Middle Cerebral Artery Occlusion (MCAO)

All animals underwent a middle cerebral artery occlusion (MCAO) at the culmination of the behavioral training (Markus et al., 2005; Papadopoulos et al., 2002; Papadopoulos et al., 2006; Seymour et al., 2005; Tsai et al., 2007). Isoflurane (inhalant 5% with oxygen) was used to initially anesthetize the animals for the procedure. For the duration of the procedure, animals received Isoflurane (3% with oxygen) to maintain the anesthetized state. First, the animal’s head and neck were shaved and cleaned with iodine. The animal then was placed in a stereotaxic instrument, and a 2 centimeter vertical incision was made on the head, between the eye and the ear. The temporalis muscle was pulled back to expose the skull. A burr hole to the skull, specifically the parietal bone of the skull, exposed the parietal cortex. The middle cerebral artery (MCA) was visualized where it transverses the rhinal sulcus. The MCA was permanently ligated with a 10-0 monofilament suture. The MCA was also transected above the suture with
microscissors. The occlusion of the MCA was done on the side opposite to the preferred forelimb (i.e. right hand; left MCAO). The temporalis muscle was sutured with a 4-0 absorbable suture, and the skin was closed with 4-0 monofilament nylon sutures.

After the MCAO, the animal was placed in the supine position. A midline longitudinal incision was made on the animal’s neck to expose the common carotid artery on both sides (CCA). The CCA were freed carefully from the vagus nerve and surrounding tissues. The CCA ipsilateral to the side of the MCAO was permanently ligated with a 4-0 silk suture. The contralateral CCA was occluded for thirty minutes by the use of an aneurysm clip in some animals. The temporary occlusion was eliminated from the procedure after mortality in several animals in the surgeries to allow a better survival rate in the animals. After the CCA’s were occluded and ligated, the incision was closed with 4-0 monofilament sutures.

Animals were placed on a warm water circulating heating pad covered with a towel. They also received a subcutaneous injection of buprenorphine (0.1 mg/kg) upon awakening for pain management and then put back into their home cages. The animals received injections of buprenorphine (0.1 mg/kg) twice a day for three days after the surgery for proper pain management. The sutures to the skin were removed 14 days after surgery.
E. Traumatic Brain Injury (TBI)

All animals in this study underwent a traumatic brain injury (TBI) at the culmination of the behavioral training (Miller et al., 2001). Isoflurane (inhalant 5% with oxygen) was initially used to anesthetize the animals for the procedure. For the duration of the procedure, animals received Isoflurane (3% with oxygen) to maintain the anesthetized state. First, the animal’s head and neck were shaved and cleaned with iodine. Then the animal was placed in a stereotaxic instrument. A midline incision to the head exposed the skull.

A craniotomy of 4 mm was performed at position 0.5 mm anterior and 4 mm lateral to the bregma. This is estimated to be directly over the forelimb sensorimotor cortex. A controlled cortical impact injury device was used to deliver the traumatic brain injury. The device consists of a small bore, double acting, pneumatic piston cylinder with a 40 mm stroke mounted on a stereotaxic micro-manipulator. The accurate determination of the piston coordinates is performed by referencing a sharp concentric tip on bregma and moving it to the previously mentioned coordinates. The concentric tip is then replaced by the impactor tip (3 mm diameter). The pneumatic piston cylinder is angled 22.0° away from vertical so that the flat impactor tip is perpendicular to the surface of the brain. The impactor tip strikes the brain at 1.7 m/sec to a depth of 1.7 mm below the cortical surface for 250 msec. After impact, the scalp was closed with a
4-0 monofilament nylon suture. Post-operative procedures were similar to those used after MCAO surgery.

F. Rehabilitation Methods

a. Nogo-A Immunotherapy

One week after MCAO and TBI surgeries Alzet mini-osmotic pumps, (Durect Corporation, Cupertino, CA) were implanted in the animals to promote recovery in the injured brain as described in previous work (Gillani et al., 2009; Markus et al., 2005; Papadopoulos et al., 2002; Papadopoulos et al., 2006; Papadopoulos et al., 2009; Ramic et al., 2006; Seymour et al., 2005; Tsai et al., 2007). Animals were anesthetized for the implantation procedure with isoflurane, as described above. The head and back of the animal were shaven, cleaned with iodine, and then the animal was placed in the stereotaxic instrument.

A small incision was made in the midscapular area where a subcutaneous pocket was surgically created with blunt scissors. This pocket housed the osmotic pump. The osmotic pump was placed in the pocket, and the catheter was led subcutaneously to the cranial site for cannula placement. A mid-line scalp incision was made to expose the skull, and one burr hole was made on the same side of the skull (ipsilateral to the MCAO) to receive the cannula. The cannula was placed at coordinates: lateral 1.3 mm; posterior 0.8; and dorsoventral 3.8, relative to the bregma cranial landmark. The external portion of the cannula was secured to the
skull using Cryanoacrylate Gel (recommended by Alzet). The scalp was then closed over the cannula apparatus with 4-0 monofilament nylon sutures. The incision made to house the pump in the back was sutured with 4-0 monofilament nylon sutures. After surgery, the incisions were closed up and animals received post-operative care as described above.

These pumps provided an intracerebroventricular (icv) administration of either purified anti-Nogo-A Ab 11c7 (monoclonal mouse IgG), or a purified control mouse IgG Ab. The pumps infused 5 mg of the Ab (2.5 mg/ml) at the rate of 15 µg/hour to the affected area for two weeks (Markus et al., 2005).

After two weeks of this antibody therapy, the pumps were removed. The rats were anesthetized and prepared for surgery as described above. Two incisions were made just large enough to remove the pump, cannula, and all tubing. After the items were removed, the incisions were closed up with 4-0 monofilament sutures. Post-operative procedures are similar to those described above.

b. **Focused Activity Sessions**

After the completion of all surgical procedures, only the MCAO animals participated the in focused activity sessions, which were geared toward forcing the animals to use and rehabilitate the affected limbs. The focused activity consists of a playground with three apparatuses that encouraged animals to use their forelimbs. The three apparatuses were located in a 5’X5’ enclosed space
equipped with a 45 degree inclined ladder (200 cm X 5 cm), vertical rope (100 cm), and a vertical cylindrical grid (100 cm X 10 cm; 1-cm$^2$) (Papadopoulos et al., 2009). Figure five shows an illustration of our focused activity playgrounds.

These sessions were held twice a day for four weeks and then only held once a day for the duration of the study. Sessions that were held twice daily were done in the morning and afternoon. Sessions held once a day were done in the afternoon. All sessions were done after behavioral testing (skilled forelimb reaching task and skilled ladder rung walking test) to ensure maximum performance on the behavioral tests. At first, animals were doing twenty minute sessions but were subsequently reduced to ten minutes per session, as the animals appeared to tire with 20 minute sessions. Every week, one minute was added to the session time, to promote endurance and rehabilitation. Animals were rotated on the three apparatuses at one minute intervals for the time allotted for the session. Animals were not allowed to be idle in the activities but were encouraged to keep exploring and moving around.

G. Golgi Analysis

Four MCAO animals and six TBI were sacrificed for Golgi analysis. All animals were 21-24 months of age when sacrificed. The animals were overdosed with sodium pentobarbital (100 mg/kg, i.P.) and transcardially perfused with 0.9% heparinized saline. The whole brains were dissected out and placed in
Golgi-Cox solution (Glaser and van der Loos, 1981). The brains were left in the solution for 14 days and then in 30% sucrose for 1-2 days. The brains were cut on a vibratome into 200 μm coronal sections and placed on frosted, pre-cleaned microscope slides.

Brains were reacted using a procedure described by Gibb and Kolb (1998). Slides with brain sections were placed in a slide rack and then placed in a tray containing ammonium hydroxide solution for 30 minutes. They were washed in distilled water for a minute and then transferred to a tray with Kodak Fix (Eastman Kodak Company) for 30 minutes. After a one minute wash in distilled water, the slides were placed in 50%, 70%, and 95% ethanol for 1 minute each. Then the slides were placed in two different 100% ethanol trays for two five minute sessions. After the ethanol washes, the slides are then transferred into a tray that contains equal parts of 100% ethanol, chloroform (≥ 99%) and Citrisolv (Fisherbrand) for 10 minutes. Then, the slides are transferred into a tray containing Citrisolv for 15 minutes. This is done twice, using a clean tray filled with Citrisolv each time. In the process, several things are important to ensure a good reaction. One is that slide racks should be blotted with Kimwipes (Kimtech) before each transfer. Also, before each session, the slide rack is dipped up and down quickly several times in the solution and then placed in the tray for the time allotted for that session. After the reaction, the bottoms of the slides are blotted with Kimwipes and the tops are blotted with Bibulous Paper (Scientific Products),
and then the slides are coverslipped using Permount (Fischer Scientific). Slides must be stored on a flat surface in a dark space for 3-4 days to allow for the tissue to be mounted properly.

The slides were then viewed with a Leica DM 4000 B light microscope outfitted with a CX9000 camera (MicroBrightfield Inc.). Pyramidal neurons were selected from cortical layer V in the forelimb area (S1FL) of the brain, in accordance to measurements seen in the Rat Brain Atlas (Paxinos and Watson; 4th Edition, 1998). According to the atlas, area S1FL is found in the range of 1.20 mm to 2.12 mm from the bregma. Other criteria for selecting a neuron for analysis included: a tear drop cell body, strong apical dendrite and good branching seen apically and basally. Eight neurons per brain were traced and analyzed using the Neurolucida (Microbrightfield Inc.) program. The neurons were selected on the side opposite of the lesion in both the MCAO and TBI brains to determine dendritic plasticity that may have occurred. The data from this technique was further analyzed statistically and graphically. In our analysis we looked at the apical and basal dendritic lengths and the number of branch segments. In the normal aged control group, both hemispheres were examined, and therefore 16 neurons per brain were traced.
Lesion analysis was done on the brains that were processed for the Golgi technique. The stroke volume was quantitatively analyzed using the Scion Image 1.63 Alias software. The software allowed us to trace lesions and calculate the surface area of the lesion. Stroke size was calculated by subtracting the area of the lesioned size from the non-lesioned side, dividing it by the non-lesioned side and multiplying it by 100 (Papadopoulos et al., 2002).

In analyzing the behavioral tasks (skilled forelimb reaching task and skilled ladder rung walking test), we used GraphPad Prism 4 to perform a repeated measures ANOVA for comparison of control Ab/MCAO & control Ab/TBI, 11c7 Ab/MCAO & 11c7 Ab/TBI, 11c7 Ab/MCAO & control Ab/MCAO, and 11c7 Ab/MCAO & control Ab/TBI. For analysis of the Golgi-Cox results, we used SigmaStat to perform a T-Test on the following groups: 11c7 Ab/MCAO & 11c7 Ab/TBI, control Ab/MCAO & control Ab/TBI, 11c7 Ab/TBI & control Ab TBI, and 11c7 Ab/MCAO & control Ab/MCAO. For the lesion analysis, we used GraphPad to perform a T-Test on the following groups: 11c7 Ab/TBI and control Ab/TBI. P values less than 0.05 were considered significant.
Table 1: **Animal Groups**: Shows the group sizes for the various studies in our experimental design. (N is group size).

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial N</th>
<th>N for Behavioral Studies</th>
<th>N for Golgi Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAO</td>
<td>12</td>
<td>2</td>
<td>Control Ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
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<td></td>
<td></td>
<td>4</td>
<td>11c7 Ab</td>
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<td></td>
<td></td>
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<td>2</td>
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<tr>
<td>TBI</td>
<td>20</td>
<td>4</td>
<td>Control Ab</td>
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<tr>
<td></td>
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<td>5</td>
<td>11c7 Ab</td>
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</table>
Figure 1: **Time-line for MCAO Experiments.** Abbreviations are as follows: FL (forelimb reaching task), BW (skilled ladder rung walking test), Ab Rx (antibody treatment), EE (environmental enrichment). Animals were 23 months of age at the time of perfusion.
Figure 2: **Time-line for TBI experiments:** (as designed by Ian Vaagenes).

Abbreviations are as follows: FL (forelimb reaching task), BW (skilled ladder rung walking test), Ab Rx (antibody treatment). Animals were 23 months of age at the time of perfusion.
Figure 3: **Enriched housing**: in which the MCAO rats lived in after surgeries. Toys, cat scratcher, igloos and food-bowls provided as form of enrichment.
Figure 4: **Behavioral Tasks:** (A) F344 Rat, 5 days post-MCAO attempting the Skilled Forelimb Reaching Task. (B) Same rat, 5 days post-MCAO attempting the Skilled Ladder Rung Walking Test. The foot-slip seen in the image denotes a deficit in the animal after MCAO.
Figure 5: **Focused activity playground**: in which the MCAO rats did daily rehabilitation exercises after surgeries. Apparatuses, in an enclosed space, on the playground include: ladders, wire mesh grid and vertical rope ladder.
CHAPTER 4
RESULTS

A. Animals/Housing

This study proved to be very challenging because of the fragile nature of the Fisher 344 rats. In the first couple of MCAO surgeries, the animals died due to surgical complications. Therefore, the MCAO procedure was amended to promote survival in the remaining animals. Instead of occluding and ligating both of the CCA, and prolonging the surgery, we decided to permanently ligate the CCA ipsilateral to the lesioned side. Of the ten animals in the MCAO group, only 6 survived stroke surgeries. In view of our increasing awareness of the fragility of these animals, we constantly checked their weights and looked for other signs of declining health. Of the 20 animals in the TBI group, 9 animals survived the TBI surgeries.

B. Lesion Analysis

Analysis of the stroke lesions of the MCAO/11c7 Ab showed a mean size of 14.15%. The mean size of the stroke lesions in the MCAO/control Ab animals was 7.31%. In the TBI/11c7 animals, the mean size of the cortical lesion was 3.42%. The TBI/Control Ab animals showed a mean size of the cortical lesion to
be 6.61%. Figure 13 shows the stroke volume of all groups. The size of the lesion did not differ between the TBI/11c7 Ab and TBI/control Ab groups (p= 0.3985, T-Test).

C. Behavioral Tasks

a. Skilled Forelimb Reaching Task

This task compared four groups of animals: MCAO/11c7 Ab (N=4), MCAO/Control Ab (N=2), TBI/11c7 Ab (N=4) and TBI/Control Ab (N=4). All animals experienced a significant deficit after the MCAO and TBI surgeries (Figure 6).

As seen in figure 6, the TBI animals appeared to recover better over time in this task as compared to the MCAO animals, but no statistical significance was observed. In the TBI group, the animals receiving 11c7 Ab tended toward better performance over time, as compared to the control Ab animals. Also, the TBI animal that received 11c7 Ab nearly recovered back to baseline levels.

The MCAO group of animals seemed to perform about the same over time regardless of what treatment they received. It is important to note that none of the stroke animals recovered back to their baseline level at the time point of 7 weeks after MCAO surgery. This was also true for the TBI animals at the time point of 10 weeks after TBI surgery. Both groups of animals did, however, recover from a profound deficit of in reaching performance (0 pellets out of 20), to a decent score (11-15 pellets out of 20 in TBI; 7-8 pellets out of 20 in MCAO). In comparing the
11c7 Ab/MCAO animals to the control Ab/MCAO animals, there is almost a significant difference between the groups (P= 0.055).

b. **Skilled Ladder Rung Walking Test**

In this test, we compared four groups: the 11c7 antibody/MCAO group, the control antibody/MCAO group, the 11c7 antibody/TBI group and the control antibody/TBI group (Figure 7).

At baseline, the MCAO animals had an average of about 0.6 foot-slips per 10 steps. Five days after the MCAO, this increased to about 0.8 slips per 10 steps in the control antibody animal group and about 1.3 slips per ten steps in the 11c7 antibody group. Preceding the stroke surgery, there was no statistically significant difference between the two groups of animals. A decrease in the number of slips over time was observed after MCAO, but this did not return to baseline levels. Also, the behavior of these animals was notable. After stroke, the animals had a very crouched posture and walked across the bars very gently as if they were afraid to slip. They seemed to put more weight on the non-affected side, as well as on the hind paws. This led to more slips in the hind paws. Over time, the animals stopped this compensatory posture and developed a more normal posture for crossing the bars.

In the TBI group, the baseline average was about 2 foot-slips per 10 steps in both groups. After the TBI surgery, this rate rose to an average of about 5 foot-slips per 10 steps in both groups. After the TBI surgery, there was little difference
seen in the performance of the 11c7 Ab group as compared to the control Ab group over time. A general decrease of foot-slips was observed in both groups over time that returns fairly close to baseline levels. Statistics were not done at this time due to low numbers in groups as well as incomplete data.

D. Rehabilitation

c. Immunotherapy

In the MCAO experiments, a total of six animals received immunotherapy. They all survived the surgeries, except for one who died two days after mini-pump removal. This animal did receive the 11c7 antibody. Of the remaining five animals, 3 animals received the 11c7 antibody and 2 animals received the control antibody.

In the TBI experiments, a total of nine animals received antibody treatment. Four animals received the control antibody, while five animals received the control antibody treatment. Eleven animals in the original group of 20 died before the pump surgeries.

d. Environmental Enrichment

Animals were placed in environmental enrichment conditions (focused activity and enriched housing) after they received immunotherapy treatments. Initially, they received 20 minute sessions on the focused activity playground, twice a day. This was following the behavioral tasks. It was quickly noted, after a week of rehabilitation, that 40 minutes per day of rehabilitation appeared too
stressful for the animals. The animals were falling off the different apparatuses and appeared exhausted. The following week, the sessions were reduced to 10 minutes each, for a total of 20 minutes per day. One minute was added to the total time each week to push for greater functional recovery. (i.e. week two was 11 minutes, week three was increased to 12 minutes). The animals performed two sessions a day for four weeks and then one session a day for the remaining five weeks. At first the animals were inactive on the playgrounds, but over time they learned how to interact with the equipment and started exploring more. They also developed better climbing skills on the rope and vertical wire mesh. These changes could be attributed to functional recovery of the limbs. There was no apparent difference between the two groups as far as their activity on the playground. When animals appeared sick, their activity was greatly hampered.

As for the housing conditions, the animals spent most of their time in a plastic igloo, sleeping. When they were first placed in the enriched housing, they did not explore as much. Over time, the animals explored more and sought out the objects placed in their cages. The toys that the animals liked best were: twigs, bag ‘o’ chews, build ‘n’ bites, and the bag of wooden chew toys. Affinity was measured through daily observation of which toys the animals moved around, and by the condition of the toys when the week was over. The best liked toys were consumed or gnawed on. There are four different levels in the housing that the animal could explore. Generally, the animals stayed on the first two levels. Where the animals had been was also noted by whether animals had moved the toys
around in that location, as well as general observations made throughout the week of their whereabouts. The animals were more active at night, which fits with their nocturnal nature.

E. Golgi Analysis

Six TBI brains, two naïve brains and four MCAO brains were analyzed via the Golgi-Cox method. Layer V pyramidal neurons in the forelimb area of the sensorimotor cortex were analyzed. The basal dendrites and the apical dendrites were analyzed for the following: individual length of branches, individual branch segments, total lengths, and total branch segments. The MCAO animals show increased numbers in the total number of branch segments, while the TBI animals show decreased numbers, compared to the normal aged animals. However, only a few comparisons between groups were significant. In comparing the 11c7 Ab/MCAO group to the 11c7 Ab/TBI group, we get a P value of 0.017 in the apical branches (Figure 8) and a P value of 0.018 in the basal branches (Figure 9). This shows that there is a significant difference in the branch segments of dendrites in animals with two different types of lesions, and the same type of immunotherapy. Figure 10 shows the total dendritic length for the apical dendrites across groups. Figure 11 shows the total dendritic length for the basal dendrites across groups. Both the MCAO animals and TBI animals show decreased numbers in the total dendritic length as compared to the normal aged animals. Statistically, the 11c7 Ab/MCAO group compared to the 11c7 Ab/TBI group was statistically significant for both the apical (P value = 0.045) and basal dendrites (P
value = 0.058). Anti-Nogo-A immunotherapy seems to have a different effect on the dendritic length of neurons in brains affected by different lesions. Figure 12 shows representative tracings for the following groups: 11c7 Ab/MCAO, 11c7 Ab/TBI, control Ab/MCAO, control Ab/TBI, and normal adult control.
Figure 6: Forelimb reaching scores over time. The data shows the number of successful reaches out of twenty pellets. Brain injury refers to either TBI or MCAO. Error bars denote standard error of the mean. Mixed ANOVA was done to determine statistics but no significance was found at this time. The following abbreviations used are: Ab Rx (antibody treatment), Ab (antibody), TBI (traumatic brain injury), MCAO (middle cerebral artery occlusion).
Figure 7: **Skilled Ladder Rung Walking Test**: Data measures the number of slips out of every ten steps that the animal takes across the ladder. Brain injury refers to either TBI or MCAO. Statistics were not done at this time. Abbreviations are as follows: Ab (antibody), Ab Rx (antibody treatment), TBI (traumatic brain injury), MCAO (middle cerebral artery occlusion).
Figure 8: **Golgi-Cox analysis of Total Branch Segments in the Apical Dendrites of the animals.** Brain injury designates either MCAO or TBI. Error bars denote the standard error of the mean. *P<0.05 for 11c7 treated MCAO and TBI groups (T-Test P values reported).
Figure 9: **Golgi-Cox analysis of Total Branch Segments in the Basal Dendrites of the animals.** Brain injury refers to either MCAO or TBI. Error bars denote the standard error of the mean. *P<0.05 for 11c7 treated MCAO and TBI groups (T-Test P values reported).
Figure 10: Golgi-Cox analysis of total Dendritic Lengths of the Apical Dendritic trees of the animals. Brain injury refers to either MCAO or TBI. Error bars denote the standard error of the mean. *P<0.05 for 11c7 treated MCAO and TBI groups (T-Test P values reported).
Figure 11: **Golgi-Cox analysis of total Dendritic Lengths of the Basal Dendritic Trees of the animals.** Brain injury refers to either TBI or MCAO. Error bars denote the standard error of the mean. *P<0.05 for 11c7 treated MCAO and TBI groups (T-Test P values reported).
Figure 12: **Box-plots comparing Golgi staining of MCAO group (n= 4 animals; n= 32 neurons) to TBI group (n=6 animals; n= 48 neurons).** The neurons in the MCAO group have significantly improved dendritic plasticity in both the apical and basal dendrites, as well as in both the branch segments and the dendritic length.
Figure 13: **Representative Golgi-Cox stained layer V pyramidal neurons from the FL area in the motor cortex.** Drawings are from Neurolucidia program. All neurons were traced at 10x magnification under a light microscope. (A) Representative neuron from the normal aged animal. (N = 32 neurons) (B) Representative neuron from MCAO/11c7 animal. (N= 32 neurons) (C) Representative neuron from a MCAO/control animal. (N= 16 neurons) (D) Representative neuron from TBI/11c7 animal. (N= 24 neurons) (E) Representative neuron from a TBI/control animal. (N= 24 neurons)
Lesion analysis of the TBI and MCAO brains after injury. Shows the lesion size, which is represented as a percent of the intact hemisphere. There was no significant difference between the two TBI groups (p= 0.3985, T-Test). Error bars denote the standard error of the mean. Statistics was not done in the MCAO groups because of low N (11c7 Ab had N=1 and Control Ab had N=2).
Aged Brain and Injury

Perhaps the most significant problem in studying cortical injury in the aged brain is how to effectively stimulate plasticity in order to promote functional recovery. The complexity of the aged brain is multiplied when considering that the elderly often also suffer from a vast amount of interconnected co-morbidities associated with aging. Sometimes, it is impossible to separate aged-related diseases such as adult-onset diabetes and stroke because they are so intertwined. A key question is how to manipulate the injured aged brain in such a way to allow for functional recovery.

The aged population is known to recover poorly from brain injury (Lindner, Gribkoff, Donlan, & Jones, 2003; Rosen et al., 2005) and also suffers from greater disabilities after stroke as compared to younger populations (Kelly-Hayes et al., 2003). A variety of therapies have been suggested for the two types of cortical injury discussed in this paper—ischemic stroke and TBI. The lack of available clinical drugs and therapies approved and on the market shows just how complex brain injury is and that we have a long way to go to understand how to treat the complex processes in brain injury.
Age and Stroke

The risk of acquiring ischemic stroke increases with age (Lloyd-Jones et al., 2009). The brain undergoes many changes with increasing age that make it more susceptible to stroke. Studies in the aged rat induced with ischemic stroke have revealed factors such as increased cardiac arrhythmias and myocardial insults (Rosen et al., 2005), lack of autonomic influences on cerebral blood flow (Hachinski, Oppenheimer, Wilson, Guiraudon, & Cechetto, 1992), increase in proliferating and apoptotic cells (Popa-Wagner et al., 2007), and excessive release of glutamate leading to nerve damage (Rothman, 1984). It is important to take into account all the various factors that are involved in stroke in the aged when studying the course of the disease and treatment.

Age and Traumatic Brain Injury:

Aged individuals recover very poorly after TBI and are likely to suffer from increased rates of TBI due to falls ("Rates of Hospitalization Related to Traumatic Brain Injury--Nine States, 2003," 2006). Perhaps the biggest obstacle to recovery in patients with traumatic brain injury is that axonal regeneration is inhibited in the CNS. This inhibition is prevalent across all age groups but is especially present in the aged brain. The elderly brain is not as plastic as it is at a younger age and thus causes recovery from TBI to be slow and often incomplete (Marklund et al., 2009).
Brain Injury Treatments:

Treatment options for ischemic stroke are quite scarce and most treatment is directed toward general rehabilitation after injury. Many clinical trials and experiments have targeted various factors and processes involved in cerebrovascular disease and have generally failed for several reasons. Treatment for TBI also is very hard to treat clinically and most treatment is geared toward alleviating the symptoms. As the world-wide elderly population increases, the need for more effective treatment is needed.

General conclusions-- MCAO experiments:

The results of the present study show that immunotherapy combined with environmental enrichment does not significantly improve functional recovery nine weeks after MCAO. Also, these treatments did not have a significant effect on dendritic plasticity in layer V pyramidal neurons in the sensorimotor cortex. However, the total branch segments showed a slight increase in the 11c7 Ab and control Ab groups compared to the normal aged animals, but no difference was seen between the antibody groups. Furthermore, our findings suggest that the anti-Nogo-A antibody did not have an effect on the dendritic plasticity of the MCAO animals. Possibly the increase in dendritic plasticity seen in the antibody groups could be attributed to the environmental enrichment therapy. Perhaps environmental enrichment is acting to spare the dendrites after MCAO. Compared to normal aged controls for total dendritic length, there is an overall slight decrease in dendritic plasticity as compared to the normal aged control.
Overall, the little difference seen in the animals who received the control antibody was compared to the animals that received the Anti-Nogo-A antibody across all experiments in this study. The results are not significant, probably due to the low number of animals in each treatment group. Therefore, we cannot conclude that environmental enrichment alongside immunotherapy does not lead to functional recovery or an increase in dendritic plasticity in the aged rat after MCAO. With a larger group size, an increase (or decrease) in both functional recovery and dendritic plasticity is a possible outcome in future experiments. Also, a post lesion period beyond nine weeks, could provide more significant results.

*Nogo-A immunotherapy and MCAO:*

Previous work from our lab showed that aged rats demonstrate recovery in the skilled reaching task after 9 weeks. A significant difference was seen in the animals receiving control antibody versus the anti-Nogo-A antibody (Markus et al., 2005). Parallels can be drawn to previous work and the current experiments, but one must consider the differences in the studies. The Markus study used Long Evans black-hooded rats (Markus et al., 2005), while our study used Fisher 344 rats. It is possible that different rat strains can lead to different results. This may be comparable to humans, whereby each elderly stroke patient has a different pattern of stroke and recovery (Gillani et al., 2009). Also, the previous study used the 7B12 anti-Nogo-A antibody (Markus et al., 2005) while we used the 11c7 antibody. In contrast, the monoclonal 11c7 antibody is directed against
amino acids 623-640 in the Nogo-A specific region in the rat. The monoclonal 7B12 antibody is specific for amino acids 760-820 in the Nogo-A specific region (Weinmann et al., 2006). While both antibodies provide neutralizing effects to Nogo-A, they may act in different ways. Both experimental methods demonstrate that functional recovery can be found in aged animals with the use of immunotherapy, but this takes a long time. Further experiments using different paradigms are needed to help promote functional recovery in a shorter time period in aged rats.

*Environmental enrichment and MCAO:*

Other work in our lab examined environmental enrichment paired with amphetamine as a treatment for recovery from MCAO (Papadopoulos et al., 2009). This previous study used a similar environmental enrichment paradigm to that of the present study and found that short-term treatment of amphetamine administration paired with environmental enrichment led to marked improvement in forelimb reaching compared to control animals. Although the experiments are quite similar to ours, some important differences exist. One obvious and perhaps most important difference is the use of amphetamine instead of immunotherapy. In several studies, amphetamine has been found to improve functional recovery in the forelimb task after brain injury (Adkins & Jones, 2005; Gilmour et al., 2005; Goldstein & Davis, 1990; Hovda & Fenney, 1984; Ramic et al., 2006; Schmanke, Avery, & Barth, 1996; Stroemer & Rothwell, 1998). Moreover, this previous work (Papadopoulos) used young adult Long-Evans black-hooded rats and the
use of young animals in previous work represents another important difference. Taken together, results suggest that environmental enrichment is a good form of therapy for the aged population after brain injury, but more experiments will have to be done to achieve a shorter recovery time in functional motor skill level.

*Dendritic Plasticity and MCAO:*

Previous work on the possible effect of Nogo-A neutralization on dendritic plasticity in the adult rat after MCAO (Papadopoulos et al., 2006) found an increase in dendritic complexity and spine density in layer V pyramidal neurons of the forelimb motor cortex in the contralesional hemisphere. This finding is somewhat consistent with our current experiments in that a slight, although statistically not significant, increase in the total branch segments in our animals receiving MCAO. The previous work used a different strain and age of rat (Long-Evans black-hooded; young adult), as well as a different Nogo-A antibody (IN-1), and they did not employ environmental enrichment. Perhaps the differences in age, strain of rat, lack of environmental enrichment, and antibody led to the differences in results in the two groups. Again, the low numbers of animals in our experimental studies, as well as their general fragility could have contributed to dissimilar results. Overall, these experiments indicate that Nogo-A immunotherapy has an effect on dendritic plasticity after MCAO in the rat.
**Stem cell therapy and MCAO:**

In other work, human adult bone marrow-derived somatic cells (hABM-SCs) were used to promote functional recovery after MCAO in the Long-Evans black-hooded (Andrews et al., 2008). Specifically, animals with hABM-SCs treatment showed a marked improvement in the forelimb reaching task one week after stroke. The combination of stem cell therapy with immunotherapy or environmental enrichment may prove to be interesting in future studies to determine if there is better functional recovery in rats receiving MCAO. Moreover, it is deemed interesting to see whether stem cell therapy improves functional performance in the aged rat model.

**General Conclusions—TBI Experiments:**

In recent TBI experiments animals showed improved functional recovery at four weeks after stroke compared to the MCAO animals that took longer to recover. However, no significant difference was found between the TBI animals receiving the control and anti-Nogo-A antibody treatments. Analysis of dendritic plasticity in these animals showed less branching, and the numbers were similar and slightly decreased as compared to the normal aged animals. As for total dendritic length, an overall decrease in length was found compared to the normal aged controls. Overall, the results show a decrease in dendritic plasticity in the contralesional hemisphere compared to the normal aged animal. No significant difference was seen in the two antibody groups for the TBI animals. However, as noted with the MCAO experiments, the small group sizes des not enable
statistical analysis. Therefore, only speculations may be made about how immunotherapy may act on dendritic plasticity and functional recovery in the aged rat after TBI.

**Comparison of MCAO and TBI Experiments:**

Statistical significance was found when comparing animals receiving 11c7 Ab after either MCAO or TBI in the Golgi analysis. A significant difference was seen in the two groups (11c7 Ab/MCAO & 11c7 Ab/TBI) in total dendritic length (apical and basal), and total branch segments (apical and basal). The MCAO group showed an increase in dendritic plasticity in the contralesional hemisphere, while the TBI animals exhibited a decrease in dendritic plasticity compared to the normal aged animals. Perhaps the environmental enrichment combined with the anti-Nogo-A treatment spared dendrites in the MCAO animals. Conclusions between the two studies are difficult because many variables must be considered. An obvious example is the fact that MCAO and TBI are two vastly different kinds of lesions that may have different effects on dendritic plasticity, and also antibody treatment may differentially affect the two types of lesions.

**Cortical lesions and rehabilitation:**

Previous work in our lab showed that the combination of amphetamine treatment with rehabilitation (focused activity and enriched housing) led to an enhancement in neuronal plasticity which correlated with improved functional recovery after a cortical lesion (Ramic et al., 2006). Important differences are to be seen between the current and previous experiments. Perhaps the biggest difference is the treatment paradigms. In
previous work with TBI, immunotherapy was used to promote rehabilitation in the animals while amphetamine treatment was used in the other work (Ramic et al.). The amphetamine experiments parallel Papadopoulos’ (2008) experiments in the MCAO model. Also a different strain of rat and age group was used. They also used the sensorimotor cortical aspiration lesion model for their brain injury model, whereas our lab used a controlled cortical impact injury (CCI) model. Combining the previous and current work, we have proven that functional recovery can be seen in rats after a brain lesion. An interesting experiment would be to compare amphetamine treatment versus immunotherapy in the aged rat, as well as with and without environmental enrichment. Much more work must be done to investigate how to achieve a shorter recovery time after a cortical lesion.

Other work examined the affects of IN-1 immunotherapy after a cortical lesion on corticostriatal plasticity (Kartje, Schulz, Lopez-Yunez, Schnell, & Schwab, 1999). Animals treated with IN-1, as compared to control antibody promoted the sprouting in corticostriatal projections to in the dorsolateral striatum contralateral to the injection site. This study is comparable to recent work in that both cortical plasticity after traumatic brain injury. However, the Kartje study used male and female adult Lewis Rats, whereas we used aged male Fisher rats. The lesion model used for inducing the TBI was also different, since the Kartje collaboration used a cortical aspiration lesion. Also, they used the IN-1 antibody as compared to our use of 11c7 antibody. Even though there were
differences seen between the two studies, they both proved that immunotherapy was effective in promoting cortical plasticity.

Other work examined on the functional motor outcome in aged Nogo-A/B deficient aged mice after traumatic brain injury (Marklund et al., 2009) demonstrated that such mice showed poor performance on behavioral tasks, as well as an increase in white matter loss in the brain. These results are contradictory studies using pharmaceutical means to suppress Nogo-A. For example, Nogo suppression with IN-1 treatment demonstrated functional recovery in animals with TBI. (Kartje 1999) Possibly, the genetics of the Nogo-A/B deficient mice played a role in their overall brain development and thus led to a more severe injury after TBI. (Marklund et al., 2009). Other than the obvious difference in the way Markland’s group and the way we blocked Nogo-A, the animal species, age, and functional tasks were dissimilar in the two experimental paradigms. The TBI model however was the same. Comparison of the two studies shows that Nogo-A plays an important role in the functional recovery of animals after TBI.

**Overall conclusions:**

Overall, we have learned much from our experiments as well as the review of studies done by other investigators. The major finding is that cortical injury in the aged brain is very complex and many factors are to be considered. The effect of various inhibitors in the brain that make it perhaps less plastic for repair must also be taken into
account. The aged brain is especially susceptible to damage and is less capable of repair in comparison to younger brains.

Our experimental design is considered interesting but, several things must change in order to promote a more successful study. A larger group of animals is needed to improve statistical analysis, although this is difficult in studies of aged, fragile animals. Possibly more robust rat strains may be available for such work. On the other hand, perhaps the Fisher rat is appropriate in resembling the fragile elderly human population. Use of the appropriate animal model is perhaps one of the biggest problems in research. The large variability in the human population represents an additional problem, as does the reduced plasticity of the aged brain. However, numerous recent studies showing the adult brain to be less rigid than previously thought does provide optimism. Additionally our rehabilitative paradigm may have been too strenuous and tiring for the aged rats and thus did not promote good health. On the other hand, rehabilitation may have been more effective if started earlier than the four weeks after the animals received MCAO. Perhaps, the physical therapy sessions should be introduced at the same time as the immunotherapy to provide a more powerful challenge against the deficits of MCAO. The introduction of physical rehabilitation examined the effect physical rehabilitation combined with immunotherapy on animals with TBI may be a more beneficial.


inhibitor permits immunochemical detection and shows enhanced neutralizing activity. *Protein Eng, 15*(11), 931-941.


Contributions as Author

MCAO Experiments:
I designed and interpreted all of the experiments. My experiments were performed with the help of several collaborators. Dr. Shih-Yen Tsai performed the MCAO and antibody pump surgeries. Vicki Husak aided me in the behavioral tasks, as well as filming and analyzing the data from those tasks. Anneisha Elerby assisted me in the Golgi-Cox procedure by helping with the tracings and analyzing the data. Melanie Bollnow also helped with the Golgi-Cox procedure, in reacting and prepping the brains for analysis.

TBI Experiments:
All experiments were designed by Ian Vaagenes. I interpreted his results from his experiments. His experiments were conducted with help from several collaborators as well. Dr. Shih-Yen Tsai performed the TBI and antibody pump surgeries. Mateo Tole, Anneisha Elerby, Dan Nockels and Katherine Podraza aided Ian with carrying out and filming the behavioral tasks. Anneisha Elerby also aided Ian in the Golgi-Cox procedure, in tracing and analyzing neurons.
VITA

Sarah J. Hein was born to Elizabeth S. Hein and Ronald L. Hein in Livonia, Michigan on September 18th, 1983. She graduated from Marian High School, with honors, in Bloomfield Hills, Michigan in 2002. She then went on to become a student in the Residential College of the school of Literature Science and the Arts at the University of Michigan in Ann Arbor. It was here that she majored in Brain, Behavior and Cognitive Sciences and developed a real passion for research. In her sophomore year, she became part of the Undergraduate Research Opportunity Program (UROP) and was placed in the otolaryngology laboratory of Dr. Yehoash Raphael. In this laboratory, she studied gene therapy on deafness and balance disorders. She wrote a senior thesis on her project studying the effect of diet presbycusis. In 2004, she presented her work at the UROP Spring Symposium. In 2005, she presented her work at the Psychology Department’s Research Day. She attended the 2006 Mid-Winter Meeting for the Association of Research in Otolaryngology where she presented an abstract and poster. She graduated from the University of Michigan in 2006.

Sarah entered her first year of graduate school at Loyola University Chicago in 2006, in the Neuroscience program. In 2007, she joined the laboratories of Dr. Evan Stubbs Jr. and Dr. Gwendolyn Kartje. She did her thesis work on cortical injury and recovery in the aged rodent. She was awarded a Diversity Supplement to NS40960 from
the NINDS/NIH in 2009 to support her work. She presented her current work at the 2009 Edward Hines Jr. VA Hospital Research Day. Sarah is a student member of Society for Neuroscience. Her future plans include working in a clinical research setting while she applies for medical school. She would like to either specialize in otolaryngology, pediatrics or neurology.
The dissertation submitted by Sarah J. Hein has been read and approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Master’s in Science.

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Date

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