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Effects of Neuronal Nogo-A on Properties of Excitatory Synapses of the Sensorimotor Cortex

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Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

EFFECTS OF NEURONAL NOGO-A ON PROPERTIES OF EXCITATORY SYNAPSES
OF THE SENSORIMOTOR CORTEX

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

PROGRAM IN NEUROSCIENCE

BY
ALICIA M. CASE

CHICAGO, ILLINOIS

MAY 2011
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Final thanks go to my family for their support and encouragement on this project, as with all things.
For all the teachers who have inspired my love of science through the years, particularly Susan Osterlie, Susan Sarejko, Tom Bahl and James Doyle.
I think, at a child’s birth, if a mother could ask a fairy godmother to endow it with the most useful gift, that gift should be curiosity.

-Eleanor Roosevelt
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<tr>
<td>AAV2/8</td>
<td>Adeno-associated virus serotype 2/8</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotropic factor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CA1/3</td>
<td>cornu Ammonis 1/3, regions of the hippocampus</td>
</tr>
<tr>
<td>CamIIK</td>
<td>Ca2+/calmodulin-dependent protein kinase 2</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tract</td>
</tr>
<tr>
<td>CT</td>
<td>Threshold cycle # where PCR amplification becomes linear</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer, one known receptor for Netrin-1</td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubecco’s Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory Post-synaptic potential</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>FMRP</td>
<td>Fragile X mental retardation protein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GPR50</td>
<td>G protein-coupled receptor 50</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MHCI</td>
<td>Major histocompatibility complex 1</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Munc18</td>
<td>Mammalian uncoordinated-18</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
</tr>
<tr>
<td>NgR1</td>
<td>Nogo-receptor 1</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>Nogo-66</td>
<td>66-amino acid loop in Nogo-A/B/C that inhibits outgrowth</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NR2A</td>
<td>aka GRIN2A; Glutamate receptor subunit epsilon-1</td>
</tr>
<tr>
<td>NR2B</td>
<td>aka GRIN2B; Glutamate receptor subunit epsilon-2</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocye-myelin glycoprotein</td>
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</table>
P           postnatal day
p75         Neurotrophin receptor
PCR         Polymerase chain reaction
PFA         Paraformaldehyde
PirB        Paired immunoglobin receptor B
PIP2        Phosphatidylinositol 4,5-bisphosphate
PIPES       piperazine-N,N'-bis(2-ethanesulfonic acid)
PKC         Protein Kinase C
PKR         Protein Kinase R
PLC         Phospholipase C
PNS         Peripheral Nervous System
PSD-95      Postsynaptic density-95
qRT-PCR     Quantitative reverse-transcriptase polymerase chain reaction
RHD         Reticulon Homology Domain
RISC        RNA-induced silencing complex
RhoA        Ras homolog gene family, member A
RNAi        RNA Interference
SD          Standard deviation
SDS-PAGE    Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM         Standard error of the mean
SH2/3       Src homology domain 2/3
siRNA       short interfering RNA
shRNA       short interfering hairpin RNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>shNogo</td>
<td>shRNA directed against Nogo-A amino acids 856-874</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TROY</td>
<td>orphan receptor TAJ</td>
</tr>
<tr>
<td>UNC</td>
<td>Uncoordinated, one known receptor for Netrin-1</td>
</tr>
<tr>
<td>vGlut1</td>
<td>Vesicular glutamate transporter 1</td>
</tr>
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</table>
ABSTRACT

Recovery after central nervous system (CNS) injury has long been a challenge for clinical investigators. Blockade of the oligodendrocyte-associated inhibitor Nogo-A has shown great promise in promoting neuronal regeneration, sprouting, and plasticity, as well as functional recovery in rodent and primate models of CNS injury. The high expression of Nogo-A in neurons of the postnatal CNS led us to look for potential roles of this protein in this stage of development. We hypothesized that postnatal, neuronal NogoA influences the density and morphology of dendritic spines in the developing CNS, in part, by regulating the maturation and stability of glutamatergic synaptic input.

To examine the roles of Nogo-A at the excitatory synapse of the neocortex, we used RNAi directed against Nogo-A and delivered it to the developing rat sensorimotor cortex via AAV2/8, a neurotropic vector. This resulted in lowered density of dendritic spines, which are known to house over 90% of excitatory connections onto pyramidal neurons. This decrease was particularly evident with thin- and mushroom-shaped spines in dendrites of the apical arbor. A decrease in protrusions with moderately-wide head widths and very long necks was also noted. We then used vesicular glutamate transporter 1 (vGlut1) as a marker for potential excitatory synapses. Knocking down Nogo-A in postnatal pyramidal neurons of the sensorimotor cortex led to a decrease in the number of vGlut1.
puncta identifying a potential presynaptic partner, in opposition to the apical
dendritic shaft. The decreased vGlut1 in the apical arbor likely represents a loss
of potential synapses that may have a strong influence on direct current injected
into the dendrite.

We further examined regions of transduced cortex via qRT-PCR for
message levels of molecules important for plasticity at the excitatory synapse,
including Neuroligin-1, NMDA receptor subunits NR2A and NR2B, and PSD-95.
We found that mRNA of Neuroligin-1 and NR2B was substantially reduced, with
no changes to NR2A or PSD-95 expression. These results suggest that neuronal
Nogo-A may act to maintain elements of the neocortical excitatory synapse
during development. This finding represents a novel role for Nogo-A in the intact
CNS.
CHAPTER ONE
OVERVIEW AND HYPOTHESIS

Recovery after brain and spinal cord injury has long been a challenge for clinical researchers. Neurons in the adult brain and spinal cord cannot grow more than a millimeter past a lesion site without aid. We now know that the environment in the injured brain and spinal cord prevents axonal regrowth and plasticity. This environment is formed by astroglial scarring, chondroitin sulfate proteoglycans, and oligodendrocyte-associated inhibitory proteins (Gonzenbach and Schwab, 2008). One of these inhibitory proteins, the first to be discovered, and the focus of work in our laboratory, is Nogo-A (Caroni and Schwab, 1988).

Thus far, therapies directed against Nogo-A have shown the most promise in in vivo studies that use animal models of spinal cord injury and stroke. Over the last twenty years, blockade of the Nogo-A protein has consistently resulted in neuronal regeneration, sprouting, and plasticity, as well as functional recovery in animal models of injury (Gonzenbach and Schwab, 2008). Today, Phase II clinical trials are underway to examine the potential of the anti-Nogo-A antibody as a treatment for spinal cord injury (Zorner and Schwab, 2010). An estimated 250,000 Americans are currently disabled by spinal cord injury, and there is no current treatment available. Anti-Nogo-A immunotherapy may also aid in
enhancing neuroplasticity in people suffering from other injury-induced neurological conditions, including stroke.

Despite these advances in preclinical and clinical studies, there is a prevailing deficit in our understanding of the normal physiological roles of Nogo-A in the intact central nervous system, and specifically in neurons. A few reports suggest Nogo-A signaling stabilizes synapses in the intact central nervous system (CNS) (Chivatakarn et al., 2007; Mingorance-Le Meur et al., 2007; Lee et al., 2008), including neuronal Nogo-A (Aloy et al., 2006), though the influence of neuronal Nogo-A at the neocortical excitatory synaptic phenotype has yet to be investigated. This dissertation will focus on three different properties that comprise the normal synaptic phenotype: dendritic spines, molecules that are key for normal synaptic function, and potential presynaptic sites opposing dendritic spines and dendrites. We will study the effect of Nogo-A on these properties by altering the amount of Nogo-A in excitatory neurons in the neocortex of normal post-natal rats.

**HYPOTHESIS**

Postnatal expression of neuronal NogoA influences the density and morphology of dendritic spines in the developing CNS, in part, by regulating the maturation and stability of glutamatergic synaptic input.
Specific Aim 1. To determine whether knockdown of neuronal Nogo-A in postnatal development will alter spine density and influence spine morphology in neocortical pyramidal neurons. Changes in dendritic spine density and morphology serve as an indicator of broad changes in cognitive capacity, both directly, in terms of excitatory postsynaptic potential (EPSP) processing and calcium buffering, and indirectly, as a predictive correlate of general cognitive functioning and environmental enrichment. To examine these parameters, anesthetized neonatal, male rat pups will receive intracerebral injections of AAV expressing enhanced green fluorescent protein (EGFP) and short hairpin siRNA (shRNA) directed against Nogo-A (shNogo). At nine weeks of age, rats will be sacrificed and dendritic spines will be visualized by confocal laser scanning microscopy. Control rats will receive AAV encoding EGFP only.

Specific Aim 2. To determine whether knockdown of neuronal Nogo-A in postnatal development will alter the message levels of mature excitatory synaptic markers. The candidate genes to be analyzed are key mediators of synaptic functioning and include Postsynaptic density-95 (PSD-95), N-Methyl-D-Aspartate (NMDA) receptor subunits NR2A and NR2B, Neuroligin-1, and Cofilin. The quantity of these molecules has been shown previously to change in response to synaptic alterations or changes in Nogo-A levels. Adult rats that have been neonatally injected with AAV will be sacrificed and microdissected regions of sensorimotor cortex analyzed via quantitative reverse-
transcriptase polymerase chain reaction (qRT-PCR) to determine the relative fold changes in mRNA levels of candidate genes.

Specific Aim 3. To determine whether knockdown of neuronal Nogo-A in postnatal development will alter the number of potential excitatory presynaptic sites opposing EGFP-filled dendrites. This direct study of the molecules that synapse onto transfected neurons will allow for an examination of presynaptic changes and an approximation of the number of spines that contain synapses. Adult rats that had been neonatally injected with AAV will be sacrificed, and the density of vesicular glutamate transporter 1 (vGlut1)-immunopositive puncta will be determined in dendrites of the sensorimotor cortex.
CHAPTER TWO
REVIEW OF LITERATURE

NOGO-A

Introduction

The CNS environment has long been understood to be inhibitory to regeneration after injury (Aguayo et al., 1981; Richardson et al., 1984), and myelin comprises a large part of that inhibitory environment (Schwab and Caroni, 1988). The Nogo-A protein was the first of these myelin-associated inhibitory molecules to be isolated, when components of CNS myelin were separated by polyacrylamide gel electrophoresis; fractions were cut from the gel and used to generate hybridoma cells that secreted antibodies that would bind to molecules within the gel fraction (Caroni and Schwab, 1988). Antibodies against one fraction in particular, named IN-1, neutralized neurite inhibition over myelin substrates in vitro and facilitated regeneration and recovery after CNS injury in vivo (Caroni and Schwab, 1988; Bandtlow et al., 1990; Rubin et al., 1994; Guest et al., 1997; Papadopoulos et al., 2002). The protein that IN-1 was directed against was eventually purified, sequenced, and named Nogo-A (Chen et al., 2000).

In vitro studies have shown that Nogo-A destabilizes growth cones and inhibits cell spreading and migration (Oertle et al., 2003). Cells that are able to
migrate through the CNS, such as stem cells, also degrade myelin-associated inhibitors (Wright et al., 2007). In the case of migrating tumor cells, this degradation is accomplished by means of a matrix-metalloprotease (Paganetti et al., 1988; Belien et al., 1999).

Antibody treatment against Nogo-A has been shown effective after animal models of CNS injury in a battery of behavioral sensory tests, motor tasks, reflex responses, and anatomical studies by several independent laboratories (Gonzenbach and Schwab, 2008). IN-1 immunotherapy has led to axonal regeneration in rodent and primate models of spinal cord injury via hemisection (Brosamle et al., 2000; Fouad et al., 2004). Axonal sprouting has been observed following cortical aspiration lesions and IN-1 administration, as evidenced by anatomical and electrophysiological studies (Bareyre et al., 2002; Emerick et al., 2003). IN-1 treatment has also led to axonal sprouting as well as improved sensory and motor function in rat pyramidotomy and stroke models of CNS injury (Thallmair et al., 1998; Seymour et al., 2005).

In addition to IN-1, other purified antibodies have also been raised against Nogo-A and found efficacious in treatment of CNS injury models. Application of 11C7 antibody has resulted in functional recovery and regeneration in primate models of spinal cord injury (Freund et al., 2006), and recovery when administered up to two months after occlusive stroke in rats (Tsai et al., 2010). Use of the 7B12 antibody after photothrombotic and occlusive stroke in rats has led to behavioral recovery and another mechanism of repair, neuroplasticity
Administration of any of the three anti-Nogo-A antibodies (IN-1, 11C7, or 7B12) has led to neuroplastic sprouting and improved cognitive recovery from a rodent model of contralateral neglect (Brenneman et al., 2008). As defined in this dissertation, neuroplasticity after injury involves the recruitment of spared neurons to the denervated regions, whereas regeneration is the regrowth of injured axons to their previous target.

**Axon guidance**

Nogo-A is one of many axon guidance molecules in the CNS. Several of these molecules, such as Netrins, Slits, and Semaphorins aid in developmental pathfinding. These molecules increase the efficiency and accuracy with which axons locate their synaptic targets (Hong et al., 1999; Galko and Tessier-Lavigne, 2000; Polleux et al., 2000; Whitford et al., 2002). To accomplish this, these molecules have rigid expression patterns at specific stages of development. For example, the Slit receptors, named Robo1-3, are expressed on corticospinal tract (CST) axons in the dorsal funiculus of the spinal cord from E14 to P4, on axons during CNS midline crossing, with expression levels peaking after decussation (Sabatier et al., 2004; Sundaresan et al., 2004). Expression patterns of many of these molecules differ between development and adulthood, forming another obstacle to repair after CNS deafferentation (Harel and Strittmatter, 2006).

Another class of axon guidance molecules was characterized in the adult nervous system as myelin-associated inhibitors of axonal outgrowth. These
molecules include myelin-associated glycoprotein (MAG), oligodendrocyte-
myelin glycoprotein (OMgp), EphrinB3, and Nogo-A (Mukhopadhyay et al.,
1994; Chen et al., 2000; Wang et al., 2002a; Benson et al., 2005).

To date, therapies directed against all of these molecules have been
examined for treatment of CNS axonal injury. Though anti-Nogo-A
immunotherapy has shown the most promise in animal models of brain and
spinal cord injury, these molecules do work in concert, and ultimately a
combination therapy may lead to the most efficacious recovery from CNS
damage.

Interestingly, several molecules known to repel axons have also been
shown to enhance dendritic plasticity, through various mechanisms specific to
different receptor-ligand systems (Polleux et al., 2000; Whitford et al., 2002;
Morita et al., 2006; Rodenas-Ruano et al., 2006; Aoto et al., 2007; Zagrebelsky et
al., 2010). It is possible that future advances in the field of Nogo-A receptor-
ligand signaling may show Nogo-A to function similarly.

**Nogo-A is a member of the Reticulon family**

The protein Nogo-A is a member of the reticulon 4 subfamily. This
subfamily has three members, named Nogo-A, -B, and -C. All three isoforms
share the Nogo-A c-terminal structure of 188 amino acids, known as the reticulon
homology domain, which contains a 66 amino-acid sequence known as Nogo-66
(Oertle et al., 2003). Nogo-A, -B, and -C each contain N-termini of decreasing
length, so that Nogo-A has the longest N-terminus, with over 800 amino acids.
Nogo-A and B are both found in the CNS and, to a lesser extent, in the peripheral nervous system (PNS). Nogo-B is also found in blood vessels, where it promotes vascular cell adhesion and migration of endothelial cells during vascular remodeling events (Acevedo et al., 2004; Miao et al., 2006; Kritz et al., 2008). Nogo-C is primarily found in skeletal muscle (Oertle and Schwab, 2003).

The reticulons are an evolutionarily conserved family containing a characteristic endoplasmic reticulum (ER) retention sequence. Members of this family have roles in exocytosis (Steiner et al., 2004) and cellular stress response (Tagami et al., 2000; Kuang et al., 2006; Wan et al., 2007; Wojcik et al., 2007). The majority of Nogo-A molecules present in a cell are found within the ER (Voeltz et al., 2006).

The N and C-termini of Nogo-A have been shown to be highly unstructured, possibly existing in different membrane topologies, as shown by bioinformatics (Li and Song, 2007) and immunohistochemistry (Oertle et al., 2003; Dodd et al., 2005). As many as 30-60% of eukaryotic proteins may have long, disordered regions (Ward et al., 2004; Tompa et al., 2006; Li and Song, 2007). This has led to the speculation that different Nogo-A structures, taken together, may permit “functional moonlighting” within the same cell, i.e. shaping ER and inhibiting neurite outgrowth (Li and Song, 2007). Nogo-A and –B may house SH2, SH3, and PDZ domains in their N-termini, potentially allowing Nogo-A to mediate downstream signaling, similar to the bidirectional signaling
reported between ephrins and Eph “receptors” (Rodenas-Ruano et al., 2006; Li and Song, 2007).

**Nogo-A Receptors**

The Nogo-66 region, found in all three Nogo isoforms, can bind to the Nogo receptor (NgR1) (Fournier et al., 2001), as can MAG and OMgp. A portion of the Nogo-A N-terminus also binds NgR1 with high affinity and has been postulated to modulate Nogo-66 binding and subsequent downstream signaling in an agonistic fashion (Fig. 1) (Hu et al., 2005). NgR1 has no intracellular component, and must signal through a multisubunit complex with p75 or Lingo-1 to activate RhoA, which in turn leads to activation of RhoA associated kinase (ROCK), and increase cytoskeleton contractility through Myosin II (Fig. 1) (Bandtlow et al., 1993; Loschinger et al., 1997; Schweigreiter et al., 2004; Sivasankaran et al., 2004; Lowery and Van Vactor, 2009). Studies in dorsal root ganglion and SH-SY5Y cells have shown Nogo-NgR1 signaling can be suppressed by NgR1 phosphorylation (Fig. 1) (Atwal et al., 2008). Animals that have had PirB genetically deleted show delayed closure of the ocular dominance critical period (Syken et al., 2006), similar to NgR1 and Nogo-A/B knockout mice (McGee et al., 2005). PirB knockout mice also display normal baseline long-term potentiation (LTP), as do NgR1 knockout and PirB/NgR1 double knockout mice.
Figure 1. Nogo-A signaling events. Activated forms of molecules are shown in black, inactive forms shown in grey. For details, see text. Inset: Domains for Nogo-A/B/C. For list of abbreviations, see page xiv. Modified from (Lowery and Van Vactor, 2009; Schwab, 2010).
(Lee et al., 2008; Raiker et al., 2010). There is some evidence suggesting PirB may mediate OMgp signaling in the hippocampus, but the efficacy of PirB signaling in the neocortex may be impaired by its low expression in this region (Raiker et al., 2010).

Blockade of NgR1 does not eliminate myelin inhibition of neurite outgrowth (Kim et al., 2004). Furthermore, the Nogo-66 loop that binds NgR1 is also present in Nogo-B, which Nogo-A immunotherapy does not target. Finally, studies using directed mutations have shown the N-terminal region of Nogo-A is a more potent inhibitory region than the Nogo-66 loop (Chen et al., 2000; Fournier et al., 2001; Oertle et al., 2003; Hu et al., 2005; Gonzenbach and Schwab, 2008), indicating that at least one other unidentified receptor or subunit may inhibit axonal outgrowth and cell spreading (Fig. 1). The Amino-Nogo receptor may also inhibit neurite outgrowth by transactivating Epidermal growth factor receptor (EGFR) in a calcium-dependent manner, although treatment with EGF or heparin-bound EGF failed to affect outgrowth (Koprivica et al., 2005) (Fig. 1).

To a limited extent, the N-terminal region of Nogo-A can achieve these results by signaling through integrins α3, α4, α5, or β3 (Hu and Strittmatter, 2008). This interaction has either a weak binding affinity, or requires indirect binding aided by fibronectin or other non-integrin cell surface receptors. The molecules implicated in the NogoA-NgR1 and integrin pathways have been studied within the context of the axonal growth cone. Although these presynaptic
molecules and their downstream mediators are known to affect spine density, morphology, and the actin cytoskeleton (Gerrow and El-Husseini, 2006; Shi and Ethell, 2006; Webb et al., 2007; Cingolani and Goda, 2008; Carlson et al., 2010), no published reports exist to date that specifically investigate Nogo-A-integrin signaling or downstream NgR1 messengers through the context of Nogo-A signaling at the spine or synapse. G protein-coupled receptor 50 (GPR50), a recently characterized protein, has recently become another candidate for an N-terminal specific Nogo-A receptor (Grunewald et al., 2009).

**Nogo-A at the Synapse**

A few published reports suggest involvement of Nogo-A at synapses and in development. Nogo-A is localized at pre- and post-synaptic terminals (Lee et al., 2008; Grunewald et al., 2009; Raiker et al., 2010). Loss of Nogo-A has also been shown to increase neocortical branching, hasten neurite polarization *in vitro*, and delay migration of cortical interneurons *in vivo* (Mingorance-Le Meur et al., 2007). Functionally, Nogo-A/B and NgR1 knockout mice have both shown an extension of the visual critical period in development, as shown by ocular dominance testing (McGee et al., 2005). Nogo-A has been shown to have a stabilizing effect on dendritic structure of hippocampal pyramidal neurons (Zagrebelsky et al., 2010; Delekate et al., 2011).
Inhibitory synapses in the cerebellum in vivo have been shown to decrease in density in response to Nogo-A (Aloy et al., 2006), though interestingly, this change was observed after overexpressing levels of Nogo-A protein postnatally in Purkinje neurons of the cerebellum. Purkinje cells showed abnormal synapse morphology and decreased number of axonal contacts onto deep cerebellar nuclei (Aloy et al., 2006). These effects may be due to downregulation of synaptic anchoring molecules (Aloy et al., 2006).

NgR1 knockout mice have shown decreased FGF-induced neurite branching in vitro, enhanced FGF2-dependent long-term potentiation, decreased hippocampal long-term depression, and altered spine morphologies in vivo (Lee et al., 2008). Specifically, neurons from the hippocampal CA1 region in the NgR1 knockout mice displayed a decrease in mushroom and thin spines and an increase in stubby spines, but no change in total spine density (Lee et al., 2008). This same laboratory recently showed that Brain-derived neurotrophic factor (BDNF) activation of the pAKT/mTOR pathway may be inhibited by Nogo-66 (Raiker et al., 2010). Other laboratories also showed that the actions of BDNF can be opposed by Nogo signaling (Endo et al., 2007; Chytrova et al., 2008). Neurotrophins can also override the inhibitory effects of Nogo on neurite outgrowth by cAMP-PKA-CREB mediated mechanism (Hannila and Filbin, 2008; Joset et al., 2010). These findings have led to the hypothesis that Nogo-A signaling works in equilibrium with growth factors to fine tune the crucial process of use-dependent neuronal plasticity.
Neocortical Expression of Nogo-A

Throughout all stages of development, Nogo-A is expressed on oligodendrocytes (Huber et al., 2002). Nogo-A is also present in embryonic development within radial glia and at the leading process of migrating neurons (Mingorance-Le Meur et al., 2007). Human embryos also express Nogo-A in postmitotic cells of the cortical plate (Al Halabiah et al., 2005). Expression in the white matter decreases after early postnatal development, though protein expression persists in pyramidal neurons of the adult neocortex, in both humans and rats (Buss et al., 2005; Cheatwood et al., 2008). Within these neurons, Nogo-A is present in postsynaptic sites in dendrites and spines of cortical neurons (Pradhan, 2007; Grunewald et al., 2009).

DENDRITIC SPINES

Dendritic spines house the majority of excitatory inputs onto pyramidal neurons (Gray, 1959). Spines act as individual compartments on the dendrite, buffering calcium dynamics and regulating electrophysiological and molecular signaling. Long-term potentiation (LTP), a form of memory on the cellular level, has been shown to induce actin to change spine shape, increasing the width of the spine head and decreasing the length of the spine neck. At the same time, the concentration of glutamate receptors increases in the postsynaptic density (Yuste and Bonhoeffer, 2001).
Spine density per length of dendrite and individual spine morphology have been shown to change in response to development, environmental changes such as enrichment or hibernation, usage and experience, and hormones (Popov et al., 1992). At the same time, large spines have been shown to change very little, and sometimes not at all throughout the adult life of an animal (Grutzendler et al., 2002). Various forms of developmental disability, such as Down’s syndrome and Fragile X syndrome (Dierssen and Ramakers, 2006), are associated with deficits in spine density and with an increased number of filopodial protrusions (Fig. 2) on pyramidal neurons of the neocortex and hippocampus.

Several theories exist with regards to formation of new spines. The first holds that after repeated exposure to an LTP-inducing stimulus, over a period of hours the spine head can form a second synapse on an axon, and this second synapse may in turn lead to the formation of another spine (Segal, 2005). This conjecture is not without controversy, as synapses onto bifurcated spines are rarely from the same neuron (Fiala et al., 2002b). A second mechanism for new spine development is that spines may arise from synapses onto the dendritic shaft (Fiala et al., 1998; Ethell and Pasquale, 2005). A third possibility is that filopodial extensions probe the environment for molecular signals resulting from changes in neurotransmitter release, and then form synapses with active axon terminals (Holtmaat et al., 2006; Nagerl et al., 2007).

The question of whether changes in spine morphology are a direct or indirect cause of plasticity is another area of scientific debate. There is no
Figure 2. Common morphologies of dendritic protrusions. (A) Shown from top to bottom is an idealized filopodium, stubby, thin, mushroom, and bifurcated spine. Classification is based on the relationship of the spine head to the neck diameter, adapted from (Lippman and Dunaevsky, 2005) (B) Representative EGFP-filled dendritic segment with spine morphologies, from AAV-transduced tissue imaged at 63x magnification on laser scanning confocal microscope. Note that filopodia were not counted as spines, and that spine morphology is truly a continuum. The categories shown here are used to generalize the differences between spines.
question that the shape of a spine will affect its function by controlling current, calcium dynamics, and by filtering membrane potential. Larger spines inject larger currents into the dendrite, thereby affecting downstream signal processing (Matsuzaki et al., 2004). Also, the length of the spine neck controls calcium diffusion from spines and electrically filters postsynaptic potential (Yuste, 2010). However, the environmental and developmental conditions known to affect spine density listed above are likely to also lead to wide-scale changes in synaptic plasticity. The same endocytic machinery that controls the amount of membrane at the postsynaptic site is also essential for insertion of proteins such as AMPA receptors into the postsynaptic density (PSD) (Wang et al., 2008). The concentration of AMPA receptors is a known requirement of synaptic maturation (Nusser et al., 1998). It is possible that spines act as diffuse computational devices, endowing circuits with the ability to perform Boolean logic, but at the very least the density and morphology of spines can serve as an indicator of general synaptic function.

**POSTSYNAPTIC MOLECULES IMPORTANT FOR SYNAPTIC FUNCTION**

PSD-95 is localized to active synapses and serves to anchor many key elements of synaptic transmission, including glutamate receptors and the actin cytoskeleton (Blanpied et al., 2008; Chen et al., 2008). Also known as DLG4, it is considered an excitatory synaptic marker.
The NMDA receptor is a type of ionotropic glutamate receptor whose participation in the post-synaptic density substantially regulates synaptic plasticity, including LTP. NMDA receptors have the highest calcium permeability of all glutamate receptors, but they require voltage-activation to remove a magnesium block of the ion channel. The channel will only open at a synapse that has recently received an excitatory post-synaptic potential, making the NMDA receptor a molecular coincidence detector and allowing for a kind of computation in the postsynaptic neuron (Yuste, 2010).

The regulation of NMDA receptor channel activity occurs through widely varied means, suggesting that this receptor has a significant role in information processing. The complexity of this regulation includes, but is not limited to, magnesium blockade, localization to synaptic or extrasynaptic sites, phosphorylation by molecules such as Ca2+/calmodulin-dependent protein kinase 2 (CamIK), and expression of different subunits. There are five NMDA receptor subunits: NR1, NR2A, NR2B, NR2C, and NR2D. Changes in NR2 subunit expression levels have been reported in the developmental maturation of synapses in the thalamus, somatosensory cortex, and cerebellar granule neurons (Liu et al., 2004; Fu et al., 2005). For example, from the peak to the closure of the visual cortex critical period, the expression of NR2A predominates over NR2B (Cho et al., 2009). Activity or deprivation-induced plasticity in adulthood has also been linked to expression levels of these subunits (Yashiro and Philpot, 2008; Corson et al., 2009).
Neuroligins are postsynaptic anchoring molecules that help maintain the synaptic cleft through the presynaptic binding to presynaptic Neurexins. Many such pairs of adhesion molecules collectively maintain the synaptic cleft, much like the many weak interactions that hold two pieces of Velcro together. Additionally, Neuroligins have demonstrated transsynaptic control of vesicular release via Neurexins (Wittenmayer et al., 2009; Stan et al., 2010). For example, Neuroligin-1, which is enriched at excitatory synapses, may control presynaptic vesicle release via β-Neurexin’s links to Munc18 via Cask-Mint1 interactions (Futai et al., 2007).

Altered Neuroligin-1 expression in rodents has been linked to changes in LTP, short-term plasticity, spatial memory, fear memory and social behavior (Futai et al., 2007; Kim et al., 2008; Blundell et al., 2010; Dahlhaus and El-Husseini, 2010; Dahlhaus et al., 2010), probably due to Neuroligin-1’s ability to expedite activity- and growth factor-mediated synaptogenesis (Nam and Chen, 2005; Xu et al., 2010). Expression levels of Neuroligin-1 have also been shown to directly affect the ratio of excitatory to inhibitory synapses; similar effects have been produced with altered levels of PSD-95 (Gerrow et al., 2006; Dahlhaus et al., 2010; Levinson et al., 2010). This role in the specification of synapses is largely thought to be mediated by interaction between Neuroligin-1 and PSD-95—or gephryn, in the case of Neuroligin-2—(Chubykin et al., 2007; Barrow et al., 2009; Levinson et al., 2010). Neuroligin-2, which is concentrated in inhibitory synapses (Graf et al., 2004; Varoqueaux et al., 2004), is one of the synaptic
markers that displayed downregulation in cerebellar Purkinje neurons that overexpressed Nogo-A (Aloy et al., 2006).

**RNA INTERFERENCE**

RNA interference (RNAi) suppresses or degrades RNA transcripts, thereby hindering production of the encoded proteins. RNAi allows for higher levels of regulation in producing the vast amount of differentiation seen in the adult. The study of endogenous RNAi has expanded in recent years, and the molecules have been found to have major roles in nervous system disorders and development. Various neurological conditions are linked to disruption of RNAi machinery, including Tourette's syndrome, various brain tumors, and the alterations in spine development and Fragile X mental retardation protein (FMRP) function in fragile X syndrome (Jin et al., 2004; Abelson et al., 2005; Chen and Stallings, 2007; Corsten et al., 2007). In development, RNAi regulates neural patterning, outgrowth, spine development, apoptosis, circadian rhythms, lineage differentiation, and proliferation (Schratt et al., 2006; Krichevsky, 2007; Visvanathan et al., 2007). RNAi can suppress alternate mRNA splice variants. For example, downstream effectors of the MicroRNA (miRNA) miR-124 can suppress non-neuronal genes expression and activate neuronal gene transcription, including their splice variants, through an intricate negative feedback cycle (Visvanathan et al., 2007; Makeyev and Maniatis, 2008).
The RNAi process is evolutionarily conserved across kingdoms, and has been documented in animals, plants, and, to a limited extent, fungi (Saumet and Lecellier, 2006). RNAi has two primary roles in the cell, as a defense mechanism against viruses, and as a means of post-transcription gene regulation, where a cell can transcribe both a message and a means to break down that message. In keeping with these two well-documented functions of RNAi, there are several methods by which RNAi may work, including nonspecific degradation of all mRNAs (Sledz and Williams, 2004). Most often, researchers use RNAi to silence the transcripts of one specific gene via short RNA duplexes.

**RNAi Machinery**

As seen in Figure 3, the first step of the RNAi pathway involves transcription from DNA of pri-miRNA, a stem-loop structure typically flanked by nucleotides of varying lengths. These flanking nucleotides on the pri-miRNA are cleaved at the stem-loop in the microprocessor complex by the enzyme Drosha, to form a hairpin structure of ~70 nucleotide (nt), known as pre-micro-RNA (pre-miRNA). Pre-miRNA can be shuttled out of the nucleus via exportin-5-RANGTP (Rana, 2007). Once released into the cytoplasm, the enzyme Dicer can further process pre-miRNA into a 22nt duplex structure known as mature miRNA. This cleavage by Dicer leaves a 2nt overhang at the 3’ end and a phosphate group at the 5’ end (Kim et al., 2005). Mature miRNA is unwound by RNA-induced silencing complex (RISC) at the end of the duplex with the lowest thermodynamic
Figure 3. **Endogenous intracellular RNAi pathway** and tools that can utilize it for therapeutic purposes or to study neuronal function. Modified from (Davidson and Boudreau, 2007).
stability, typically the end with the highest A-T to G-C ratio (Khvorova et al., 2003). The 3’ strand at this end is degraded, while the 5’ strand is irreversibly bound to RISC, where it can then bind complementary segments of mRNA. If the strand is a perfect match, Dicer then cleaves the message strand into 23nt segments. These segments are recognized by cellular nucleases and further cleaved into individual nucleotides and, in so doing, prevent protein synthesis (Davidson and Boudreau, 2007).

By leaving the miRNA strand intact and irreversibly bound to Dicer, a single miRNA duplex can inhibit protein synthesis of numerous mRNA transcripts. RISC-miRNA complexes can sometimes be passed down to progeny cells and even germline cells (Carmell et al., 2003). This amplification can ideally silence a large proportion of message copies, and the protein is considered ‘knocked down’. mRNA strands with a small number (less than three) of imperfect matches have been documented to bind the complimentary strand in the RISC but are usually not cleaved (Bagga et al., 2005). In this instance, the miRNA-RISC can bind a single copy of the message, thereby halting access to ribosomes. However, if the cell has other copies of the same mRNA, then these messages are still available for protein synthesis. When this occurs, the message is termed repressed, but not silenced.
Synthetic RNAi as a Tool for Neuroscientists

RNAi is frequently used in neuroscience and other fields as a technique to study effects of proteins, and as gene therapy, aiming to suppress proteins that cause or exacerbate diseases. Synthetically introduced pri-miRNA, pre-miRNA, or miRNA duplexes are referred to as artificial miRNA, short hairpin (shRNA), or short interfering (siRNA) RNA duplexes, respectively. These exogenous forms of RNAi can utilize the same machinery as mature miRNA, though they may require modification to achieve efficacy, either by design or by the cells transfected. For example, chemically synthesized siRNA duplexes are capable of undergoing phosphorylation by cellular enzymes at the 5’end (Rana, 2007). However, the cell has no machinery to form the 2nt overhang at the 3’end. This change must be introduced in the design of the siRNA. The overhang is not required for silencing but aids incorporation into the RISC, and usually results in higher knockdown efficacy (Kim et al., 2005; Marques et al., 2006; Rana, 2007).

The neuroscience community has capitalized on RNAi applications in many ways, using the technique to study effects of specific genes (Shalizi et al., 2006) and gene therapy studies for inherited neurological conditions (Cearley and Wolfe, 2007; Denovan-Wright et al., 2008). Therapeutic RNAi studies have been performed in rodent models of Huntington’s disease, lipid storage diseases, Alzheimer’s disease, and amyotrophic lateral sclerosis, amongst others (Xia et al., 2004; Harper et al., 2005; Rodriguez-Lebron et al., 2005; Denovan-Wright et al., 2008). With several phase I and II clinical trials investigating siRNA in
neuroscience-related applications, such as macular degeneration (Cearley and Wolfe, 2007; Denovan-Wright et al., 2008), RNAi remains a possibility in the field of genetic therapy.

Another prominent application includes the knockdown of specific genes to study their functions. For example, those interested in identifying various regulators of dendritic spine development and maintenance have utilized RNAi to identify many cytoskeleton effectors necessary for those roles, such as the Rac1 GEF Tiam1 (Tolias et al., 2007), p190 RhoGAP and Rho GTPase (Zhang and Macara, 2008), drebrin and Ras (Biou et al., 2008), Arf GAP centurin alpha1 (Moore et al., 2007), ARF6 and EFA6A (Choi et al., 2006), the synaptic adaptor protein GIT1 (Zhang et al., 2005), and the PKC target MARCKS (Calabrese and Halpain, 2005). The Sabatini group found that activation of the interferon response can decrease spine density and length (Alvarez et al., 2006). On the other hand, loss of RNAi machinery itself has been found to result in abnormally long dendritic spines, up to 12 microns in length (Davis et al., 2008). This suggests that the endogenous miRNA pathway and gene-specific knockdown are critical for normal spine maintenance.

Predicting efficacy of siRNA sequences can be complicated. Different segments of a single mRNA transcript will have different accessibility to siRNA sequences, due to attached proteins or mRNA folding into secondary or tertiary structures (Brown et al., 2005; Westerhout and Berkhout, 2007). This has led to
the common practice of screening different siRNA sequences \textit{in vitro} to identify a sequence with optimal knockdown efficiency (Overhoff et al., 2005). 

A considerable caveat when using siRNA as a tool to investigate protein function involves potential changes to activation states of off-target genes, which include any gene other than those intended for knockdown. Experimental interpretation can be confounded by suppression of unintended genes due to sequence homology. siRNA can also induce expression of off-target genes through the interferon cascade, in a dose-dependent manner (Persengiev et al., 2004; Judge et al., 2005). Other off-target effects can be induced by detection of double stranded (ds) RNA, either by the PKR or 2,5-oligoadenylate synthetase pathways. Both of these latter enzymes can suppress transcription while simultaneously activating genes such those of the interferon pathway, the first line of viral defense in a host cell (Dong and Silverman, 1997; Sledz et al., 2003). Segments of dsRNA over 33nt long can activate an enzyme known as ds-RNA-dependent protein kinase (PKR) (Manche et al., 1992). Once active, PKR can then phosphorylate eIF2, prohibiting methionine-tRNA from binding to ribosomes and, as a result, globally inhibit all protein production (Zamanian-Daryoush et al., 1999).

Interferons are a family of cytokines that can activate immune cells such as macrophages, promote immunity against viruses in nearby cells, and influence cellular proliferation and apoptosis. Interferons transduce signals through receptors at the cell surface and on endosomes, using the Janus Kinase-Signal
Transducer and Activator of Transcription (JAK-STAT) pathway of transcription factors to induce changes in at least 300 genes (Der et al., 1998). Interferon activity can be measured by a variety of methods, including systemic cytokine levels, microarrays, proteomics, and, in the CNS, microglia activation. General rules of thumb have been elucidated for specific structures and sequences that tend to be more predisposed to set off the Interferon response than others (Birmingham et al., 2006). For example, siRNA sequences of over 23nt have been shown to induce the Interferon response more than shorter siRNAs (Hornung et al., 2005). It has also been reported that blunt-ended structures were worse than structures with 2nt 3’ overhangs (Marques et al., 2006), which Dicer-mediated cleavages normally produce. siRNA duplexes with weak thermodynamic strand preference double the risk of these events by allowing RISC to unwind both strands with equal ease (Khvorova et al., 2003).
CHAPTER THREE

POSTNATAL KNOCKDOWN OF NEURONAL NOGO-A LEADS TO A DECREASE IN APICAL DENDRITIC SPINE DENSITY IN THE NEOCORTEX

ABSTRACT

The myelin-associated protein Nogo-A is a well-known inhibitor of axonal regeneration and compensatory plasticity, yet functions of neuronal Nogo-A are not as clear. The present study examined the effects of decreased levels of neuronal Nogo-A on dendritic spines of developing neocortical neurons. Decreased Nogo-A levels in these neurons resulted in lowered spine density, particularly with thin- and mushroom-shaped spines in the apical arbor. Spine density and morphology in the basilar dendritic tree was unaltered by loss of Nogo-A. These results suggest a role for neuronal Nogo-A in maintaining a spine phenotype in neocortical pyramidal cells.
INTRODUCTION

The Nogo-A protein is a potent suppressor of neuronal outgrowth in the intact (Montani et al., 2009) and injured (Gonzenbach and Schwab, 2008) CNS. Anti-Nogo-A immunotherapy after ischemic stroke in the adult rat induces axonal remodeling and functional recovery, and also leads to enhanced dendritic arborization and increased spine density in pyramidal neurons of the contralesional neocortex (Papadopoulos et al., 2006). This finding of effects on spine density led us to investigate the role of Nogo-A in neocortical pyramidal neurons of the undamaged CNS, to determine if neuronal Nogo-A plays a role in dendritic spine development.

Earlier studies of neuronal Nogo-A have shown that expression levels are high in embryonic and neonatal development, particularly in populations such as Purkinje neurons of the cerebellum, pyramidal cells of the hippocampus, and in layer V pyramidal neurons of the sensorimotor cortex of the rodent (Huber et al., 2002; Mingorance et al., 2004; Mingorance-Le Meur et al., 2007). Temporal expression patterns of Nogo-A throughout embryonic and neonatal development indicate that the molecule may participate in processes beyond inhibition of outgrowth and plasticity (Josephson et al., 2001; Caltharp et al., 2007). Indeed, recent work has suggested that Nogo-A is involved in neuronal branching, neuronal migration, and synapse stabilization (Aloy et al., 2006; Mingorance-Le Meur et al., 2007). Specifically, cultured neurons from mice lacking expression of Nogo-A as well as the other Nogo isoforms B and C showed enhanced axonal
branching *in vitro* (Mingorance-Le Meur et al., 2007). *In vivo*, these mice displayed delayed interneuron migration during neocortical development (Mingorance-Le Meur et al., 2007). Also, Nogo-A overexpression in cerebellar Purkinje neurons from postnatal day 7 to adulthood led to a decrease in stability of inhibitory synaptic contacts, a decreased number of synapses overall, and deficits in motor learning and coordination (Aloy et al., 2006).

In order to directly assess the role of neuronal Nogo-A in dendritic spine development *in vivo*, we used a Nogo-A knockdown approach mediated by shRNA, delivered by an adeno-associated virus serotype 2/8 (AAV2/8). AAV2/8 is ideally suited for targeting neuronal Nogo-A because approximately 95% of transduced cells in the CNS are neurons (Allocca et al., 2007; Dodiya et al., 2009). Transduction of oligodendrocytes, the only other cell in the intact CNS that is Nogo-A positive, is negligible (Broekman et al., 2006). Our study demonstrates that shRNA knockdown of neuronal Nogo-A in early postnatal development leads to lowered spine density, particularly with thin and mushroom-shaped spines in apical dendrites of adult neocortical pyramidal neurons. We saw no changes to spines in the basilar arbor of these neurons.
METHODS

AAV production

Plasmids

Plasmids to be used included Nogo-A shRNA, helper plasmid, and AAV2/8. One μg of each plasmid was added to Escherichia coli DH5α bacteria (Invitrogen). Transformation involved a 20 minute incubation period over ice, followed by 45 seconds at 37°C. The bacteria were grown in Luria broth (LB), and mixed in eppendorf Thermomixer at 600 rpm for 30-90 minutes. Bacteria were plated and incubated overnight at 37°C. The following day, a single clone was transferred to 2 mL LB containing ampicillin (100 μg/mL), incubated with agitation overnight, transferred to flasks containing 200 mL amp-LB, and incubated with agitation overnight again. Plasmids were isolated by anion-exchange columns, per maxiprep protocol (Qiagen). Plasmid concentration was estimated by absorbance at UV 260:280 λ. Plasmid identity was verified with restriction enzyme digestion followed by agarose gel electrophoresis and sequencing.

Production

HEK-293 cells (Invitrogen) were grown in Dulbecco’s modified Eagles medium (DMEM) with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin to 70-90% confluence on 15 cm dishes. Cells were washed twice in Phosphate-buffered saline (PBS), and 18 mL of DMEM:5%
Modified Bovine Serum (Virapak transfection kit, Stratagene) + penicillin/streptomycin (100 units/mL) media added to each plate. Briefly, 25 μg of each of the three plasmids were mixed with 100μL of 2.5 M CaCl₂, followed by 750 μL of 2X 2×N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline. The mixture was incubated for 10 minutes at room temperature. 1.8 mL of this solution was added drop-by-drop to each plate, and plates were gently swirled. To assist transfection, incubator CO₂ levels of were dropped to 3% for 12-18 h. After this time, cells were washed twice and media was replaced. Three days post transfection, cells were washed, harvested, and lysed by 3 cycles of freeze/thaw. After the third thaw cycle, lysates were incubated at room temperature for 30 minutes in benzonase (50 U/mL), and then centrifuged at 3700 g for 20 minutes.

**Purification**

An iodixanol purification gradient was created according to (Zolotukhin et al., 1999). Briefly, Quick-seal Ultra-clear 28x89 centrifuge tubes were filled with 5 mL of 60% iodixanol with 30 μL of a 0.5% phenol red stock solution, 5 mL of 40% iodixanol in PBS-MK buffer, 6 mL of 25% iodixanol in PBS-MK buffer with 60 μL of phenol red stock, 9 mL of 15% iodixanol, and 15 mL of the crude lysate. All solutions were added dropwise, by Pasteur pipette, with special care taken to avoid mixing. Tubes were sealed and centrifuged at 18° for 1 hour at 350,000 g. An 18-gauge needle was used to aspirate 5 mL of the 60:40 interface. The virus
was then concentrated by centrifugation through Amicon Ultra-15 100 K filter (Millipore). The buffer was exchanged to PBS through repeated dilutions and centrifugations.

_Titre estimation_

Primate fibrosarcoma HT-1080 cells were cultured in 6-well plates to 80% confluence. AAV2/8 was serially diluted and added to cultures. On the fourth day, media was replaced. Fluorescent cells were counted at four, five, and six days post infection with aid of a hemacytometer. Viral titre was estimated from an average of counts on these three days. Stocks of AAV-Nogo, AAV-Luciferase, and AAV-EGFP were estimated to be $2 \times 10^9$ infectious particles / µL.

_Anhimals_

Long-Evans Black-Hooded male rats (Harlan) were used throughout the study. At postnatal day 5 (P5), litters were size-matched to 8 pups. At weaning (P23), males were housed two or three to a cage, allowed free access to food and water, and maintained on a 10h/14h light/dark cycle. This study was conducted using protocols approved by the Edward Hines Jr. VA Hospital Institutional Animal Care and Use Committee in accordance with National Institutes of Health principles of laboratory animal care.

P3 pups were cryoanesthetized, a 1 cm incision was made in the scalp, and the pup was placed in a neonatal stereotaxic device. Two craniotomies were
performed. Injection coordinates are given in Table 1. With these coordinates, EGFP+ cells were observed in both the hippocampus and sensorimotor cortex. A 32 gauge microsyringe (Hamilton) was lowered into the brain, and left in situ for one minute. 250 nL of virus was slowly injected into each site over a period of one minute. The needle was left in place for one minute before gradual removal over the course of one minute. Multiple injections were performed simultaneously to minimize the time spent under anesthesia. Animals were warmed by a halogen lamp and skin was sutured with 5-0 monofilament. A small bubble of tattoo ink was injected under the skin of the dorsal paw(s) for identification. Each neonate was away from the dam for approximately two hours.

**Verifying Nogo-A knockdown**

HEK293 cells were transfected with decreasing amounts of shNogo plasmid and equal amounts of Nogo-A plasmid using Polyfect Transfection Reagent in accordance with manufacturer’s instructions (Qiagen). At the same time, an identical set of plates were also transfected with shLuciferase plasmid in equimolar amounts to shNogo, to control for promoter competition. Cells were lysed and harvested at three days post transfection. All experiments were replicated at least three times.

SDS-PAGE and western blotting were performed as previously described (Kelleher and Long, 1992). Briefly, 20 mg of cell protein lysates were
Needle positioned at 16° angle to allow space for both needles

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**Table 1. Neonatal stereotaxic coordinates for AAV-injection.**

Coordinates are measured in mm with respect to bregma.
electrophoresed through a SDS-polyacrylamide gel and transferred at 55 V for 90 minutes to a polyvinylidene difluoride membrane. Membranes were dried to visualize transfer efficiency, then rewetted in methanol. Membranes were incubated overnight at 4°C with primary monoclonal antibodies (1:750 diluted in TBS-Tween:5% powdered milk for mouse 11C7, 1:5000 dilution as before for rabbit anti-GAPDH antibody). The membrane was washed with PBS and incubated with an alkaline phosphatase conjugated secondary antibody specific to the primary species (1:5000 dilution; Pierce) for 2 hours at room temperature before washing and reacting the membrane with chemiluminescent substrate (Pierce). Dose-dependent trends band pattern were visualized from three replicate experiments.

To confirm Nogo-A knockdown in vivo, animals were injected as described above, anesthetized (sodium pentobarbital, 1 mg/kg, i.p.) and transcardially perfused with 4% phosphate-buffered paraformaldehyde. Brains were cryoprotected for 24 hours in 30% sucrose solution, sectioned on a cryostat at 40μm, and stored in PBS with azide. Free-floating sections were incubated with blocking solution, Tris-buffered saline (TBS)-0.1%TritonX-100:10%NGS for 1.5 hours, followed by primary antibody overnight in 5% NGS (11C7, 1:750 diluted in TBS:0.1% Triton-X:5% NGS, Novartis). Sections were washed in TBS-0.1%Triton-X-100, and incubated in secondary antibody (Rabbit-anti-mouse Alexa Fluor 594, Molecular Probes A21125, diluted 1:1000, as above) for 2 hours
at room temperature. Sections were again washed, mounted onto slides, and coverslipped.

Ten to twelve 0.5 µm-thick stacks were taken at 63x magnification on a laser-scanning confocal microscope. Three animals were examined per group, with at least fifty EGFP+ cortical cells counted per animal. Of these transduced cells, the total number of Nogo-A immunopositive cells was also counted. The number of double-labeled cells was expressed as a percentage of the total number of transduced cells.

**Dendritic spine imaging via laser-scanning confocal microscopy**

Rats were anesthetized (sodium pentobarbital, 1 mg/kg, i.p.) and transcardially perfused with 4% phosphate-buffered paraformaldehyde. Brains were removed, cut into 200 µm thick sections with a vibratome, mounted onto slides and coverslipped. Images were captured with a confocal microscope and analyzed using NIH ImageJ. Five terminal, 2nd order or higher, EGFP-filled pyramidal dendrites were imaged per animal at 63x water-immersion objective on a scanning laser confocal microscope with 4x zoom. Dendrites from the sensorimotor cortex were chosen from the basilar arbor and from the apical arbor proximal to the tuft of layer V neurons. All protrusions along a 10 µm segment of dendrite were counted from five different neurons per animal. Filopodia dendritic protrusions that did not show a clearly identifiable spine head, as described in (Harris et al., 1992), were counted and each listed separately. For
morphology analysis, maximum head diameter and length were measured for each spine. Protrusions were classified according to (Lippman and Dunaevsky, 2005), as seen in Figure 2. Six rats per group were utilized for basilar spine analysis. To analyze apical spines, 6 and 10 rats were used from the AAV-EGFP and AAV-shNogo groups, respectively. For basilar spine evaluation, 6 animals were used per group.

**Statistical analyses**

Unless otherwise stated, Prism 4 for Windows (GraphPad Software, Inc.) was used to analyze all data via the two-tailed Student’s t-test. In all cases, p < 0.05 was regarded as statistically significant.
RESULTS

Recombinant virus, AAV-shNogo, mediated in vitro and in vivo Nogo-A gene knockdown.

Neocortical layer V pyramidal neurons were transduced following AAV injection (Figs. 4 and 5) into the presumptive primary sensorimotor cortex of P3 rat pups. While we did occasionally observe EGFP-positive cells that resembled astrocytes, approximately 95% of cells transduced had a neuronal morphology (Fig. 6A-H). Pyramidal neurons transduced with the control vector, AAV-EGFP, showed immunoreactivity for Nogo-A, while very few pyramids from the AAV-shNogo group showed any Nogo-A immunostaining (Fig. 6). The average percent of double-labeled cortical cells was 38.3 ± 3.8 in the control vector group, and 9.7 ± 7.7 in the AAV-shNogo transduced group (p=0.029; Fig. 7B), indicating a 75% decrease in the number of transduced, Nogo-A positive neurons. HEK-293 cells transduced with Nogo-A and shNogo-A showed reduction in Nogo-A protein as the dose of shNogo-A plasmid increased (see Fig. 7A for a representative blot).
Figure 4. Representative viral spread from two rAAV2/8 treated rats. Examples of moderate and high viral spread shown at left, and right, respectively. Blue color indicates regions of EGFP signal, as detected at 5x magnification. Figure adapted from Paxinos and Watson, 2006.
Figure 5. Representative AAV2/8-EGFP transduction in sensorimotor cortex. The green color indicates a virally transduced neuron (EGFP). Note the layer of transduced pyramidal neurons. Pictures were taken at 5x, 20x, and 63x magnification. 63x photos were taken on scanning confocal microscope. Modified from Paxinos and Watson, 2006. Scale bar indicates 10 micrometers.
**Figure 6.** Representative *in vivo* knockdown mediated by recombinant virus in neurons. A-D, J-L When injected in the primary sensorimotor cortex of neonatal rats, AAV-EGFP transduced neurons (arrows point to examples) showed Nogo-A immunoreactivity 6 weeks after injection. However, the AAV-shNogo transduced neurons showed negligible Nogo-A immunoreactivity (E-H, M-O). j-o Scanning confocal microscope images, at a higher magnification, demonstrate loss of Nogo-A. Scale bar: D, H: 100 µm, O: 10 µm.
Figure 7. Diminished Nogo-A protein levels following knockdown in vitro and in vivo. (A) Plasmid transduced HEK293 cells show dose-dependent decrease in Nogo-A protein levels. Blot shown is representative of a trend observed in three separate replications. Numbers indicate the molar ratios of shRNA:target. (B) When assessing cells from neocortical tissue sections labeled for viral transduction via EGFP, AAV-EGFP-shNogo transduced neurons show a reduction in Nogo-A immunoreactivity, when compared to AAV-EGFP control virus. Data pooled from 50 cells/animal, 3 animals/group. * indicates p < 0.05, two-tailed Student’s t-test, error bars indicate SEM.
Neuronal Nogo-A knockdown in vivo results in reduced apical dendritic spine density and alterations in apical spine morphology.

After a 6 week survival period, EGFP-positive layer V pyramidal neurons from rats transfected with AAV-EGFP had an average spine density of $10.7 \pm 0.6$ per 10 µm of dendrite length (see Fig. 2 for representative image). A 26% reduction in neuronal dendritic spine density was detected in neurons transduced with AAV-shNogo, with an average spine density of $7.9 \pm 0.6$ per 10 µm of dendrite length ($p = 0.006$, Fig. 8A).

Neurons from animals transduced with AAV-shNogo exhibited a significant decrease in the proportion of thin-type spines ($0.8 \pm 0.2$; $p = 0.03$) as compared to neurons transduced with the control virus AAV-EGFP ($1.7 \pm 0.4$; Fig. 8B). A decrease in mushroom-shaped spines was also observed in adult neocortical pyramidal neurons following loss of Nogo-A ($6.2 \pm 0.5$ per 10 µm of dendrite length for AAV-EGFP and $4.2 \pm 0.5$ for AAV-shNogo, $p = 0.01$, Fig. 8C).

Densities of filopodia protrusions ($1.0 \pm 0.2$ per 10 µm of dendrite length for AAV-EGFP and $1.1 \pm 0.1$, $p = 0.62$, data not shown), stubby ($2.1 \pm 0.1$ per 10 µm of dendrite length for AAV-EGFP and $2.6 \pm 0.3$ for AAV-shNogo, $p = 0.14$, data not shown) and bifurcated spines ($0.5 \pm 0.2$ per 10 µm of dendrite length for AAV-EGFP and $0.3 \pm 0.1$ for AAV-shNogo, $p = 0.22$, data not shown) were similar between groups.

Spine length ($0.59 \pm 0.03$ µm for AAV-EGFP and $0.62 \pm 0.03$ µm for AAV-shNogo, $p = 0.43$) and maximal spine head width ($0.89 \pm 0.05$ µm for AAV-EGFP
and 1.00 ± 0.10 µm for AAV-shNogo, p = 0.29) were measured, with no significant differences observed between groups (data not shown). When measurements were sorted by bins of 0.4 microns, a decrease in the number of very long protrusions was observed in the Nogo-A knockdown group (longer than 1.2 µm, p = 0.002, Fig. 9A). Similarly, when spine width was binned, a slight decrease in the number of wide protrusions was observed (width between 0.6-0.849, p = 0.047, Fig. 9B). Together, these results suggest that postnatal Nogo-A gene knockdown influences apical spine dynamics in layer V neocortical pyramidal neurons.

**Reduced levels of postnatal, neuronal Nogo-A did not alter basilar spine density or morphology.**

As seen in Figure 10, basilar spine density was unchanged following Nogo-A knockdown (9.1 ± 1.2 spines per 10 µm dendrite length for AAV-EGFP and 7.8 ± 0.7 for AAV-shNogo, p = 0.38, Fig. 10). Similarly, filopodia protrusions (1.4 ± 0.5 per 10 µm dendrite length for AAV-EGFP and 1.1 ± 0.3 for AAV-shNogo, p = 0.64, data not shown), mushroom (4.3 ± 0.5 per 10 µm dendrite length for AAV-EGFP and 3.7 ± 0.5 for AAV-shNogo, p = 0.40, Fig. 10), stubby (2.3 ± 0.4 per 10 µm dendrite length for AAV-EGFP and 1.9 ± 0.3 for AAV-shNogo, p = 0.47, Fig. 10), thin (1.8 ± 0.2 per 10 µm dendrite length for AAV-EGFP and 1.4 ± 0.1 for AAV-shNogo, p = 0.14, data not shown), or bifurcated spine morphology (0.7 ± 0.1 per 10 µm dendrite length for AAV-EGFP and 0.6 ± 0.1 for AAV-shNogo, p =
0.71, data not shown) were not altered by the loss of Nogo-A in postnatal development.
Figure 8. Apical sensorimotor cortex spine density and morphology following postnatal loss of Nogo-A. (A) Spine density was substantially decreased in response to Nogo-A knockdown. (B) A decrease in mushroom- and (C) thin-type spines was also observed. * indicates $p < 0.05$ via two-tailed Student’s t-test, $n = 6$ and 10 from EGFP and shNogo groups, error bars indicate SEM.
Figure 9. Apical sensorimotor cortex morphology following postnatal loss of Nogo—Decreases in the density of very long spines (A) and wide spines (B) were observed following postnatal loss of Nogo-A. * indicates $p < 0.05$ via two-tailed Student’s t-test, $n = 6$ and 10 from EGFP and shNogo groups, error bars indicate SEM.
Figure 10. Basilar sensorimotor cortex spine density and morphology following postnatal loss of Nogo-A. (A) No substantial differences were observed in spine density or spine morphology (B, C). Data pooled from 6 animals per group, Error bars indicate SEM.
DISCUSSION

The results of this study indicate that postnatal suppression of Nogo-A by AAV-delivered shRNA leads to a 26% reduction in spine density in apical dendrites of pyramidal neurons in layer V of the sensorimotor neocortex. Furthermore, morphological analysis of these transduced neurons showed a 52% and 32% significant decrease of in mushroom and thin spines, respectively suggesting a shift in spine morphology in the apical dendrites of neurons with decreased amounts of Nogo-A. Postnatal Nogo-A knockdown also altered morphology so there were 37% fewer protrusions with very long necks compared to control group, and 22% fewer protrusions of moderately-wide diameter. No changes were observed in basilar spine density or morphology between groups.

Nogo-A was initially isolated from CNS myelin and identified as a highly potent inhibitor of neurite outgrowth (Caroni and Schwab, 1988). Over the past two decades, numerous studies of anti-Nogo-A antibody infusion in animal models of spinal cord injury and stroke have shown that this treatment results in functional recovery, axonal regeneration, and neuronal plasticity (Gonzenbach and Schwab, 2008). In particular, contralesional layer V neurons of the forelimb sensorimotor cortex displayed increased axonal sprouting (Seymour et al., 2005), dendritic complexity, and spine density (Papadopoulos et al., 2006) following stroke and anti-Nogo-A immunotherapy in adult rats. However, after knockdown in neonatal rats, our results of decreased spine density and altered spine morphology support the hypothesis that an additional function of Nogo-A may be
to promote spine development. When considering the present result of a decrease in spine density seen after Nogo-A knockdown as compared to our previous result of an increase in spine density after stroke and immunotherapy, several explanations are possible. First, the age of the animals was different, and decreasing the levels of Nogo-A protein in developing neurons could have led to a different result than administering the anti-Nogo-A therapy in the adult animal. Second, the axonal sprouting observed after anti-Nogo-A immunotherapy may have led to a retrograde activity- or trophic-induced increase in spine formation in the adult animals. Third, mechanistic differences between an antibody-mediated therapy to neutralize global Nogo-A and direct loss of the protein in neurons through RNAi cannot be ruled out; anti-Nogo-A shRNA affected neuronal development over eight weeks’ time, where the antibody was administered to an established nervous system for only two weeks. Furthermore, the knockdown approach eliminated surface and ER-localized Nogo-A, but the antibody only affected molecules at the plasma membrane.

The changes in spine morphology seen in the apical tree are representative of a deeper change in neuron functioning. Spine morphology is known to change after LTP in hippocampal and cortical neurons, producing larger spine heads, and shorter and wider spine necks in an actin-dependent manner (Fifkova, 1985; Monfils and Teskey, 2004; Kopec et al., 2006). The decrease in both of these measures seen after knockdown may indicate alterations in the formation of LTP in neurons lacking postnatal Nogo-A. Furthermore, we also observed changes in
apical spines when morphology was assessed by manually assigning spines to different categories (Fig. 2, Chapter 2). This method is limited by the continuum that exists between forms (Arellano et al., 2007a), but it does suggest alterations in the distributed form of computing that spines perform (Yuste, 2010). Also, mushroom spines have been shown to be more stable, so the loss of mushroom spines observed here in response to neuronal Nogo-A knockdown may indicate an increase in spine turnover (Parnass et al., 2000).

A clear future direction for this work would be to examine the possibility of off-target effects. As an shRNA sequence, shNogo can avoid activating interferon pathways as siRNA duplexes that lack the 2 NT overhang would. Also, because our sequence is only 19-nt long, it is able to avoid PKR off-target pathways which are triggered by RNA sequences over 33 NT long. Previous work in our lab has shown that spine density is not affected by exogenous short hairpin delivered by AAV2/8 over a 7 week postnatal time period. This hairpin was directed against firefly Luciferase and the plasmid also encoded for the fluorophore ZS-green (Pradhan et al., 2010). This hairpin would not control for shNogo856 off-target effects because these effects are specific to the sequence used for RNAi. A BLAST search shows that shNogo856 only targets Nogo-A in Rattus norvegicus to a significant degree of sequence matches (E value < 0.1). Another recently developed algorithm has indicated that Foоцен-m1, a poorly-characterized member of the reticulon family, may be susceptible to regulation via the shNogo856 sequence, though this protein is the only match besides Nogo-A
(Ryan et al., 2008). Future studies would benefit from use of a second vector to knock down Nogo-A, directed against a different sequence, to rule out the possibility of off-target effects. Further insight into this possibility may be gained from an examination of interferon gene expression.

The lack of response in the basilar spines to the loss of neuronal Nogo-A was unexpected, but may be due to subtle differences in wiring between the different regions on the dendritic tree. For example, the proximal apical and basilar arbors are innervated by layer 5 neurons from the ipsilateral cortex. The basilar arbor receives additional inputs from layer 3 neurons from the contralateral cortex, and thus serves to also integrate regions from the different cortices (Deuchars et al., 1994; Lubke et al., 1996; Thomson and Bannister, 1998). Hippocampal slice cultures treated with anti-Nogo-A antibody display similar differences in responsiveness between different regions of the hippocampal dendritic arbor. Specifically, pyramidal neurons of the CA1 that had been treated with the anti-Nogo-A antibody 11C7 showed increase in stubby-type spines of the distal apical arbor, but no change to other dendritic regions. Pyramidal neurons of the CA3 receiving the same treatment saw no change to dendritic complexity in their proximal apical tree, but a statistically significant increase in complexity of the distal apical tuft (Zagrebelsky et al., 2010). While these slice cultures were obtained from P5 mouse pups and maintained for at least three weeks in vitro, they can not translate directly into this study on this nine-week postnatal developmental window in vivo. Nevertheless, the results
from this study support the general hypothesis that Nogo-A signaling can maintain synaptic and dendritic structures.

Several mechanisms may underlie Nogo-A’s role in plasticity and development, including the actions of intracellular Nogo-A, and interaction with a Nogo-66 receptor (NgR1) or integrins (Voeltz et al., 2006). NgR1 has been shown to mediate changes in neuronal plasticity (McGee et al., 2005), including spine morphology, synaptic function (Lee et al., 2008; Raiker et al., 2010), and length of neuronal stem cell neurites (Wang and Zhu, 2008). Integrins can interact with the amino-terminal of Nogo-A (Hu and Strittmatter, 2008), and have also been implicated in dendritic spine plasticity (Shi and Ethell, 2006; Webb et al., 2007). Nogo proteins have been implicated in migration of neocortical interneurons during embryonic development in vivo (Mingorance-Le Meur et al., 2007). Loss of these proteins in cultured cortical neurons increases both the rate of neurite polarization and the magnitude of neurite branching, potentially through intracellular mechanisms (Mingorance-Le Meur et al., 2007). Indeed, intracellular Nogo-A has been reported to induce topological changes in another membranous structure, the ER (Voeltz et al., 2006; Kiseleva et al., 2007). While the molecular mechanism of spine loss observed here has yet to be uncovered, our results suggest that endogenous levels of Nogo-A expression in postnatal development are necessary for the formation and maintenance of spine density and morphology.
CHAPTER FOUR

POSTNATAL KNOCKDOWN OF NEURONAL NOGO-A LEADS TO A DECREASE IN MESSAGE LEVELS OF GENES IMPORTANT FOR SYNAPTIC PLASTICITY

ABSTRACT

Nogo-A protein is an important regulator of neurite outgrowth, and is a recognized inhibitor of functional recovery and anatomical plasticity after CNS injury. This negative role Nogo-A plays in the injured CNS has led researchers to investigate beneficial roles that may have aided in the evolutionary conservation of this protein. To examine the roles of this protein at the synapse of the intact CNS, we used RNAi directed against Nogo-A and delivered it to the developing rat sensorimotor cortex via AAV2/8, a neurotropic vector. We examined regions of transduced cortex via qRT-PCR for message levels of molecules important for synaptic plasticity, including Neuroligin-1, NMDA receptor subunits NR2A and NR2B, and PSD-95. We found that mRNA of Neuroligin-1 and NR2B were substantially reduced, with no changes to PSD-95 and NR2A expression. These results suggest that neuronal Nogo-A may regulate synaptic plasticity by means of controlling the molecular composition at synapses. This finding represents a novel role for Nogo-A in neurons of the uninjured CNS.
INTRODUCTION

The potent neurite outgrowth inhibitory protein Nogo-A is found in myelin sheaths and in some subsets of neurons (Huber et al., 2002; Wang et al., 2002b; Liu et al., 2003; Cheatwood et al., 2008). Studies of oligodendrocyte-associated Nogo-A have shown the protein causes axonal growth cone collapse, at least in part through the downstream signaling of NgR1, which is found in neurons (Chivatakarn et al., 2007; Raiker et al., 2010). Antibody therapies directed against Nogo-A have shown promise in preclinical and clinical stage I trials for the treatment of spinal cord injury (Freund et al., 2006; Zorner and Schwab, 2010). This same treatment has also led to functional recovery in rat models of stroke (Wiessner et al., 2003; Tsai et al., 2010), even if the treatment is not administered until two months after injury (Tsai et al., 2010). An understanding of how Nogo-A regulates plasticity at the synapse may aid in the discovery of new therapeutic avenues for several neurological conditions.

To determine the role of Nogo-A at the synapse, we examined the expression pattern of molecules that are elements of the functional excitatory synapse following postnatal knockdown of neuronal Nogo-A. We chose to examine rat neuron development because this is where cortical neuron synaptogenesis occurs, and neuronal Nogo-A expression peaks at this time (Huber et al., 2002; Hunt et al., 2003; Buss et al., 2005; Mingorance-Le Meur et al., 2007). The resulting molecular phenotype may give insight into synaptic plasticity of neurons that lack Nogo-A. We examined the molecules post-synaptic
density 95 (PSD-95), the NR2A and NR2B subunits of the NMDA receptor, and neuroligin 1. PSD-95 is an intracellular postsynaptic anchoring molecule localized to active synapses that serves to stabilize many key elements of synaptic transmission, including glutamate receptors and the actin cytoskeleton (Blanpied et al., 2008; Chen et al., 2008). A shift in the predominance of NMDA receptor subunits NR2A and NR2B is an indicator of a change in the threshold of use-dependent plasticity (Tang et al., 2001; Liu et al., 2004; Fu et al., 2005; Yashiro and Philpot, 2008; Corson et al., 2009). Neuroligin-1 is an intercellular anchoring molecule, one of the final molecules to be delivered to the site of synapse assembly (Nam and Chen, 2005; Gerrow et al., 2006; Barrow et al., 2009). These molecules are all mediators of excitatory signaling and known to regulate synaptic plasticity, including long-term potentiation. We also examined levels of the Nogo-A splice variant Nogo-B and Cofilin, a known downstream target of Nogo-A signaling. This allowed us to compare our model to others that have demonstrated changes in these molecules in Nogo-A knockout mice (Dimou et al., 2006; Montani et al., 2009).

In rats with neuronal Nogo-A knockdown, we saw a decrease with respect to control group in the expression of NR2A, NR2B and Neuroligin-1, with no substantial differences to PSD-95, Nogo-B or Cofilin.
METHODS

Quantitative RT-PCR (qRT-PCR)

Isolation of mRNA

P3 rats were injected with AAV as described in Chapter Three. At P65, rats were decapitated and their brains were rapidly removed, frozen in isopentane, and stored at -80°C until further processing. Approximately 30 milligrams of tissue from the sensorimotor cortex was quickly dissected over ice and homogenized in ice-cold TRIzol (Invitrogen). During the course of microdissection, the brain was frequently rotated between the dissection plate and dry ice to prevent the tissue from thawing to room temperature. Regions were chosen based on previous studies of viral spread. Total RNA was extracted per (Chomczynski and Sacchi, 2006)). Genomic DNA was degraded and eliminated using TURBO DNA-free (Ambion) according to manufacturer’s instructions. Five µg of RNA was reverse-transcribed to synthesize first-strand complementary DNA (cDNA) using Superscript II (Invitrogen), and the resulting cDNA was diluted to 100 µL. “No template” controls were used to detect any potential contamination at the reverse-transcription step, and also with every set of PCR reactions.
**PCR reaction**

For each PCR reaction tube, 1 µL of cDNA templates (diluted as described above) was added to a solution of primer pairs (Table 2) and SYBR-green Supermix (Bio-Rad), with the exception of EGFP cDNA, which was amplified using EGFP primers (QT01171611, QIAGEN) and QuantiTect SYBR Green PCR Master Mix (QIAGEN). SYBR-green fluorescence was detected at the end of every extension step (Table 2). All real-time PCR reactions were performed in triplicate, with the average cycle threshold (Cₜ) value used to quantify cDNA. Relative cDNA amounts were calculated according to (Pfaffl, 2001), with GAPDH as the reference gene. Absolute EGFP expression was used to identify samples with high transduction, and the animals in the upper 50th percentile of EGFP expression were used in analysis. Six animals in the EGFP group and five animals in the shNogo group were used in the final analysis.

**Primers**

For each set of primer pairs, end products were run on gels to verify product base pair (bp) size until the primer set was optimized (Fig. 13), at which point the thermal melting curve analysis was used to verify specificity. Amplification efficiency of all target and reference genes was calculated from the Cₜ values of serially diluted templates. Serial dilutions of cDNA, amplified using all primer sets, replicated in a linear fashion (in all cases, r² > 0.99).
Statistical analyses

Due to the inherent variability in the qRT-PCR technique, mathematical outliers, as determined by Tukey fences, were excluded from analysis. Prism 4 for Windows (GraphPad Software, Inc.) was used to analyze all data via the F-test to compare variances. In cases where variances between groups were not significantly different, data was analyzed by the two-tailed Student’s t-test. If variances between groups were found to be different, data was then analyzed via two-tailed Student’s t-test with Welch’s correction. In all cases, p < 0.05 was regarded as statistically significant.
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Table 2. Primer sequences, anticipated product size, and cycling parameters for candidate genes.
Figure 11. Representative gel banding patterns for primer sets used in qRT-PCR experiments. Indicated PCR products from microdissected tissue were run on agarose gels. DNA ladder (bp) indicated on the left. Duplicates and triplicates in the bottom panel represent 10- and 100-fold dilutions of the original amount of cDNA, respectively.
RESULTS

AAV-shNogo leads to reduced levels of Nogo-A, with no changes to Nogo-B or Cofilin expression.

We first sought to validate the selected method of tissue sampling, which involved microdissection by hand of neocortical regions transduced with AAV. Because this method was known to contain both transduced and untransduced neurons, we chose to examine only the samples with the highest amount of AAV transduction, as assessed by EGFP expression. Using this method, we analyzed the relative amount of the shRNA target, Nogo-A. Relative expression was calculated by the differences in C_T values between the gene of interest and the GAPDH housekeeping gene, which is unaffected by Nogo-A knockdown (p = 0.34, Fig. 12). Relative message levels of Nogo-A were significantly reduced in AAV-shNogo samples (0.12 ± 0.05) with respect to the AAV-EGFP control group (1.00 ± 0.33, p = 0.047, Fig. 13A).

The next objective was to compare our neuronal Nogo-A knockdown rat model to the Nogo-A knockout mouse paradigm, which displayed two-fold increase in Nogo-B protein (Dimou et al., 2006; Montani et al., 2009). Relative quantities of Nogo-B mRNA remained unchanged between groups (0.45 ± 0.18 for AAV-shNogo and 1.00 ± 0.30 for AAV-EGFP control, p = 0.21), and Cofilin expression was similar between groups (0.56 ± 0.17 for AAV-shNogo and 1.0 ± 0.14 for AAV-EGFP, p = 0.08, Fig. 13). Therefore, our knockout model used here has the advantage of lacking at least the Nogo-B compensation issue.
Postnatal loss of Nogo-A influences expression of genes important for synaptic plasticity.

Relative mRNA levels of the post-synaptic scaffold PSD-95 (1.00 ± 0.25 for AAV-EGFP and 1.23 ± 0.39 for AAV-shNogo, p = 0.62, Fig. 14) and the NMDA receptor subunit NR2A (1.00 ± 0.45 for AAV-EGFP and 0.18 ± 0.04 for AAV-shNogo, p = 0.14) displayed no substantial differences between groups. There were significant decreases to message levels of Neuroligin-1 (1.00 ± 0.10 for AAV-EGFP and 0.40 ± 0.11 for AAV-shNogo, p = 0.003) and NR2B (1.00 ± 0.18 for AAV-EGFP and 0.14 ± 0.06 for AAV-shNogo, p = 0.005) following knockdown of postnatal, neuronal Nogo-A (Fig. 14). The ratio of NR2A/NR2B was similar between groups (1.00 ± 0.47 for AAV-EGFP and 4.79 ± 2.41 for AAV-shNogo, p = 0.22).
Figure 12. GAPDH expression was similar between groups, as determined by two-tailed Student’s t-test with Welch’s correction. This holds true even if the two mathematical outliers in the shNogo group were to be excluded. n = 6 for EGFP and n = 5 animals for shNogo, error bars indicate SEM.
Figure 13. Relative expression levels of Nogo-A, Nogo-B, and Cofilin mRNA in samples with highest AAV transduction. (A) Levels of Nogo-A were decreased in response to AAV-shNogo, while (B) Nogo-B expression was unchanged. (C) Cofilin mRNA was not appreciably different in response to loss of Nogo-A. Levels are relative to each sample’s GAPDH C\textsubscript{T} value, and results for each candidate gene are normalized to the average value of the AAV-EGFP control group. * indicates p < 0.05 via two-tailed Student’s t-test, n = 6 for EGFP and n = 5 animals for shNogo, error bars indicate SEM.
Figure 14. Relative expression levels of selected postsynaptic molecules’ mRNA in samples with highest AAV transduction. (A)
While the levels of PSD-95 and (B) NR2A were unchanged in response to loss of Nogo-A, there was a reduction in the amount of (C) Neuroligin-1 and (D) NR2B message. Levels are relative to each sample’s GAPDH C_T value, and results for each candidate gene are normalized to the average value of the AAV-EGFP control group. * indicates p < 0.05 via two-tailed Student’s t-test, n = 6 for EGFP and n = 5 animals for shNogo group, error bars indicate SEM.
DISCUSSION

The results from this study show that postnatal knockdown of Nogo-A results in an 88% reduction in Nogo-A mRNA, 60% reduction Neuroligin-1, and an 86% decrease in the NMDA receptor subunit NR2B in the sensorimotor cortex when compared to the AAV-EGFP control group. No change was detected in the message levels of Nogo-B, Cofilin, and PSD-95 compared to controls. The NMDA receptor subunit NR2A did not show a statistically significant reduction in mRNA, but there was an 82% decrease when compared to the control group.

The excitatory synaptic adhesion molecule Neuroligin-1 was decreased in rats with neuronal Nogo-A knockdown. Neuroligin-1 and -2 are found exclusively at excitatory and inhibitory synapses, respectively (Graf et al., 2004; Levinson and El-Husseini, 2005; Chubykin et al., 2007). Using a postnatal mouse overexpression paradigm of Nogo-A in cerebellar Purkinje neurons, Aloy et al. (2006) saw a decrease of Neuroligin-2 as well as several other indications of synaptic disassembly. Interestingly, our model of decreased Nogo-A protein levels resulted in a decrease in Neuroligin-1, although we did not examine Neuroligin-2 and they did not examine Neuroligin-1. However, the fact that overexpression and knockdown paradigms led to similar changes in Neuroligin expression—and synaptic destabilization—may be due to potential model-specific effects, or the difference between inhibitory and excitatory synapse regulation.

We found that Nogo-A knockdown led to a significant decrease in the message levels of the glutamatergic NMDA receptor subunits NR2B, but only a
trend toward decreased NR2A. NMDA receptors are key mediators of synaptic plasticity and an established means of coincidence detection (see Chapter Two). NR2-A and -B subunit expression represents a kind of reciprocal control of the threshold for modifying synaptic strength. Neuronal activity generally facilitates transcription and surface delivery of NR2A, but it represses transcription of NR2B and facilitates NR2B degradation (Fu et al., 2005; Yashiro and Philpot, 2008; Corson et al., 2009).

In the first weeks of postnatal development, NR2B subunits predominate over NR2A. This pattern is shifted in the young adult, where there are more NR2A subunits than NR2B (Tang et al., 2001; Liu et al., 2004; Giza et al., 2006). We did not see a shift in the ratio between NR2A/NR2B between the young adults in each treatment group. Instead, both subunits decrease to a similar degree in the AAV-shNogo group—82% decrease in NR2A and 86% decrease in NR2B—perhaps suggesting a broader decline in the expression of this receptor entirely. The growth of spines into a mushroom morphology requires the presence of NMDA receptors (Matsuzaki et al., 2004), and the decrease in NMDA receptor subunits seen here in response to loss of Nogo-A may be linked to our previous finding of fewer mushroom spines in animals after postnatal Nogo-A knockdown (Chapter three). The result may also relate to other laboratories’ observations of altered LTP in Nogo-A knockout mouse hippocampal neurons, since NMDA receptors are known mediators of the coincidence detection required for LTP (Delekate et al., 2011).
It is important to note that NMDA receptors are found at sites other than synapses. In the rat hippocampus, approximately 36% of the total dendritic NMDA receptor pool can be extrasynaptic (Harris and Pettit, 2007). Furthermore, signaling through this second pool of NMDA receptors appears to have a different functional consequence than signaling at synapses. Signaling through nonsynaptic NMDA receptors can lead to neurotoxicity (Hardingham and Bading, 2010). Potential mechanisms involved in the differential signaling between synaptic and nonsynaptic NMDA receptors may include the different types of signaling complexes available at these sites, and the nature of activation such as transient spikes at the synapse compared to chronic low-level signaling as a result of increased extracellular glutamate at nonsynaptic NMDA receptors (Hardingham and Bading, 2010).

It is possible that the decrease in NMDA receptor subunits seen here may reflect a loss of receptors at extrasynaptic sites. This would be in keeping with a pro-apoptotic role that has been proposed for Nogo proteins (Chen et al., 2006; Kuang et al., 2006; Xu et al., 2006; Teng and Tang, 2008). However, the synaptic pool of NMDA receptors is most likely affected by postnatal neuronal knockdown, given the estimated proportion of extrasynaptic NMDA receptors comprising about a third of all NMDA receptors (Harris and Pettit, 2007), and decreases upwards of 80% in NMDA receptor subunits in our samples (Figure 14).
We saw no significant changes to Cofilin or Nogo-B expression between rats with postnatal, neuronal Nogo-A knockdown and the control group. This result is in contrast to Nogo-A knockout mice, which displayed alterations in Cofilin signaling and a compensatory increase in Nogo-B expression (Dimou et al., 2006; Montani et al., 2009). This difference in results could be due to the way knockout mice are generated, i.e., all Nogo-A in all cell types would be absent, whereas our only target using the viral mediated knockdown approach is neuronal Nogo-A. Furthermore, while Nogo-B expression has not been linked to altered dendritic structure (Zagrebelsky et al., 2010), the lack of Nogo-B compensation might confer a slight advantage to our AAV-mediated knockdown model used here.

In our study, the relative mRNA expression of the excitatory postsynaptic scaffolding molecule PSD-95 remained unchanged. PSD-95 expression has been linked to the density and size of spines (El-Husseini et al., 2000; Chang et al., 2009), although synaptic plasticity has also been observed with no change to overall protein density in PSD-95 (Blanpied et al., 2008). One possible interpretation of our data is that Nogo-A knockdown may lead to an increase in PSD-95 per spine, given the decrease in spines seen in the apical arbor (Chapter three). It is also possible that the lack of change is influenced by untransduced neurons, or the continued expression of Nogo-B which can signal through NgR1 through its Nogo-66 loop (see Fig. 1). A third possible interpretation is that regulation of the PSD-95 scaffold occurs in this system by another means. The
protein has a half life of approximately 100 minutes at the postsynaptic density (Gray et al., 2006), so it is reasonable to assume that mRNA levels may serve as a sensitive indicator of protein levels. However, PSD-95 activity is known to be regulated by extrasynaptic localization and phosphorylation in addition to protein expression, and it is possible that these methods of post-translational regulation are simply more fundamental than translational means (Bence et al., 2005; Morita et al., 2006; Barrow et al., 2009). A study of PSD-95 protein levels and localization using immunohistochemistry and high-resolution imaging might clarify this matter.

In conclusion, we found that loss of postnatal Nogo-A in neurons of the sensorimotor cortex is associated with a decrease in several molecules key for glutamatergic transmission and plasticity: Neuroligin-1 and the NMDA receptor subunits NR2A and NR2B. The lack of change to PSD-95 indicates that some elements are still intact. We propose a role for neuronal Nogo-A in the distribution of molecules important for excitatory synaptic function over the postnatal window of development. Our findings give further insight into the roles of this protein in the intact CNS, although additional study is needed to determine the molecular mechanisms underlying this finding.
CHAPTER FIVE

POSTNATAL KNOCKDOWN OF NEURONAL NOGO-A LEADS TO A DECREASE IN THE APICAL COUNT OF AN EXCITATORY PRESYNAPTIC MARKER, VGLUT1, IN THE ADULT SENSORIMOTOR CORTEX

ABSTRACT

Anti-Nogo-A immunotherapy has shown great promise in promoting functional recovery and anatomical plasticity in rodent and primate models of CNS injury. Studies of Nogo-A function in the uninjured rodent have suggested a role in neuronal development. To study the role of Nogo-A in the intact neocortex, we used vesicular glutamate transporter 1 (vGlut1) as a presynaptic marker for potential excitatory synapses. We found that knocking down Nogo-A in postnatal pyramidal neurons of the sensorimotor cortex led to a decrease in the number of vGlut1 puncta identifying a potential presynaptic partner, in opposition to the apical dendritic shaft. Though we found no changes to vGlut1 density in the basilar arbor, the decreased vGlut1 in the apical arbor likely represents a loss of potential synapses that may have a strong influence on direct current injected into the dendrite.
INTRODUCTION

Our previous work has shown that loss of neuronal Nogo-A leads to a decrease in the density of apical dendritic spines in neocortical pyramidal neurons (Chapter 3). We also found that this treatment leads to a decrease in the expression of several molecules key for synaptic structure and plasticity (Chapter 4). Nogo-A’s putative roles in synaptic plasticity are thought to include the regulation of axonal outgrowth and stabilization, as well as presynaptic release of dopamine, although this latter function is less well-studied (Xiong et al., 2008; Schwab, 2010; Zagrebelsky et al., 2010). The question remains whether Nogo-A in neurons can affect presynaptic machinery as it does within oligodendrocytes.

Excitatory dendritic synapses are present at all stages of brain development and often have a strong influence on dendritic currents. While dendrite shafts are more often reserved for inhibitory synapses, estimates of glutamatergic shaft synapses can range from 4-10% of a neuron’s total excitatory synapses in adulthood (Gray, 1959; Boyer et al., 1998). In early postnatal development, this number can increase to as much as 50% (Boyer et al., 1998).

In our attempt to study the effects of neuronal Nogo-A on properties of the excitatory synapse, we used deconvolution microscopy of fluorescently-tagged, potential presynaptic sites in opposition to EGFP-filled dendrites. Deconvolution microscopy offers a similar resolution capacity as laser confocal microscopy but has the advantage of very little photobleaching associated with high-resolution images, allowing improved quantification of fluorescence. Identifying
presynaptic markers opposing spines and dendrites is facilitated by the resolution limit of deconvolution microscopy. The width of the synaptic cleft is approximately 20 nm, maintained through the collective action of many pairs of transsynaptic anchoring molecules. While fluorescently labeled proteins on either side of this 20 nm cleft are not truly in the same compartment as one another, they will appear at least partly colocalized when imaged by deconvolution microscopy, since the technique has a resolution limit of <200 nm. The color shift (yellow puncta in Figs. 17, 18) that results actually aids in colocalization analysis.

We chose to use vesicular glutamate transporter 1 (vGlut1) as a marker for potential excitatory synapses because these synapses by definition use glutamate as a neurotransmitter. We found that knocking down Nogo-A in postnatal neurons leads to a decrease in the apical density of potential presynaptic partner, vGlut1, on dendritic shafts. This likely represents a loss of potential synapses that may have a strong influence on direct current injected into the dendrite.
METHODS

Immunohistochemical staining

Rats were injected with AAV and perfused as described in Chapter Three. Brains were cryoprotected for 72 hours in 30% sucrose solution, cut at 40 µm thickness on a cryostat, and stored in PBS with azide for no more than three weeks prior to staining. Tissue sections were assessed for EGFP expression on uncoated slides at low magnification, and six optimal sections were chosen for each rat sample (Fig. 15). Selected free-floating tissue sections were permeabilized with 30 mM PIPES buffer-0.1% saponin for six hours at room temperature before postfixing in 4% PFA for five minutes. Sections underwent four washes in buffer (TBS-0.3% Triton-X-100) for five minutes each. Blocking was performed for 90 minutes at room temperature in buffer containing 10% Normal Donkey Serum (NDS).

Primary polyclonal antibody directed against guinea-pig vesicular glutamate transporter 1 (vGlut1), at 1:4000 dilution (Millipore AB5905) in buffer containing 5% NDS was added for three nights at 4 °C, with agitation. Sections were rinsed in buffer for three 20 minute washes and one 40 minute wash. DyLight649 anti-guinea pig secondary, 1:400 dilution (Jackson ImmunoResearch 706-495-148) in buffer containing 5% NDS was allowed to incubate overnight at 4 °C. Sections were rinsed for five 20 minute washes in buffer, one 60 minute wash in buffer, and a final wash for 5 mins in 0.1M phosphate buffer before mounting to gel-coated slides. Slides were dried,
coverslipped in Fluoromount-G mounting medium, and stored at 4°C for at least one day prior to imaging (Fig. 15). For each sample, a “no primary” control was utilized to assess background fluorescence as well as nonspecific staining from the secondary antibody. Sections from different rats were stained and imaged in parallel, and the experimenter was blinded with regard to treatment groups throughout all staining, imaging, and analysis procedures.

**Synaptic protein imaging via deconvolution microscopy**

EGFP-filled excitatory pyramidal neurons from the sensorimotor forelimb cortex region were identified by morphology. A total of six rats were examined per treatment group. For each rat, z-stacks of dendritic segments from five different neurons were imaged in each the basilar and proximal apical arbors. Apical and basilar arbors were differentiated by morphology, where apical arbors are characterized by a thick primary dendrite extending from the pial-most region of the neuron cell body towards the pial surface, and basilar dendrites comprise all other branches originating on the cell soma. Dendritic segments analyzed were required to be at least 10 µm in length, and their dendritic protrusions had to be completely encapsulated within the z-stack.

Z-stacks of images were taken on an inverted microscope (Olympus) using a 100x oil-immersion objective. Focal planes were spaced 0.3 µm apart on the z-axis, at 1024x1024 resolution, binning of 1x1. Image stacks were deconvoluted via softWoRx Deltavision (Applied Precision) and analyzed for density of vGlut1-
positive puncta colocalized with EGFP with ImageJ software (NIH). vGlut1+
puncta colocalized with EGFP-filled dendrites were counted and categorized
according to their placement on the dendritic shaft or spine, as identified by
morphology seen through the imaging stack (Fig. 15).

**Statistical analyses**

Prism 4 for Windows (GraphPad Software, Inc.) was used to analyze data
via the two-tailed Student’s t-test. In all cases, p < 0.05 was regarded as
statistically significant.
Figure 15. Methods used to stain and examine EGFP-filled dendrites in thinly-sliced tissue.

Brains were sectioned at 40 micrometers into PBS with azide
Rats from different groups were processed in parallel
4th series was mounted to slides, coverslipped, and used to assess overall transduction

Individual tissue sections from series 1-3 individually assessed for sensorimotor forelimb cortex L5 pyramidal neuron transduction at low magnification, on unsubbed slides
Selected 6 sections/rat (+1 for the no primary control)

Free floating sections of rats from different groups were immunostained for vGlut1 in parallel (including the no primary control for each rat)

Five apical and five basilar dendrites from L5 sensorimotor cortex pyramidal neurons were imaged at 100x magnification
Dendrites were at least 10 microns in length

Deconvolution of image stacks using DeltaVision softWoRx (Applied Precision)
Puncta were categorized according to spine type or shaft localization

Image stacks analyzed for density of vGlut1 puncta using ImageJ software (NIH)
RESULTS

vGlut1 immunohistochemistry identifies potential presynaptic terminals.

vGlut1 immunostaining patterns resembled previous published reports (Alonso-Nanclares et al., 2004; Cubelos et al., 2005; Melone et al., 2005; Kubota et al., 2007; Tabuchi et al., 2007), with punctuate arrangements seen throughout neocortical grey matter, but absent from neuronal somas (Fig. 16A). This latter effect was well demonstrated in the hippocampus, where the stratum pyramidale was negative for vGlut1 immunoreactivity (Fig. 16B). The vGlut1+ puncta were found at the distal tips of spines, where synapses are known to form (Harris et al., 1992), in approximately 87% of spines analyzed (Figs. 17, 18). Furthermore, “no primary” controls expressed little to no signal, and the maximal light intensity values were less than 5% of that seen in stained tissue sections. Given all this, the vGlut1+ puncta serve as a reliable indicator for potential presynaptic terminals.

AAV-shNogo leads to reduced number of potential presynaptic connections onto apical dendrites.

Loss of postnatal Nogo-A, in neurons, was associated with a decrease in vGlut1+ puncta colocalizing onto dendrites (13.5 ± 1.3 colocalized puncta per 10 µm dendrite for AAV-EGFP and 9.1 ± 1.1 for AAV-shNogo, p = 0.03, Fig. 19A). This change was due to a decrease in puncta at the dendritic shaft (2.5 ± 0.2 colocalized puncta per 10 µm dendrite for AAV-EGFP and 1.6 ± 0.2 for AAV-
shNogo, \( p = 0.01, \) Fig. 19B), and not due to the aforementioned decrease in dendritic spines because there were no significant changes seen in the density of colocalization with dendritic spines (10.5 ± 1.5 colocalized puncta per 10 \( \mu \)m dendrite for AAV-EGFP and 7.5 ± 0.9 for AAV-shNogo, \( p = 0.12, \) Fig. 19C).

Indeed, the percentage of spines that colocalized with vGlut1+ puncta was not different between groups (86.5% ± 3.6 for AAV-EGFP and 83.2% ± 1.7 for AAV-shNogo, \( p = 0.39, \) data not shown). This suggests a potential loss in the density of presynaptic terminals situated on the apical dendritic shaft of animals with decreased postnatal, neuronal Nogo-A expression.

**Decreased postnatal levels of Nogo-A protein does not affect vGlut1+ colocalization with basilar dendrites.**

Dendrites of the basilar arbor did not show a change in density of vGlut1+ colocalization in the total dendritic compartment (12.4 ± 1.9 colocalized puncta per 10 \( \mu \)m dendrite in AAV-EGFP, 10.8 ± 1.2 for AAV-shNogo, \( p = 0.49, \) Fig. 20A), dendritic shafts (3.6 ± 0.6 colocalized puncta per 10 \( \mu \)m dendrite for AAV-EGFP and 2.8 ± 0.4 for AAV-shNogo, \( p = 0.36, \) Fig. 20B), or dendritic spines (9.1 ± 1.6 colocalized puncta per 10 \( \mu \)m dendrite in AAV-EGFP, 7.9 ± 1.0 for AAV-shNogo, \( p = 0.53, \) Fig. 20C). As with the apical arbor, the percentage of spines that colocalized with vGlut1+ puncta was not different between groups (89.6% ± 2.2 for AAV-EGFP and 89.3% ± 2.6 for AAV-shNogo, \( p = 0.93, \) data not shown).
Thus, the concentration of potential presynaptic terminals is unchanged in dendrites of the basilar arbor in response to Nogo-A knockdown.
Figure 16. Representative vGlut1+ immunoreactivity. (A) Neocortical layer V region with punctuate staining throughout the parenchyma, and pyramidal cell soma profiles visible by their lack of staining. (B) Low-magnification image of the hippocampus with staining absent from white matter and stratum pyramidale (arrowheads). (C) Orthogonal view of stack taken on confocal microscope with 1 µm thick optical slices showing antibody penetration through the thickness of the tissue. Scale bars denote (A) 300 and (B, C) 50 micrometers.
Figure 17. **vGlut1+ puncta and EGFP colocalization.** Deconvolved high magnification z-slice image of (A) spines in green, (B) vGlut1+ puncta in red, and (C) their colocalization in yellow. Yellow arrowheads indicate dendritic colocalization; arrows indicate shaft synapse colocalization. Scale bars indicate 1 micrometer.
Figure 18. vGlut1+ puncta and EGFP colocalization. Deconvolved high magnification z-slice image of (A) spines in green, (B) vGlut1+ puncta in red, and (C) their colocalization in yellow (yellow arrowheads). Though the postsynaptic EGFP and presynaptic immunolabeled vGlut1 are present in separate cells, they appear colocalized because of the resolution restrictions of deconvolution microscopy. Scale bar indicates 1 micrometer.
Figure 19. Colocalization of vGlut1 and EGFP-filled apical dendrites.

(A) Postnatal, neuronal Nogo-A knockdown leads to a decrease in vGlut1 in apical dendrites of the sensorimotor cortex. (B) This change can be attributed to the vGlut1+ puncta opposing the dendritic shaft compartment. (C) There was no substantial change in the average number of colocalized puncta on the apical dendritic spines of these neurons. Error bars indicate SEM, data pooled from 6 animals per group, * indicates p < 0.05, two-tailed Mann-Whitney test.
Figure 20. Colocalization of vGlut1 and EGFP-filled basilar dendrites.

(A) Postnatal, neuronal Nogo-A knockdown does not affect colocalization in basilar dendrites of the pyramidal cells of the neocortex. (B) No differences in puncta density were distinguished on the dendrites of these neurons. (C) The density of vGlut in opposition to dendritic spines in the basilar arbor was unchanged following loss of Nogo-A in sensorimotor neurons. Data pooled from 6 animals per group, error bars indicate SEM.
DISCUSSION

We found that loss of Nogo-A in a subset of developing neurons affected the density of a potential presynaptic partner, vGlut1, in the apical compartment of the dendritic arbor. This decreased density of vGlut1-immunoreactive puncta was attributed to a 36% loss of potential synapses onto dendritic shafts, as opposed to dendritic spines, since the percentage of apical spines with a potential presynaptic partner was similar between groups. These results are consistent with the hypothesis that neuronal Nogo-A regulates properties of the excitatory synapse in the apical arbor of neocortical pyramidal neurons.

Our previous study of Nogo-A’s effects on dendritic spines did not address the possibility of a compensatory increase in excitatory synapses onto the dendritic shaft (Mateos et al., 2007). Moreover, dendritic spines may contain more than one synapse, and approximately 4-10% of neocortical spines have no synaptic connections (Gray, 1959; Arellano et al., 2007b). Computational models have shown inputs via shaft synapses, though they have less computational potential than spine synapses, have been predicted by cable theory to inject more direct current into the dendrite (Byrne, 2003). Shaft synapses have also been shown to have a larger PSD area than spine synapses (Rusakov et al., 1998). Dendritic shaft synapses are also less likely to have a perforated morphology than spine synapses (Anderson and Martin, 2006).

Functionally, the decreased density in potential shaft synapses seen in response to reduced Nogo-A levels may represent a loss in inputs that would have
a strong influence on dendritic integration, though further studies would be needed to confirm this. For example, defining a synaptic populations’ functional state requires ultrastructural, electrophysiological, and FM-styryl dye labeling approaches to locate synaptic assembly, confirm that the neurons are capable of communication, and identify sites of spontaneous synaptic vesicle recycling, respectively. Additionally, cross-synaptic immunohistochemistry is needed to confirm that presynaptic machinery is found in opposition to postsynaptic structures (Ahmari and Smith, 2002). Interestingly, the percentage of potential shaft synapses seen in even the control group (19% of total potential synapses) is higher than would be expected in hippocampal pyramidal neurons of adult rats and is more typical of a pre-pubertal stage of development (Boyer et al., 1998).

vGlut1 is primarily found in vesicles in nerve terminals, where it aids glutamatergic transmission. It is widely expressed throughout various brain regions and layers of the neocortex (Fremeau et al., 2001). While immunogold labeling has shown the transporter to be present in a small subset of astrocytic processes, it was estimated to be at a sevenfold lower intensity than that seen in axon terminals (Bezzi et al., 2004), and thus this background staining seems to be a minor limitation to accurately identifying potential presynaptic boutons, in light of other restrictions to defining functional synapses discussed above.

Dendritic neurotransmitter release has been reported in neocortical layers 2/3, and while it is entirely probable that neurons from layer 5 can release glutamate in a retrograde signaling mechanism, we are unaware of any such
reports at the time of this writing (Jenstad et al., 2009). Important to the current study, vGlut1 is not located in dendrites, though vGlut3 is (Fremeau et al., 2004).

Our finding of no changes to the density of vGlut1+ puncta opposing dendrites of the basilar tree in this same population of neurons is interesting, but slight differences in Nogo-A regulation between different regions of the dendritic arbor has been observed in hippocampal dendrites. In the CA1 region, n1C7 treatment led to an increase in basilar dendritic complexity, but no change to apical complexity. In the CA3, the same treatment led to a decrease in length of proximal apical dendrites, but an increase in length of distal apical dendrites, and no change to basilar dendrites (Zagrebelsky et al., 2010). Our finding of no change to the basilar arbor is also in keeping with our results on spine density (Chapter 3) and may also account for the unchanged levels in PSD-95 mRNA in regions of transduced neocortex, since the tissue microdissection attempted to isolate the deeper cortical layers (Chapter 4). The basilar arbor has both common and distinct inputs with the proximal apical arbor. In the sensorimotor cortex, the basilar tree shares inputs from intralaminar layer 5 connections as does the proximal apical tree, but the basilar arbor also receives input from layer 3 neurons as well (Deuchars et al., 1994; Lubke et al., 1996; Thomson and Bannister, 1998). Excitatory postsynaptic potential (EPSP) recordings from the soma of Layer 5 neurons of the frontal agranular cortex have shown that these connections are associated with different N MDA receptor subunit compositions. Stimulation via callosal inputs, such as those one would expect from layer 3, that
have shown to preferentially synapse onto the basilar arbor, was enhanced by an agent that preferentially promotes inputs from NR2A-containing NMDA receptors. Conversely, EPSPs elicited from stimulation of local intralaminar fibers that one could expect to synapse onto both proximal apical and basilar arbors, were dampened by a NR2B-subunit specific antagonist (Kumar and Huguenard 2003). Thus, the intracortical and intercortical neurons that have been shown to synapse onto different regions of the dendritic tree also house different subunit compositions of the NMDA receptor. Our previous work (Chapter 4) has shown these same NMDA receptor subunits to be differentially affected by Nogo-A knockdown. Specifically, we observed a decrease in NR2B with no substantial change to NR2A subunit expression. It is possible that postnatal, neuronal Nogo-A may alter dendritic connectivity, in part by differentially influencing glutamatergic input to the Layer 5 pyramidal neurons.

The changes described here in response to altered levels of Nogo-A represent a novel function for Nogo-A in influencing potential dendritic shaft synapses of the apical tree. The presence of Nogo-A within these dendrites and synapses has been verified by immunohistochemistry and immunoblot of synaptosomes (Pradhan, 2007; Lee et al., 2008; Grunewald et al., 2009; Raiker et al., 2010). The decrease in potential dendritic shaft synapses represents a means by which Nogo-A further regulates stability at the excitatory synapse. Ultimately, this work aids in our understanding of the functions of this important therapeutic target in the intact nervous system.
CHAPTER SIX
GENERAL DISCUSSION

This project has demonstrated that postnatal loss of neuronal Nogo-A via viral-mediated knockdown leads to a statistically significant reduction in the densities of apical spines on Layer V pyramidal neurons of the sensorimotor cortex. This same treatment resulted in a third fewer potential excitatory dendritic shaft synapses seen in opposition to dendrites of these neurons, as visualized by vGlut1 immunohistochemistry. Postnatal, neuronal Nogo-A knockdown also resulted in a shift in dendritic spine morphology in the proximal apical dendrites, including decreases in thin and stable mushroom spines that are important for learning and memory, respectively. Further alterations in dendritic spine morphology included fewer protrusions with very long necks when compared to the control group, and fewer protrusions of moderately-wide diameter. The decrease in very long spines translates into a population of spines with less current injection into the parent dendrite. These changes were not observed in the basilar arbor of the same neurons.

To further investigate the synaptic phenotype of these neurons, regions of AAV-transduced tissue were examined for changes in mRNA of molecules fundamental to excitatory synaptic function. Samples of tissue that had received
Figure 21. Summary of results. EGFP-filled apical dendrites are depicted in green. The magnitude of the decrease in levels of Neuroligin-1 and NMDA receptor subunits is much larger than what would predicted by the decrease in dendritic synapses alone, hence the decreased amount of these molecules at an individual synapse.
AAV-shNogo-A had less Nogo-A when compared to samples from the control group. We found that knockdown did not significantly alter the amount of Nogo-B mRNA, which indicates a lack of compensatory coupling with Nogo-B expression that has been reported in Nogo-A knockout mice (Dimou et al., 2006). However, the remaining Nogo-B may still confound our results, as it has the Nogo-66 loop that can signal through NgR1. Both groups examined had similar levels of PSD-95, but there was a decrease in the relative amounts of Neuroligin-1 and in NMDA receptor subunit NR2B message transcripts in samples lacking postnatal neuronal Nogo-A when compared to samples from control animals. There was a trend towards decreased NR2A in Nogo-A knockdown samples, and mean amounts of NR2A showed a decrease when compared to the control group, though the change was not statistically significant. Overall, we did observe a decrease in molecules known to be important at the excitatory synapse, though some elements of the synapse remained unchanged.

Synaptic structures are key for synaptic plasticity. Decreases in synapse and spine density and changes to spine morphology lead to disruptions in information processing. These features, as seen in pyramidal neurons of the neocortex, are also hallmarks of several neurological disorders, including Schizophrenia and Alzheimer’s disease (el Hachimi and Foncin, 1990; Garey et al., 1998; Fiala et al., 2002a). The time course of spine loss in Alzheimer’s disease correlates with the age of symptom onset (el Hachimi and Foncin, 1990; Scheff et al., 1990; Scheff and Price, 1993; Scheff et al., 1993). Similarly, synapse
elimination in patients with Schizophrenia peaks at adolescence, and symptoms typically emerge at late adolescence and early adulthood (Garey et al., 1998). Schizophrenia and Alzheimer’s disease are also associated with a decrease in overall concentrations of NMDA receptors (Penzes et al., 2011).

In the study of mental retardation, Down’s syndrome is characterized by decreases in spine density and a reduction in dendritic arbor branching in the neocortex (Takashima et al., 1981; Takashima et al., 1994). Autism spectrum disorders are associated with mutations in Neuroligins and their binding partners, Neurexins, as well as changes to spine density and morphology (Abrahams and Geschwind, 2008; Dahlhaus and El-Husseini, 2010). Fragile X syndrome is caused by a loss of Fragile X Mental Retardation Protein (FMRP), a regulator of protein synthesis which promotes spine growth (Jin et al., 2004; Belmonte and Bourgeron, 2006). FMRP directs the locations of mRNAs, including PSD-95, in response to metabotropic glutamate receptor activity (van Spronsen and Hoogenraad, 2010).

These conditions all have their own characteristic pathophysiology, but show these similar changes in synaptic features. These features are also seen in the neocortex after postnatal Nogo-A knockdown, raising the question of a shared underlying molecular pathway. Rho GTPases are regulators of the actin cytoskeleton, spine and synapse development, and Nogo-A signaling (Tashiro and Yuste, 2004; Lippman and Dunaevsky, 2005; Calabrese et al., 2006). One of the main candidate genes in the Down’s syndrome critical chromosomal region
regulates neuronal differentiation via RhoA (Berto et al., 2007). Three of the thirteen genes known to be associated with X-linked mental retardation encode for Rho GTPases (Ramakers, 2000; Chelly and Mandel, 2001; Negishi and Katoh, 2002; Govek et al., 2005; Nadif Kasri et al., 2009). At least two Rho GTPases and one Rho GAP have been linked to Schizophrenia (Davidkova et al., 2003; Hashimoto et al., 2005; Hill et al., 2006). A thorough behavior analysis of Nogo-A knockout mice has revealed an increase in Schizophrenia-like behavior, with alterations in startle response, perseverative behavior, and increased sensitivity to the locomotor stimulating effects of amphetamine (Willi et al., 2009; Willi et al., 2010). These changes were not seen in adult wild-type mice that had received anti-Nogo-A antibody treatment, suggesting that this phenotype is a product of alterations in development (Willi et al., 2010). Further work examining RhoA and synaptic stabilization may help understand the connections between these conditions, and eventually aid in the development of therapies for affected individuals.

The decrease in dendritic spines seen here after postnatal Nogo-A knockdown stands in contrast to our laboratory’s previous finding of increased spine density after middle cerebral artery occlusion and Nogo-A immunotherapy (Papadopoulos et al., 2006). There are several possible reasons for this: (1) In the present project, the Nogo-A knockdown was delivered at postnatal day 3, while anti-Nogo-A immunotherapy was given to an adult animal; therefore the age which Nogo-A signaling was disrupted could account for the different results
in spine density. (2) Nogo-A may have different signaling mechanisms from different cellular compartments, and while the knockdown targeted all Nogo-A molecules in the neuron—those at the surface as well as the ER—the antibody treatment was only able to affect the relatively small proportion of Nogo-A molecules that were at the plasma membrane. Finally, (3) oligodendrocyte-associated Nogo-A was still present in the knockdown paradigm but not in the immunotherapy treatment. Our Nogo-A knockdown primarily targeted neurons, and the presence of Nogo-A in other cell types such as oligodendrocytes may have resulted in the differences between this project and our earlier study (Papadopoulos et al., 2006).

We attempted a pilot study to determine if Nogo-A knockdown led to a behavioral change that could be measured using standard tests of rodent behavior. In this regard, postnatal Nogo-A knockdown in the sensorimotor cortex and hippocampus did not affect spatial navigation or swim speed as measured using the Morris water maze (Appendix). This may be due to several possible reasons. First, AAV-delivery was targeted to a large region of hippocampus, and the viral transduction was simply too low, or did not cover enough of the extent of the dorso-ventral axis. Groups examining other gene targets have found cognitive measures of behavior after AAV-mediated gene delivery, though injection was limited to nuclei or small regions of the brain (Bahi et al., 2009; Alexander et al., 2010; Spiteri et al., 2010). Similarly, non-cognitive changes to coordination were observed after injecting AAV containing a gene for
a neurotrophic factor into large regions of the cerebral cortex (Airavaara et al., 2010). It is also possible that the lack of changes seen to spatial navigation behavior in our study is the fact that Nogo-A may have a relatively minor role at the synapse, compared to essential components of vesicular release machinery such as voltage-gated calcium channels, or SNARE proteins, for example.

Overexpression of the botulism target SNAP-25 in the adult hippocampus via AAV leads to memory impairment via Morris water maze despite only 8% transfection efficiency (McKee et al., 2010). However, while we did not count cells to assess transduction efficiency, the density of EGFP-positive cells in our work was comparable to the number of neurons stained for a Golgi reaction, which would be approximately 1% of neurons. If our efficiency of transduction had been higher or more uniform, we anticipate postnatal Nogo-A knockdown would have affected spatial navigation or locomotion on the Morris water maze.

**FUNCTION OF NEURONAL NOGO-A IN PYRAMIDAL NEURONS**

**Postsynaptic Nogo-A signaling**

Together, these results show that loss of neuronal Nogo-A in postnatal development leads to a decrease in synaptic structures and associated molecules. These findings are in keeping with the hypothesis that Nogo-A functions to maintain synaptic structures, a hypothesis that has recently been demonstrated in other cell types. In the developing PNS, Nogo-A-mediated facilitation of neurite branching (Petrinovic et al., 2010). Nogo-A knockout or blockade by 11C7
anti-Nogo-A antibody lead to increased neurite length and decreased branch formation in DRG dissociated cultures. DRG explant cultures exposed to the same treatments showed increased length and fasciculation of neurites. Blockade of NgR, Lingo1, or ROCK produced similar results in both cell culture systems. In the embryonic hindlimb, injection of 11C7 led to axons that failed to grow out from the plexus area or formed aberrant pathways that did not extend through the full area of the limb, while injection of control antibodies did not alter peripheral nerve formation. Nogo-A knockout mouse embryos show decreased length and width of hindlimb nerves (Petrinovic et al., 2010). The addition of 11C7 antibodies to hippocampal slice cultures led to decrease in apical complexity in CA3, but a decrease in complexity in the basilar tree of neurons of the CA1. This treatment also led to an increase in the density of stubby spines in the apical tree of CA3 pyramidal neurons (Zagrebelsky et al., 2010).

Nogo-A and its receptor NgR1 are present at both the pre and postsynaptic structures in cortical pyramidal neurons (Pradhan, 2007; Grunewald et al., 2009; Raiker et al., 2010). We can be sure of loss of Nogo-A at the postsynaptic structure because shNogo and EGFP were expressed on the same plasmid. We did not observe EGFP-filled fibers synapsing onto or intersecting the dendrites examined, but we can not exclude the possibility of Nogo-A knockdown on the presynaptic terminal as the majority of neurons expressed EGFP only at the soma and not in their dendrites or axons (Fig. 5, 6). As such, Nogo-A may or may not have been expressed at the presynaptic terminal (Fig. 23). This may be remedied
**Figure 22. Model for synaptic stabilization via Nogo-A.** Neuronal Nogo-A may signal through NgR1 or integrins. Dark arrows indicate signal pathways Nogo-A is known to activate in neurons. Grey arrows indicate pathways that have been shown in other neuronal signaling cascades. Activated forms of molecules are shown in black, inactive forms are shown italicized, in grey. Inset shows area enlarged. “?” indicates an outcome seen in response to Integrins in other neuronal contexts, but have not been reported in Nogo-A/Integrin signaling pathways. See p. xiv for abbreviations.
Figure 23. Potential mechanism by which Nogo-A knockdown destabilizes synapses. Loss of postsynaptic Nogo-A induced NgR1 and Integrin signaling can lead to a loss of synapse-stabilizing signaling schemes, including the inactivation of Cofilin via phosphorylation. Nogo-A in axon terminal is indicated by a dashed line signifying it may or may not be deleted in our knockdown paradigm (see text). Inset indicates a lower magnification of the region that has been enlarged.
in future studies by use of a stronger, more persistent promoter.

The possibility of Nogo-A signaling through an autocrine mechanism may contribute to the findings described here. Nogo-A-mediated activation of NgR1 can mediate growth cone collapse through increasing actin contractility by way of RhoA/ROCK-mediated activation of Myosin II. Nogo-A induced RhoA/ROCK activation is also known to activate LIM kinase (LIMK), which in turn inactivates Cofilin by phosphorylation. This inactivation of Cofilin blocks its ability to sever the negative end of F-actin filaments, which slows actin turnover and helps create an environment of synaptic stability. The LIMK-Cofilin mechanism of Nogo-A signaling may account for some of our results (Figs. 22, 23). However, Cofilin is only one of many actin-binding proteins, so additional signaling pathways may be responsible for our results. Amino-Nogo-A has demonstrated the ability to signal through integrins, which are known to mediate synaptic and spine stability through actin cytoskeleton dynamics (Gerro and El-Husseini, 2006; Shi and Ethell, 2006; Webb et al., 2007; Cingolani and Goda, 2008; Hu and Strittmatter, 2008; Carlson et al., 2010).
Growth cone collapse vs. dendritic stabilization

Differences in Nogo-A induced signaling cascades may account for how reduction of an axonal inhibitory molecule (Fig. 1) can decrease spine density and expression of synaptic molecules (Fig. 23), when one would initially expect an increase in these latter parameters. At sites of established neuronal-neuronal contact, Nogo-A may stabilize the cytoskeleton through RhoA/LIM kinase/Cofilin mechanism. At the axonal growth cone, oligodendrocyte or neuronal Nogo-A communicates through RhoA/MLCK/MyosinII pathway to stabilize actin and thereby halt growth cone advancement. A careful review of the literature demonstrates that other axonal inhibitory molecules have also demonstrated the phenomenon of promoting dendritic sprouting and plasticity, such as ephrinB3, semaphorin 3A, Slit1, and Netrin-1. Various mechanisms have been proposed to account for this. In the case of the axonal outgrowth inhibitor ephrinB3, earlier reports had established mechanisms that can modulate their signaling such as forward/reverse signaling between ephrins and their ‘receptors,’ the Ephs (Aoto et al., 2007). For semaphorin3A, a guanylate cyclase gradient appears to govern the nature of the response so it can inhibit axonal outgrowth and still promote dendritic branching (Polleux et al., 2000; Morita et al., 2006).

Some axon guidance molecules have multiple effects dependent on which of its many known receptors the molecule encounters. For example, the axon guidance cue Slit1 can promote dendritic growth (Whitford et al., 2002), but it is also known to repel or attract axons based on the presence of different robo
receptors the axon encounters. Likewise, Netrin-1, which promotes dendritic growth (Suli et al., 2006), helps axons cross the CNS midline in embryonic development because it can be attractive toward the midline prior to crossing and repulsive once the axon has crossed the midline, based on the presence of a matrix metalloprotease that truncates DCC receptors (Galko and Tessier-Lavigne, 2000). The Netrin-1 molecule can also signal through Unc5h receptors, which inhibit axonal growth cones (Hong et al., 1999).

The mechanisms by which some molecules control dendritic development are yet to be discovered. For example, manipulations of Neurotrophin-3 levels can lead to opposing effects of dendritic growth in different cortical layers. Specifically, Neurotrophin-3 appears to negatively regulate dendritic branching in layer 4 and promote branching in layer 6 (McAllister et al., 1997). Further studies are needed to confirm the molecular mechanisms (Fig. 22) whereby Nogo-A can simultaneously stabilize dendritic synapses and destabilize growth cones in the injured CNS.

**FUTURE DIRECTIONS**

One of the most valuable studies of this system would be studies to clarify the molecular mechanisms responsible for the decrease in spines and synaptic molecules seen in response to reduction in neuronal Nogo-A. To study the proposed neuronal Nogo-A signaling pathways (Figs. 22, 23), a neuron primary culture model would be ideal. If plated at ideal density, many synaptic features
that are seen in vivo can also be seen in vitro, including dendritic spines (Murphy and Andrews, 2000; Vicario-Abejon, 2004). The first experiment needed to confirm the proposed mechanisms would be demonstration that Nogo-A knockdown destabilizes actin, which could be confirmed by an F/G-actin ratio assay. This would also be more useful as a more economical, non-anatomical endpoint than spine analysis or staining of synapse-associated proteins. Because the F/G-actin ratio can also be examined in vivo, this assay could also be used in our in vivo model to confirm that cell culture transfection model has similar traits.

If the F/G actin ratio could be used as a reliable endpoint of Nogo-A-mediated signaling, subsequent experiments might use a double-transfection technique with plasmids containing different fluorescent markers for different receptors and downstream effectors. Additionally, NgR1 antibodies, ROCK inhibitors, coflin inhibitory peptide, and Rho-A inhibitors could be employed to tease out the signaling pathways involved. Other endpoints such as pull-down assays or a RhoA G-LISA activation assay would be useful means to examine Nogo-A signaling.

The validation of this model and these results by a second virus that would target a different region of Nogo-A is a second potential direction. Also, a tetracycline or doxycycline on/off system would be excellent means to study if these effects are due to spine and synaptic maintenance or developmental windows that regulate these properties. Recently, the Schwab lab has developed
a line of Nogo-A knockout rats that are currently under investigation. This line has miRNA directed against Nogo-A expressed in nearly all neurons, and a comparison of our two models would be a good way to study the effects of intracellular vs. intercellular-acting Nogo-A.

It would also be interesting to investigate the role of Nogo-A at the inhibitory synapses of these neurons, although this line of inquiry does less to address the mechanisms at hand. This could be examined by immunohistochemistry of gephyrin and GABA transporters, although ultrastructural analysis would be a better means to study structural changes. As with excitatory connections, FM-styryl dye cycling and electrophysiology studies are needed in addition to fluorescent imaging methods, to completely describe a molecule’s effect on synapses.
APPENDIX:

PILOT EXPERIMENTS
APPENDIX: PILOT EXPERIMENTS

SPATIAL MEMORY TESTING FOLLOWING NOGO-A SILENCING IN THE POSTNATAL RAT HIPPOCAMPUS

RATIONALE

Synapses have been postulated to be the physical sites where molecular memory mechanisms, such as those required for LTP, are housed (Segal, 2005). A well-studied and practical paradigm to examine memory is the Morris water navigation test, although this behavioral test assesses changes on a much broader scale than the molecular level of a single synapse. Nonetheless, studies involving the Morris water maze and LTP paradigms have frequently shown the two results correlate with changes in spine density (Morris et al., 1986; Tang et al., 2001; Eyre et al., 2003; Segal, 2005; Lee et al., 2008). Furthermore, it has previously been shown that Nogo-A immunotherapy results in improved cognitive recovery following CNS lesions (Lenzlinger et al., 2005; Gillani et al., 2010). Therefore, we assessed the effect of postnatal Nogo-A silencing in the hippocampus on spatial navigation, to determine if neuronal Nogo-A is required for development of the cognitive capabilities required for the Morris water maze.
METHODS

Husbandry

P3 male rat pups were cryoanesthetized and injected with AAV2/8, as described in chapter 3. Vehicle rats treated identical to AAV-injected rats, except they received injections of sterile PBS. Vehicle injected, AAV-EGFP, AAV-Zsgreen-shLuciferase, and AAV-EGFP-shNogo groups were comprised of 10, 14, 14, and 15 rats, respectively. Morris water navigation testing commenced on P52. This study was conducted using protocols approved by the Edward Hines Jr. VA Hospital Institutional Animal Care and Use Committee in accordance with National Institutes of Health principles of laboratory animal care.

Morris water navigation testing

Place testing consisted of four trials per day, for six consecutive days, with each trial separated by at least 10 minutes. A 6’ diameter pool was filled with 22°C water, made opaque by addition of white tempera paint. A white, 2” square platform was submerged 2 cm below the surface of the water. Starting locations were randomized daily. The rat was gently lowered into the pool, facing the inside wall of the pool, and allowed to swim until he found the platform or until two minutes had expired.

One probe trial was performed, on day seven. This testing procedure was identical to hidden platform testing, except that the platform had been removed from the pool. Each rat swam for two minutes.
Matching-to-Place testing occurred over five consecutive days (days eight-twelve), in which the rat was introduced to the pool twice a day, with 20 seconds between trials. The location of the hidden platform changed daily, in a randomized manner. The rat was allowed three minutes to find the platform.

For each test, swim paths were digitized by Noldus Ethovision Software. Place and Matching-to-place paths were analyzed for trial time, total distance traveled, swim velocity, thigmotaxis, and route circuitry. Trial time, total distance traveled, and average swim velocity were determined for each trial by Noldus Ethovision. This software was also used to measure thigmotaxic behavior, by defining an inner and outer portion of the pool, and recalculating the distance traveled in each region. Route circuitry was calculated as the quotient of total distance traveled divided by the displacement distance from the trial starting point to the nearest edge of the hidden platform.

Place task paths were also analyzed for the degree of heading direction error within the first 5 seconds of each trial. Heading direction error was investigated using Oriana circular statistics program by first assessing angular variance (r) for each rat over the first six days of the Place task. Angular variance ranges from 0 to 1, with 0 indicating random heading direction, and 1 indicating all heading angles point to the same direction. Among groups where angular variance was equal, we then generated a mean heading direction angle for each rat on each day of the Place task, and then compared mean heading direction angles for each day across groups.
Statistics

For the Probe trial, the preference score, \( P = [(T-A) + (T-B) + (T-C)]/3 \), was calculated as in (Brown et al., 2000), and analyzed by ANOVA with Tukey post-hoc testing (Graphpad). Mean heading direction angles were compared using the Watson-Williams F test in Oriana (Wallace et al., 2002). All other data was analyzed by repeated-measures ANOVA with Tukey post-hoc testing (SPSS). In all cases, \( p > 0.05 \) was regarded as significant.

RESULTS

Postnatal, neuronal Nogo-A knockdown as described here does not lead to impairment in short or long term spatial memory, as measured by the Morris water maze place (\( p = 0.22 \) for distance traveled and \( p = 0.82 \) for latency to find the platform, App. Fig. 3) and matching-to place tests (\( p > 0.99 \) for time to find the platform during the second trial, App. Fig. 5), respectively. No differences were observed with regards to swim velocity on the place test (\( p = 0.34 \), App. Fig. 4). Similarly, no significant differences were observed between groups in the probe trial (\( p = 0.98 \)) or thigmotaxic behavior (\( p = 0.80 \)). Furthermore, navigation strategies appear to be unchanged, as determined by route circuitry and heading direction analysis (data not shown).
App. Figure 1. Experimental design, including behavioral testing and swim paths representative normal animals on select testing days.
App. Figure 2. Hippocampal neuron transduction with AAV2/8. The green color indicates a virally transduced neuron (EGFP). Note the layer of transduced pyramidal neurons in the CA1 region. Pictures were taken at 5x, 20x, and 63x magnification. 63x photos were taken on scanning confocal microscope. Scale bars indicate 10 microns. Adapted from Paxinos and Watson, 2006.
App. Figure 3. Long-term memory performance on the Morris water maze place task. No significant differences were observed between groups for (A) time or (B) distance traveled per trial. Data pooled from 10 (Vehicle), 14 (EGFP), 14 (shLuc), and 15 (shNogo) animals per group. Error bars indicate SEM.
App. Figure 4. **Short-term memory performance on the Morris water maze matching-to-place task.** No significant differences were observed between groups for the time it took to find the platform for either Trial 1 or Trial 2. Data pooled from 10 (Vehicle), 14 (EGFP), 14 (shLuc), and 15 (shNogo) animals per group. Error bars indicate SEM.
App. Figure 5. Swimming velocities were not significantly different between treatment groups. Data pooled from 10 (Vehicle), 14 (EGFP), 14 (shLuc), and 15 (shNogo) animals per group. Error bars indicate SEM.
CONCLUSION

Previous reports have noted a change to cognitive behavioral testing following CNS injury and Nogo-A immunotherapy (Brenneman et al., 2008; Gillani et al., 2010). While the results here do not support the hypothesis that Nogo-A is required for development of spatial memory, there are a few points to consider. First, interpretation is limited by the fact that the virus did not transfet the entire hippocampus (App. Fig. 2), and redundancy within the structure may compensate for any loss in function. Second, the behavioral test may not be sensitive enough to detect changes that may have occurred in this system on a molecular scale. Third, the peak of Schaffer collateral synaptogenesis at P7 would fall earlier than the expected peak in AAV2/8 expression in the CNS at P17 or later (Reimsnider et al., 2007). As such this experiment does not address the potential role of synaptogenesis, merely the possibility of Nogo-A in a maintenance role in later postnatal development.
APPENDIX: PILOT EXPERIMENTS

HIPPOCAMPAL SPINE DENSITY AND MORPHOLOGY FOLLOWING NOGO-A SILENCING IN THE POSTNATAL RAT

RATIONALE

Given our result of decreased spine density in the pyramidal neurons of the sensorimotor cortex, we sought to examine the pyramidal neurons of the hippocampus due to several similarities between the two neuronal populations. Both sets of excitatory pyramidal neurons have been well-studied as models of synaptic plasticity. Additionally, both populations show similar Nogo-A expression patterns, with high levels of protein expressed in development and lower levels in adulthood (Meier et al., 2003; Hasegawa et al., 2005). As such, hippocampal neurons appeared ideally suited for studies on the effect of Nogo-A in spine density and morphology.

The striatum radiatum is a band running throughout the hippocampus that houses the proximal dendrites of the pyramidal neuron’s apical tree. A single neuron in the CA1 region receives inputs from as many as 5000 axons traveling through Schaffer collateral pathway, originating in the CA3 region of the hippocampus (Kandel, 2000). We found no change in spine density and a decrease in filopodial spine morphology following postnatal, neuronal Nogo-A knockdown.
METHODS

Rats that had been injected at P3 and had been examined for behavioral deficit by Morris water maze (App., above) were used for this study. An inclusion criterion was based on neuron transduction, as assessed by EGFP. AAV-EGFP and AAV-shNogo groups were comprised of 8 and 7 rats, respectively. Spines were analyzed as also described in Chapter 3, with the exception that dendrites were imaged in the striatum radiatum from pyramidal neurons of hippocampal region CA1.

RESULTS

Spine density and morphology in the Hippocampal CA1 region following loss of postnatal, neuronal Nogo-A

Nine weeks after AAV delivery, no changes in spine density were observed between groups (p = 0.27, App. Fig 6A). A 53% decrease in filopodia-type protrusion density was seen in AAV-shNogo group (1.3 ± 0.2 filopodia/10 µm dendrite), when compared to the AAV-EGFP group (0.7 ± 0.1 filopodia/10 µm dendrite, p = 0.02, App. Fig. 6B). However, no other changes in spine morphology were observed between groups, including measurements of spine length (0.89 µm ± 0.05 for AAV-EGFP and 1.00 µm ± 0.10 for AAV-shNogo, p = 0.29) and head diameter (0.59 µm ± 0.03 for AAV-EGFP and 0.62 µm ± 0.03 for AAV-shNogo, p = 0.50).
App. Figure 6. Hippocampal CA1 region spine density and morphology following postnatal loss of Nogo-A. (A) No substantial differences were observed in spine density. (B) A decrease in filopodia-type protrusions was observed. Data pooled from 8 (EGFP) and 7 (shNogo) animals per group. Error bars indicate SEM, * indicates p < 0.05, two-tailed Student’s t-test.
CONCLUSIONS

The region of the dendritic arbor studied here, apical segments at least 100 µm distal to the soma, has been shown to be more sensitive to changes in spine density and morphology (Gould et al., 1990; Pyapali and Turner, 1994). We also targeted both populations of neurons involved at the CA1 spine for Nogo-A knockdown. Nevertheless, our finding of no change to spine density in the postnatal hippocampus following Nogo-A knockdown is in keeping with similar work in the adult following blockade of Nogo-A (Zagrebelsky et al., 2010) and NgR1 (Lee et al., 2008).

The reported spine density of CA1 neurons of the intact adult, male rat ranges from 9 spines/10 micrometers of dendrite (Knafo et al., 2004) to more than twice the density we have seen here, as assessed by the Golgi method (Norrholm and Ouimet, 2000; Gonzalez-Burgos et al., 2004; Stranahan et al., 2008) and DiI labeling (Cunningham et al., 2007). In an effort to transfect as many neurons as possible, in the hopes of seeing behavioral effects, it is possible that we may have raised the background level to the point where signal was lost to high background noise.

One possible reason for similar spine densities between groups may be the remaining levels of Nogo-B (Chapter 4). Nogo-B, like Nogo-A, can signal through NgR1 via the Nogo-66 loop. Inducible, Nogo-A/B knockout models may aid in future investigation of neuronal Nogo-A signaling in postnatal development.
APPENDIX: PILOT EXPERIMENTS

DENDRITIC MORPHOLOGY AND SPINE DENSITY IN THE FORELIMB SENSORIMOTOR CORTEX OF NOGO-A KNOCKOUT MICE

RATIONALE

Alterations in dendritic plasticity have been shown in response to Nogo-A antibody therapy in rats that had received middle cerebral artery occlusion, an experimental model of stroke (Papadopoulos et al., 2006). Naïve rats that receive the same antibody regimen demonstrate transient changes to spine density (Papadopoulos et al., 2006). Similarly, cultured neocortical neurons of Nogo-A knockout mice display altered neurite morphology (Mingorance-Le Meur et al., 2007), and the dendritic structure of hippocampal neurons is altered in response to Nogo-A knockout, knockdown, and antibody occlusion (Zagrebelsky et al., 2010).

We chose to examine the dendritic structure of neocortical neurons of Nogo-A knockout mice that had over 10 generations of backcrossing to the 129X1/SvJ strain. This rigorous backcrossing reduces experimental variation by eliminating genetic influence of the C57BL/6 strain. 129X1/SvJ Nogo-A knockout mice differ from Nogo-A knockout mice with C57BL/6 genetic background in that the former strain has a slight increase in regenerative potential, and a reduced immune response after injury (Dimou et al., 2006).
METHODS

Golgi-Cox Staining and Analysis

Adult Nogo-A knockout mice that had been backcrossed to the 129X1/SvJ line were anesthetized with an overdose of pentobarbital, transcardially perfused, and their brains were prepared for Golgi-Cox stain. After coronal slicing at 200 µm on a vibratome, brains were stained and coverslipped as previously described as in (Gibb and Kolb, 1998; Papadopoulos et al., 2006). Large pyramidal neurons from the forelimb sensorimotor cortex were located in layer V by morphology and with the aid of a mouse stereotaxic brain atlas (Paxinos and Franklin, 2001). Neurons that were intact, unobstructed, and fully impregnated were traced via camera lucida (see App. Fig. 7 for representative tracings). Five neurons were traced per animal, for a total of seven mice in the wild-type group and eight mice for the Nogo-A knockout group.

Dendritic length was approximated via the Sholl method, which counts the number of lines that intersect a series of concentric circles. Dendritic complexity was calculated by counting the total number of branch points for each arbor. For spine density analysis, the number of protrusions was counted for a 50 µm length of dendrite.

Statistical Analysis

Data was analyzed by two-tailed Student’s t-test. In all cases, p > 0.05 was regarded as significant.
RESULTS

Nogo-A knockout mice were similar to wild-type mice with regards to total dendritic arbor length (1537 ± 270 µm of dendrite for EGFP and 1465 ± 167 µm, p = 0.66). We divided this data into apical (556 ± 72 µm of dendrite for wild-type and 532 ± 75 µm for knockout, p = 0.69) and basilar arbor (981 ± 224 µm of dendrite for wild-type and 933 ± 164 µm for knockout, p = 0.70, App. Fig. 8) compartments and saw no differences between groups. Also, the complexity of the apical and basilar dendritic trees displayed no change between wild-type and Nogo-A knockout neurons (App. Fig. 9).

There was a slight trend toward increased average spine density in the Nogo-A knockout animals when examining both the apical and basilar compartments together (7.9 ± 2.1 spines per 10 µm dendrite for wild-type and 9.0 ± 1.9 spines per 10 µm for knockout, p = 0.08). We further broke this data down into apical (8.5 ± 2.5 spines per 10 µm dendrite for EGFP and 8.0 ± 2.0 spines per 10 µm for Nogo-A knockout, p = 0.25) and basilar (7.9 ± 1.5 spines per 10 µm for EGFP and 8.5 ± 1.8 spines per 10 µm for knockout, p = 0.16, App. Fig. 10) compartments and saw no significant differences between groups.
**App. Figure 7.** Representative Layer V pyramidal neuron tracings for wild-type and Nogo-A knockout mice. Small numerals next to dendrites indicate the branch order. Each time the dendrite split, both branches were counted for dendritic complexity analysis.
App. Figure 8. Average length of dendrites in Layer V pyramidal
neurons of Nogo-A knockout mice. Length of dendritic trees was analyzed
total arbor and by apical and basilar compartments. Results were similar
between groups. Data pooled from 7 (wt) and 8 (KO) animals per group. Error
bars indicate SEM.
App. Figure 9. Dendritic complexity of Nogo-A knockout mice.

Dendritic bifurcation was similar between groups, at any level of branch order analyzed (two-tailed Student’s t-test analyzed at each branch order point). Data pooled from 7 (wt) and 8 (KO) animals per group. Error bars denote SEM.
App. Figure 10. Spine densities of Nogo-A knockout mice neurons of the neocortex. (A) Representative segment of Golgi-Cox stained dendrite. (B) Shown are the average spine densities per all dendritic segments, and for the apical and basilar arbors. No significant differences were observed between groups. Data pooled from 7 (wt) and 8 (KO) animals per group. Error bars denote SEM.
CONCLUSION

We observed a slight trend of increased spine density in the Nogo-A knockdown group when compared to wild-type mice, though the p value did not cross the 0.05 threshold. No significant differences were observed in gross dendritic structure, as assessed by length of dendrites or number of branches at any order.

It is important to note that this model targets Nogo-A from all cell types, and at all stages of development. The use of mutant mice to study the effects of Nogo-A has created controversy for nearly eight years (Woolf, 2003; Teng and Tang, 2005). Recently two of the three investigators that originally developed Nogo knockout mice have questioned the use of these animals to study CNS injury (Tuszynski and Schwab, 2010). The challenges mainly center on effects of genetic background, the continued presence of other inhibitory molecules such as ephrin and semaphorin, and developmental compensation. For example, a two-fold upregulation of Nogo-B protein in these animals may be a form of compensatory response to the genetic deletion of Nogo-A (Dimou et al., 2006).

Phenotypically, Nogo-A knockout mice with C57BL6 genetic background display alterations in midbrain dopaminergic signaling and schizophrenia-like behavior (Willi et al., 2010). Nogo-A/B knockout mice have shown delayed closure of the optical dominance critical period (McGee et al., 2005). While we have not observed significant, large-scale changes in plasticity at the level of dendritic architecture, it is possible that further segregation of spines into mid- and distal-apical trees would reveal changes in density, such as seen in the CA1
neurons of Nogo-A knockout mice with the C57BL/6 genetic background (Zagrebelsky et al., 2010). Furthermore, alterations in neuronal plasticity in the neocortex may be detected by methods other than gross structural features, or may only be observable at certain stages of development. Future studies in this area would benefit from addressing these possibilities as well as a combination of Nogo-A knockdown rats and antibody therapies directed against Nogo-A.
NINDS fact sheet on spinal cord injury.


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VITA

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In August of 2005, Alicia entered the graduate program in Neuroscience at Loyola University Chicago. In spring of 2006, she joined the laboratory of Dr. Wendy Kartje at Edward Hines, Jr. VA Hospital to investigate the effect of neuronal Nogo-A on properties of the excitatory synapse. While at Loyola, she served on the Graduate Student Committee as Program Representative for three years and Treasurer for one. She has presented her work at local Chicago Chapter meetings as well as national meetings for the Society for Neuroscience. Alicia is currently a student member of the American Association for the Advancement of Sciences and the Society for Neuroscience.

After obtaining her Ph.D, Alicia plans to join the laboratory of Dr. Michelle Hastings at Rosalind Franklin University. There, she will work as a Postdoctoral Fellow to examine treatments for RNA splicing errors in neurological disorders.