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Using Gene Knockout and Transgenic Approaches to Evaluate *in vivo* Functions of CNS Regeneration Inhibitors: *How Important is Nogo?*

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Summary

Nerve cells of the central nervous system (CNS) are distinct from the peripheral nervous system (PNS) in that they are not capable of regenerating after injury (Purves, D. *et al.* 2001). In recent studies, scientists have discovered that the agents causing this inability to regenerate are myelin-derived protein inhibitors that form three families—MAG, OMgp, and Nogo with a common receptor, NgR (GrandPre, T. *et al.* 2000). Through alternative splicing and use of two promoters, the Nogo gene accounts for three proteins: Nogo-A, Nogo-B, and Nogo-C. Recent *in vivo* studies on Nogo-A^{-} (Simonene, M. *et al.* 2003) and Nogo-A/B$^{-/-}$ mice (Kim, J. *et al.* 2003) have shown potential for regeneration of axons after injury. However, in the field of neuroregeneration, the Nogo controversy has led to a race to define exactly what role Nogo and other inhibitory molecules play. The controversy's underlying basis is the fact that axonal regeneration in Nogo-A^{-} mice created by Martin E. Schwab's lab (Simonene, M *et al.* 2003) is only modest, while axonal regeneration in Nogo-A/B$^{-/-}$ mice created by Stephen M. Strittmatter's lab (Kim, J. *et al.* 2003) is significant yet decreases upon age. In this proposal, we will develop a Nogo-B knockout from Schwab's Nogo-A^{-} mice to alleviate the discrepancies in levels of axonal regeneration seen in Schwab's and Strittmatter's mice. Next, we will address the issue of minimal axonal regeneration in the older mice (>11 weeks) from Strittmatter's lab (Kim, M. et al. 2003). The functionality of MAG has been reported to have a minor role as a secondary inhibitor of axonal regeneration *in vitro* and *in vivo*. However, the effect of OMgp has not yet been studied *in vivo,* and we hypothesize that the minimal axonal regeneration seen in the older mice (>11 weeks) from Strittmatter's lab is caused by OMgp expression in the older mice. We will first assess levels of OMgp expression in younger (6-11 weeks) and older (>11 weeks) wild type mice. We will also create knock out mice for OMgp^{-} and OMgp/Nogo-A/Nogo-B$^{-/-}$ to test the extent of axonal regeneration. Finally, the effect of OMgp overexpression in the inhibition of axonal regeneration will be tested using conditional transgenic Nogo-A/B$^{-/-}$ mice that overexpress OMgp.

Specific Aims

One: ***Creation of double knockout Nogo-A/B$^{-/-}$ mouse from Schwab's Nogo-A^{-} mice:*** Is Nogo-B acting as a secondary inhibitor of axonal regeneration?

Two: ***Test for differential expression of OMgp in younger and older mice:*** Does a difference exist in the expression of OMgp in young (6-11 weeks) and old (>11 weeks) mice?

Three: ***Creation of OMgp and Nogo-A/Nogo-B/OMgp$^{-/-}$ knock out mice:*** Will inactivation of Nogo-A, Nogo-B, and OMgp increase axonal regeneration after spinal cord injury?

Four: ***Creation of OMgp transgenic mice to evaluate if axon regeneration is reduced further:*** Will overexpression of OMgp in Nogo-A/B$^{-/-}$ mice inhibit axonal regeneration?

Introduction

Spinal Cord Injury (SCI) in CNS is the clearest example of a condition in which axonal damage leads to a significant functional disability despite minimal neuronal death (Kim, J. et al. 2003). In the US, 7,600-10,000 cases of SCI accumulate each year (Woolf, C. 2003). Once severed, major axon tracts such as those in the spinal cord never regenerate (Purves, D. et al. 2001). The devastating consequences of these injuries (loss of movement and the inability to control basic bodily functions) have lead neuroscientists to seek ways of restoring the connections of severed axons (Purves, D. et al. 2001).

Aforementioned, axons in the mammalian central nervous system (CNS) do not spontaneously regenerate following injury; and consequently, there is little functional recovery (Purves, D. et al. 2001). This differs from the response of injured axons in the adult peripheral nervous system (PNS), which do regenerate after injury (Purves, D. et al. 2001; Fournier, A. et al. 2001). Damage to axonal tracts in adult CNS triggers a very different response than in the PNS (Fournier, A. et al. 2001). As axons and their myelin sheaths break down distal to the site of injury, the removal of their debris is relatively slow (sometimes persisting for many weeks) and inefficient (Purves, D. et al. 2001). Proximal to the site of injury, neurons fail to activate growth related genes and associated proteins that are expressed during development and successful regeneration in the PNS (Purves, D. et al. 2001). The lack of regeneration is due to a combination of factors including death of injured neurons, reduced capacity of

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adult neurons to grow when injured, lack of the necessary trophic factors to support growth, and the presence of an environment hostile for any growth (Woolf, C. 2003). Components of this hostile environment include astrocytes and the myelin membrane, which interfere with regeneration at the site of injury (Woolf, C. 2003). Astrocytes form the growth associated astroglial scar consisting of tenascin, keratin, and CSPGs, all of which have been shown to inhibit axonal growth *in vitro* (Fournier, A. et al. 2001). Central myelin produced by oligodendrocytes is also a powerful suppressor of axonal growth (Fournier, A. et al. 2001).

The role of axonal environment in regeneration was first explored by Albert Aguayo's lab (Vidal-Sanz, M. et al. 1987). They showed that the optic nerve located in CNS regenerates and restores the appropriate synaptic connections to the superior colliculus after injury when segments of the sciatic peripheral nerve were grafted into CNS sites of the optic nerve (Vidal-Sanz, M. et al. 1987). Failure of CNS neurons to regenerate is thus not due to an inability to sprout new axons, but rather due to something in the local environment that inhibits growth cones from forming and axons from extending (Purves, D. et al. 2001). This demonstration that CNS axons can sometimes regenerate successfully sparked an intensive effort by many labs to discover axonal regeneration inhibitors in the local environment (Purves, D. et al. 2001).

In recent studies, axonal regeneration inhibitors have been identified as myelin-derived protein inhibitors forming three families--Myelin-Associated Glycoprotein (MAG), Oligodendrocyte-Myelin glycoprotein (OMgp), and Nogo with a corresponding Nogo receptor (NgR) (Kim, J. et al. 2003). MAG inhibits axon growth *in vitro* (Liu, B. et al. 2002). *In vivo*, MAG has been shown to limit axon regeneration under certain conditions, although regeneration remains poor in the absence of MAG (Kim, J. et al. 2003). Rather than limiting axonal growth, the function of MAG physiologically appears to be the maintenance of myelin stability through axoglial contact (Kim, J. et al. 2003). Mice lacking MAG have delayed myelination, myelin splitting and redundancy, and decreased thickness of periaxonal cytoplasmic collar of oligodendrocytes, which leads to axon degeneration over time (Kim, J. et al. 2003). OMgp is predominantly localized on the surfaces of oligodendrocytes (Wang, K. et al. 2002). It is a glycosylphophatidylinositol (GPI)-anchored CNS myelin protein and a potent inhibitor of neurite outgrowth *in vitro* (Wang, K. et al. 2002). In addition, an OMgp-enriched myelin fraction has been shown to inhibit neurite outgrowth more than a MAG-enriched myelin fraction (Wang, K. et al. 2002). The effect of OMgp has not yet been studied *in vivo* (Wang, K. et al. 2002). Another myelin-derived axon growth inhibitor is Nogo or Reticulon 4 (GrandPre, T. et al. 2000). Analysis of Nogo expression reveals three mRNA species derived from two promoters and alternative splicing of one gene (GrandPre, T. et al. 2000). Nogo-A, found in neurons, is the major protein species in oligodendrocytes, and is produced embryonically by skeletal muscles (Kim, J. et al. 2003). Nogo-B is a less abundant form found in the brain (Kim, J. et al. 2003). Nogo-C is found in neurons and produced in adult skeletal muscles, but it is not thought to be a major axonal regeneration inhibitor as it is not found in oligodendrocytes and myelin (Kim, J. et al. 2003). All isoforms of Nogo share a common C-terminal domain of 188 amino acids (He, X.L. et al. 2003). A 66 amino acid sub domain, expressed on the surface of the three forms can potentially inhibit axon growth by binding to an axonal Nogo-66 receptor (NgR) expressed by CNS neurons (He, X.L. et al. 2003). MAG, OMgp, and Nogo have been shown to bind with high affinity to NgR (He, X.L. et al. 2003). The three ligands for NgR are structurally quite unrelated—Ig-like domains for MAG, a curved leucine-rich repeat array for OMgp, and a compact globule for Nogo-A, B, C— all converge on a selection of distinct binding hotspots on NgR (Fournier, A. et al. 2001). *In vitro* activity of Nogo and selective expression of Nogo in CNS but not PNS myelin are consistent with its role as a myelin-derived inhibitor of axonal regeneration after injury (Purves, D. et al. 2001). Several lines of evidence support the role for Nogo-A/B/C in limiting CNS axon regeneration (Kim, J. et al. 2003).

However, three studies on Nogo, along with the aforementioned, report a stark divergence in regeneration phenotype leading to the Nogo controversy in defining exactly what role Nogo and other inhibitory molecules play (Woolf, C. 2003). The controversy's underlying basis is the fact that axonal regeneration in Nogo-A^{-} mice created by Martin E. Schwab's lab (Simonene, M. et al. 2003) is only modest, while axonal regeneration in Nogo-A/B$^{-/-}$ mice created by Stephen M. Strittmatter's lab is significant but decreases upon age (Kim, J. et al. 2003; Zheng, B. et al. 2003; Woolf, C. 2003).

We hypothesize that differences in the extent of axonal regeneration in Martin E. Schwab lab (Simonene, M. et al. 2003) and Stephen M. Strittmatter lab (Kim, J. et al. 2003) mice respectively are due to the presence of Nogo-B, which may be serving as a compensatory, secondary axonal inhibitor. Therefore, we will create a knock out of Nogo-B from Schwab's Nogo-A^{-} mice to resolve the discrepancy in levels of axonal regeneration seen in Schwab's and Strittmatter's mice. We also propose that the age-dependant inhibition seen in Strittmatter's mice is due to increased inhibition of OMgp in older (>11 weeks) Nogo-A/B$^{-/-}$ mice. Therefore, we will first assess the expression of OMgp in younger and older mice. Secondly, we will create OMgp$^{-/-}$ and OMgp/Nogo-A/Nogo-B$^{-/-/-}$ mice to completely alleviate this age dependant discrepancy in axonal regeneration. Finally, we will create transgenic mice from Nogo-A/B$^{-/-}$ mice that overexpress OMgp to determine whether it can inhibit axonal regeneration upon overexpression.

Aim One: Creation of double knockout Nogo-A/B$^{-/-}$ mouse from Schwab's Nogo-A^{-} mouse

***Rationale*:** Martin E. Schwab's laboratory (Simonene, M. et al. 2003) demonstrated a compensatory increase in Nogo-B axonal inhibitor expression following knockout of Nogo-A. Considering that axonal regeneration following spinal chord injury (SCI) in these Nogo-A^{-} mice was minor compared to that observed in the Nogo-A/B$^{-/-}$ mice of Strittmatter's laboratory (Kim et al., 2003), it is possible that Nogo-B may be inhibiting axonal regeneration. To demonstrate that Nogo-B, in addition to Nogo-A, significantly inhibits axonal regeneration following SCI, a Nogo-A/B$^{-/-}$ double knockout mouse will be created from Schwab's existing Nogo-A^{-} knockout mouse. We expect that axonal regeneration following SCI will be more extensive in our Nogo-A/B$^{-/-}$ mice as compared to Schwab's Nogo-A^{-} mice. The double knockout mouse will be created using a gene replacement approach similar to that employed by Schwab's laboratory (Simonene, M. et al. 2003), which will selectively abolish Nogo-B expression. Southern, Northern, and Western blot analyses will be

performed to ensure successful knockout of Nogo-B. The extent to which Nogo-B is responsible for a significant fraction of myelin-dependant inhibition *in vitro* will be determined by examining the growth of wild type axons in the presence of Nogo-A^{-} and Nogo-A/B$^{-/-}$ derived myelin. The inhibitory affect of Nogo-B will be assessed *in vivo*, by examining the extent of axonal regeneration after dorsal hemisection injuries (Kim et al., 2003) in both knockouts. Expected improvement in locomoter recovery by Nogo-A/B$^{-/-}$ mice compared to Nogo-A^{-} mice will solidify the characterization of Nogo-B as an inhibitory agent of post SCI axonal regeneration.

Strategy A: Creation of the Double Knockout Mouse

The technique used by Schwab, M.E. et al (Simonene, M. et al. 2003) to create the Nogo-A knockout mice will be adapted to create the Nogo-A/B$^{-/-}$ mice. Nogo-A^{-} mice will be obtained from Schwab's lab, and they will serve as controls as well as tools for creating the Nogo-A/B$^{-/-}$ double knockout mice. A Nogo-B specific replacement vector will be obtained from the Omnibank Sequence Tag database (Lexon genetics Inc.). The vector will then be used to design a plasmid containing a fusion gene (Kim et al., 2003) of IRES-β-geo and neomysin genes such that it could be targeted to the Nogo-B locus. Thus, when the genomic DNA of Nogo-A^{-} mouse is transformed in a neomycin selection media, the Nogo-B gene will be replaced by our gene construct (Figure 1). This newly synthesized DNA fragment will be added to a targeting vector, which will in turn be introduced into a culture of Nogo-A^{-} mouse embryonic stem (ES) cells. Following growth selection with neomysin, the surviving ES cells will be injected into a blastocyst and introduced into pseudopregnant female mice. The resulting chimeric male offspring will be bred with Nogo-A^{-} female mice to produce heterozygous Nogo-A/B$^{-/-}$offspring, which will then be identified and interbred to generate the homozygous Nogo-A/B$^{-/-}$ mice.

(*Homologous Recombination method (and Knockout Mouse)*, 9/24/03)
(http://www.bio.davidson.edu/courses/genomic/method.html

Expected Results
The A1 generation is expected to be 50% heterozygous mice (GA) for the fusion gene construct (Fig. 2A) after crossing wild type mice (G) and mice with cells containing the fusion gene (A). The A1 generation, a cross between the heterozygous mice, is expected to generate 25% homozygous mice (AA) for the fusion gene construct (Fig. 2B).

Strategy B: Verifying knockout mice *in vitro*

Method 1: Southern Blot Analysis
Homologous recombination will first be confirmed through Southern blot analysis of the mouse DNA. Various restriction enzyme sites on the mouse DNA will be determined. Enzymes with restriction sites specific to the locus of the gene (Enzyme A or B) that is also present on the Nogo-A/B$^{-/-}$ mouse DNA (Enzyme A at 3' end or Enzyme B at 5' end) (Figure 1). Genomic DNA will be extracted using the DNeasy Tissue Kit (Qiagen), and techniques given by Amersham Biosciences will be applied to perform a restriction enzyme digest with either enzyme A or B, and probing of the DNA with the ^{32}P-labeled respective 3' or 5' probe. The mixture of DNA fragments, cleaved by the specific restriction enzymes, will be run on an agarose gel and transferred to a nylon membrane. A probe that is complementary to either the 3' or 5' DNA sequence will be generated using PCR and allowed to undergo hybridization (Amersham Biosciences).

Expected Results
Results should reveal that the β-geo gene is present only in the Nogo-A/B$^{-/-}$ knockout mouse, while the Nogo-B gene is present both in the wild type and Nogo-A^{-} control mice. This will be indicated by the different fragment sizes generated by the restriction enzyme digest using either Enzyme A or B. The large enzyme fragment (_-geo) should only be generated for both the wild type and control mice, while the small fragment (Nogo-B) should be generated only for the knockout Nogo-A/B$^{-/-}$ mice.

Method 2: Northern Blot Analysis
A Northern blot analysis will be done to confirm whether or not the Nogo-B gene is expressed. A ^{32}P-labeled probe will be developed (Amersham Biosciences) from a cDNA library of Nogo-B (Origene Technologies) that will hybridize with the Nogo-B mRNA. Total brain RNA extracts will be isolated and purified from each of the 3 types of mice (WT, Nogo-A^{-}, and Nogo-A/B$^{-/-}$) using the RNeasy Midi Kit (Qiagen). Liver cell mRNA not expressing Nogo-B will also be extracted and used as the negative control. RNA extracts will be isolated, eloectrophoresed on an agarose gel, and transferred to a nylon membrane, as described by Amersham Biosciences. β-actin mRNA will be used as the loading control (Kim, J., et al., 2003), considering that it is found in all eukaryotic cells including nervous system cells, and its quantification is not dependant upon age (Alberts, B. et al. 1998).

Expected Results
Results from the Northern blot analysis should demonstrate complete absence of Nogo-B in the negative control mRNA and in the mRNA of the adult brain of Nogo-A/B$^{-/-}$ mice. Nogo-B mRNA should be expressed in the brains of both wild type and Nogo-A^{-} control mice. All mouse mRNA extracts should express β-actin.

Method 4: Western Blot Analysis
A Western blot will be performed on adult cerebral cortex extracts from each of the three mice. The primary anti-Nogo N terminus antibody, followed by an HRP conjugated goat anti-rabbit secondary antibody, used by Zheng, B., et al., 2003, will be used for the Western blot. Protein samples will be electrophoresed on a polyacrylamide gel, transferred onto a nitrocellulose membrane, and hybridized with the primary and secondary antibodies (Amersham Biosciences). A liver cell native control will be used, and myelin-associated glycoprotein (MAG) antibody will be used as the loading control (Kim, J., et al., 2003).

Expected Results
The Western blot should confirm that Nogo-B protein is synthesized in both wild type and Nogo-A^{-} control mice and not in Nogo-A/B$^{-/-}$ knockout mice. Considering that MAG protein is produced in all cell types, the Western blot should confirm its expression in all control and experimental tissues.

Strategy C: Verifying knockout mice *in vivo*

Method 1: Analysis of Brain histology
Effects of an absence of the Nogo-B gene and presence of the β-geo- neomysin fusion gene construct in knockout mice will be determined by comparing brain

size and gross brain anatomy of the recombinant mice to wild type and Nogo-A$^-$ mice through staining and imaging specific brain sections. Major brain nuclei in addition to neuronal layers of the cerebral cortex and cerebellum will be specifically analyzed through the staining of coronal sections with hematoxylin and eosin (Kim et al., 2003). Because the Nogo gene insertion places Lac-Z (consistent with IRES-β-geo) under the control of the Nogo-B regulatory elements, those cells that would normally express Nogo-B can be identified by examining the cells for β-galactosidase activity in X-gal cultures. Luxol fast blue will be used to stain for total myelin content and location in the cerebral and cerebellar cells of Nogo-A/B$^{-/-}$, wild type, and Nogo-A$^-$ control mice to determine if oligodendrocyte formation and survival following recombination are consistent with that of wild type and Nogo-A$^-$ mice, marked by CNPase (Strittmatter, S.M. et al., 2003).

Expected Results
Coronal sections of the cerebral cortex, stained with hematoxylin and eosin (Fig. 6A and 6B) or X-gal (Fig.6C) should reveal brain nuclei that are indistinguishable from those of wild type and Nogo-A$^-$ control mice. The number and position of β-galactosidase-positive cells in this section should be very similar to that of Nogo-B-positive cells in the cerebral cortex of wild type and control mice (Grandpre, et al., 2000; Wang, et al., 2002a), indicating that neuronal placement and survival is normal in adult Nogo-A/B$^{-/-}$ mice. Parasaggital cerebellar sections from Nogo-A/B$^{-/-}$ mice, stained with hematoxylin and eosin (Fig. 6D and 6E) should be indistinguishable from wild type and control mice. Patterns of oligodendrocyte distribution and location in sections of young adult mice lacking Nogo-B, stained with Luxol fast blue (Fig. 6G and 6H), are expected to be similar to those of wild type and Nogo-A$^-$ mice (Grandpre, et al., 2000; Wang, et al., 2002a). Cells in sections stained with X-gal (Fig. 6C, 6F and 6I) should all indicate blue color thereby indicating the expression of the β-galactosidase gene.

Method 2: Behavioral Analysis
To determine whether there are any effects on axonal performance that might change neuronal function and mouse behavior, we will perform a qualitative neuronal examination on control and experimental mice using the rotarod test, in which mice will be placed on the Technical Scientific Equipment rotating rod, moving at constant acceleration (Kim, J., et al., 2003). After initial training, on consecutive days, the length of time that the mice are able to remain on the rod will be recorded (Kim, J., et al., 2003).

Expected Results
The experiment should reveal no observable difference between the wild type/Nogo-A$^-$ control and Nogo-A/B$^{-/-}$ experimental mice (Fig.7). No differences should be observed between mice with and without Nogo-B in the rotarod experiment.

Strategy C: Assessing whether Nogo-A/B$^{-/-}$ Myelin Inhibits Axonal outgrowth *in vitro*
Nogo-B is produced by oligodendrocytes, which are present in the myelin. In order to assess the effects of Nogo-B deletion on axonal outgrowth, we will compare the growth of wild type axons in cultures containing myelin derived from Nogo-A$^-$ control mice and Nogo-A/B$^{-/-}$ experimental mice. Two different assays will be used for this investigation:

Method 1: Neurite Outgrowth Assay
The neurite outgrowth assay is used here to determine the effect of an absence of Nogo-B on the extent to which wild axons sprout. The protocol for the assay will be adapted from Kim, J., et al., 2003.

Expected Results
The absence of Nogo-B in Nogo-A/B$^{-/-}$ myelin (Fig. 8C) and lack any inhibitor in control (Fig. 8A) should result in extensive neurite outgrowth, while the presence of Nogo-B in Nogo-A$^-$ myelin (Fig. 8B) should leads to modest axonal regeneration.

Method 2: Growth Cone Collapse Assay
The growth cone collapse assay is used here to measure the effect of the absence of Nogo-B on the branching of wild axons by investigating the extent to which myelin derived from Nogo-A$^-$, and Nogo-A/B$^{-/-}$ mice cause wild type axons to maintain growth cones, which is an indication of axonal regeneration capacity. The protocol for the assay will be adapted from Kim, J., et al., 2003.

Expected Results
The absence of Nogo-B any in Nogo-A/B$^{-/-}$ (Fig 9C) and any inhibitor in the control (Fig 9A) derived myelin, is expected to lead to a larger number of growth cones when compared to axons cultured in Nogo-A$^-$ myelin (Fig 9B).

Strategy D: Investigation of Axonal Sprouting After Spinal Chord Injury in Nogo-A/B$^{-/-}$ Mice

Method
The effects that a lack of myelin dependent inhibition, due to the absence of Nogo-B, may have on axonal regeneration following SCI will be explored in Nogo-A$^-$ and Nogo-A/B$^{-/-}$ mice, as was done by Kim, J., et al. 2003. A dorsal hemisection injury will be introduced at the T7 level of the spinal chord in accordance with Animal Care and Use Committee guidelines. Resulting axonal sprouting will be investigated using Biotin dextran amine (BDA) injections at sites rostral and caudal to the SCI (Kim, J., et al., 2003). Female mice will be anesthetized, and laminectomies performed at the T7 level, exposing the spinal cord. A dorsal hemisection will be performed using the tip of a 32-gauge needle, which should cause complete interruption of the dorsal and dorsolateral cortispinal tracts (CSTs). Severed skin and muscle layers will be sutured. BDA molecular probes will be injected through a hole drilled in the skull of the mice into the brain area overlying the sensorimotor cortex in order to trace the corticospinal tract (Kim, J., et al., 2003. Mice will be cared for and treated with antibiotics for several days following operation. Spinal cords will be dissected at approximately 20 days post SCI, processed as described by Kim, J., et al. (2003), sectioned, and examined with a 20x objective lens to obtain counts and projection patterns of regenerated fibers highlighted through BDA staining. For rostral and caudal investigations, coronal sections at the T4 and C8 vertebrae of the spinal cord will be observed respectively. Sagittal Sections of the injury site will also be observed.

Expected Results
a) Coronal Studies
Coronal sections at C6 and T2 should reveal an absence of axonal sprouting rostral and caudal respectively to SCI in wild type mice (Fig.10A and 10D). The red arrows indicate the corticospinal tract stained

with BDA, injected from the cerebrum at the contralateral position of the spinal cord. Moderate axonal sprouting (indicated by black arrows) should be observed in Nogo-A^{-} mice rostral and caudal to SCI (Fig.10B and 10E), while extensive sprouting should be observed rostral and caudal to SCI in the Nogo-A/B$^{-/-}$ mice (Fig.10C and 10F). Such results may consolidate Nogo-B as a key player in myelin-derived inhibition of CNS neuronal growth.

b) Sagittal Studies
Sagittal sections are expected to reveal an absence of axonal sprouting rostral and caudal to SCI in wild Type mice (Fig.11A). The sagittal sections of the spinal cord of Nogo-A^{-} mice however should reveal moderate axonal sprouting (indicated by black arrows) (Fig.11B) as was demonstrated by Kim, J., et al., 2003, and extensive axonal sprouting is expected in the spinal cord of Nogo-A/B$^{-/-}$ mice (Fig.11C). The red and green arrows indicate the injury site at T4 and the corticospinal tract stained with BDA respectively.

Strategy E: Assessment of Functional Recovery of Locomotor Activity Following Spinal Chord Injury

Method
Correlation of extensive long-distance axonal regeneration with functional recovery after the hemisection injury in the recombinant mice will be assessed using the BBB score, a standardized open-field measure of locomotor function after SCI (Basso, et al., 1996). The test is designed to rate the movement of a mouse on a scale from 0 to 21, 0 being the lowest and 21 being the highest. It is comprised of placing a mouse inside of a pool with a diameter of approximately 90cm and walls 10cm high (Scheff et al, 2002). The mouse is observed for a 4 minute period by two separate observers, who base the score on a variety of critera (Scheff et al, 2002). The animals are tested starting as soon as 1 day post-injury, testing typically occurs at 1 week intervals for 6-9 weeks, but can be monitored as frequently as daily. (Scheff et al, 2002). A score of 0 is awarded to a mouse who shows no hind limb movement, and a score of 21 is awarded to a mouse who displays coordinated limb movement, consistent planar stepping, consistent toe clearance, parallel paw position throughout the step cycle, consistent trunk stability, and the tail constantly pointed upwards (Scheff et all, 2002).

Expected Results
Comparison of open field locomotor activity between Nogo-A^{-} and Nogo-A/B$^{-/-}$ mice should demonstrate significantly lower BBB scores achieved by Nogo-A^{-} mice than by Nogo-A/B$^{-/-}$ mice. Hence, recovery of locomoter performance should be accelerated in Nogo-A/B$^{-/-}$ mice, which would be most likely attributed to increased axonal growth if observed.

Aim Two: Test for differential expression of OMgp in younger (6-11 weeks) and older (>11 weeks) mice

Rationale: It is possible that age affects axonal sprouting in Nogo-A/B$^{-/-}$ mice. All of Strittmatter's young adult Nogo-A/B$^{-/-}$ mice exhibited pronounced CST axon sprouting proximal to the site of injury (Kim, J. et al. 2003). However, preliminary studies indicate that sprouting may be restricted at older ages in the Nogo-A/B$^{-/-}$ mice (Kim, J. et al. 2003). Thus, CST sprouting may occur only when SCI occurs at the young adult stage (6-11 weeks) (Kim, J. et al. 2003). If substantiated, this could indicate that neurons in older animals have either a reduced capacity to grow or that some non-Nogo growth inhibitory factor is expressed increasingly with age (Woolf, C. 2003). However, any implication of this in human adult CNS lesions remains unknown (Woolf, C. 2003). Therefore, we propose that the differential axonal sprouting in young (6-11 weeks) and adult (>11 weeks) mice is due to an inhibitor that is expressed increasingly with age. A recent study conducted by Wang, K. et al. 2002 reports that OMgp-enriched myelin fraction inhibits neurite outgrowth more than a MAG-enriched myelin fraction. OMgp is highly expressed by mature oligodendrocytes positive for myelin basic protein (MBP) which are enriched with axon-adjacent myelin layers (Wang, K. et al. 2002). The effect of OMgp has not yet been studied *in vivo* (Wang, K. et al. 2002). In order to determine if OMgp expression is age-dependant, we will perform Northern and Western analyses, immunohistochemistry, and *in situ* hybridization to evaluate wild type OMgp levels and locations *in vivo* within mice ages 6 weeks, 11 weeks, and 14 weeks.

Strategy A: Northern Blot analysis of mice

Method
A Northern blot will be done (as explained in Aim 1, Strategy B, Method 2) to evaluate whether or not the OMgp gene is expressed in wild type mice of ages 6 weeks, 11 weeks, and 14 weeks. A probe will be developed for the OMgp gene from a cDNA library (Origene Technologies), where a fragment of OMgp DNA sequence will be used to create a ^{32}P-labeled probe through PCR, which will hybridize with the OMgp mRNA (GrandPre, T. et al. 2000). ß-actin probe (Kim, J. et al. 2003) will be used as the loading control.

Expected Results
Northern blotting should reveal expression of OMgp in all ages (6, 11, and 14 weeks) of the wild type mice. Strength of hybridization of the probe should increase with age to account for age-dependant inhibition seen in Strittmatter's Nogo-A/B$^{-/-}$ mice (Figure 13). ß-actin expression should be consistent through all ages of mice.

Strategy B: Western Blot analysis of mice

Method
A Western blot will be done to reveal the presence or absence of the OMgp protein within wild type mice of ages 6 weeks, 11 weeks, and 14 weeks. Anti-OMgp antibodies will be used to detect enrichment of OMgp protein in brain extracts (Wang, K. et al. 2002). Myelin-Basic protein will be used as the loading control considering that it is found in nervous system cells, and its quantification is not dependant upon age (Kim, J. et al. 2003).

Expected Results
Western blotting is expected to indicate the occurrence of protein synthesis at a greater rate in correlation with increased age in wild type mice (Figure 14). The OMgp antibody should give a stronger signal in adult compared to young mice. Myelin Basic Protein should indicate consistent expression through all ages of mice.

Strategy C: Immunohistochemistry analysis of mice

Method
Immunohistochemistry analysis will be used to detect OMgp expression in spinal cord and several brain tissues (cerebral cortex, cerebellum, and corpus callosum) of the wild type mice of ages 6 weeks, 11

weeks, and 14 weeks. Protein expression will be assessed using the primary and secondary antibodies developed by Wang, K. et al. 2002. Tissue samples will be prepared and sections developed from anesthesized mice, and OMgp expression will fluoresce from staining by antibodies (Wang, X. et al. 2002). Lung tissue from wild type mice will be used as a control since OMgp is an oligodendrocyte-myelin glycoprotein and should only be found in nervous system cells (Kim, J. et al. 2003).

Expected Results
The immunohistochemistry analysis should indicate evident OMgp protein expression in the spinal cord, cerebral cortex, cerebellum, and corpus callosum of all ages of wild type mice. OMgp should immunofluoresce by primary and secondary antibodies in larger quantities in correlation with increasing ages of mice to account for age-dependant inhibition. OMgp should not immunofluoresce in lung tissue considering that OMgp expression should only occur in the CNS (Figure 15).

Strategy D: *In Situ* Hybridization analysis of mice

Method
In situ hybridization analysis will be used to detect OMgp expression in the spinal cord and several brain tissues (cerebral cortex, cerebellum, and corpus collasum) of wild type mice of ages 6 weeks, 11 weeks, and 14 weeks. *In situ* hybridization reveals whether the mRNA is translated into protein even if the protein, in that cell is not expressed (Nieto, M. et al. 1996). In a previous study, OMgp was reported to be highly expressed by mature oligodendrocytes positive for myelin basic protein (MBP), and enriched in the axon-adjacent myelin layers (Wang, K. et al. 2002). Hence, an *in situ* hybridization should reveal similar results. Protein expression will be assessed using the same probe as aforementioned (GrandPre, T. et al. 2000). Tissue samples will be prepared similar to aforementioned (Nieto, M. et al. 1996). Lung tissue from wild type mice will be used as a control (Kim, J. et al. 2003).

Expected Results
The *in situ* hybridization analysis is expected to demonstrate OMgp expression in oligodendrocytes and myelin evident in the spinal cord, cerebral cortex, cerebellum, and corpus callosum of all ages of wild type mice. OMgp should be hybridized by the probe and expressed in profile in a similar manner to the CNS tissue in immunohistochemistry analysis. The tissue should not express in lung tissue (Figure 16).

Aim Three: Creation of OMgp and Nogo-A/Nogo-B/Omgp$^{-/-}$ knockout mice

Rationale: Based on the expected results from aim 1, the most plausible reason for the modest axonal regeneration in the mice from Schwab's lab (Simonen et al., 2003) is the action of Nogo-B acting as a compensatory protein causing inhibition of axonal regeneration. The expected results from aim 2 suggest the possibility that OMgp acts as a potential inhibitor in older mice. Therefore we propose to construct two types of knockout mice to demonstrate the inhibitory activity of Omgp on axonal regeneration in older mice specifically. The Omgp gene will be knocked out, and both younger (6-11 weeks) and older (>11 weeks) mice will be analyzed for potential axonal regeneration after SCI. The main objective of this experiment is to determine whether OMgp is the prominent inhibitor in older mice. We expect axonal regeneration after SCI in older mice (>11 weeks), but not in younger mice (6-11 weeks). We will then design a Nogo-A/Nogo-B/Omgp$^{-/-/-}$ mice by knocking out OMgp in the mice designed in aim 1. The purpose of this experiment is to investigate the role of Nogo-A and Nogo-B as potential inhibitors of axonal regeneration besides OMgp in older mice. We hypothesize that Nogo-A & B act as prominent inhibitors in younger mice (7-11weeks) and that OMgp is the prominent inhibitor in older mice (>11 weeks). The success of both aims would be tested by *in vitro* and *in vivo* techniques. *In vitro* studies include neurite outgrowth and growth cone collapse assays, while *in vivo* studies consist of histological analyses of coronal and sagittal sections of the spinal cord following SCI.

Step: OMgp^{-} Knockout Mice

Strategy A: Designing of the OMgp Knockout Mice

Method
Wild type mice will be obtained from Strittmatter's lab (Kim et al., 2003) and will serve as tools for designing the triple knockout mice. The OMgp knock mice will be generated by introducing a fusion gene construct will be obtained from Strittmatter et al (Kim et al., 2003), which expresses the β-geo and the neomycin resistant genes. The gene construct will be placed before the OMgp promoter through homologous recombination, by modifying the gene construct will be further with sequences of the actual OMgp gene (Wang, K. et al. 2002). The homozygous mice will be generated using the gene construct as explained in aim 1, strategy A.

Strategy B: Verifying the genotype of the Knockout Mice *in vitro*

Method 1: Northern Blotting
The OMgp gene, if disrupted by the neomycin-galactosidae fusion gene, is not expected to produce mRNA strand. Total brain RNA will be isolated from the OMgp knock out mouse and hybridized with the same OMgp specific probe used in Aim 2, Strategy A. Cell extracts from the liver cells of the knockout mice will be used for the loading control, and a β-actin specific probe that will be obtained from Strittmatter (Kim et al., 2003) will be used as the loading control.

Expected Results
Omgp protein is expected to be present in wild type mice and absent in the brain extract and liver cells of the OMgp$^{-/-}$ mice (Fig.17). Equal amounts of mRNA content are expected to arise in both wild type and knock out mice for the loading control (Fig. 17).

Method 2: Western Blotting
The western blot analysis will be conducted on total brain lysate. The OMgp antibody for the western blot will be obtained from Wang et al (Wang et al., 2003) and will be hybridized as explained in aim 1, strategy b, method 3. Cell extracts from liver cells will be analyzed for the negative control, and expression of MBP protein will be analyzed using the MBP specific antibody obtained from Strittmatter lab (Kim et al., 2003) for the loading control.

Expected Results
If the neomycin gene successfully replaces the target OMgp gene, the OMgp protein should not be produced and will not bind to the OMgp specific antibody. Therefore, we expect the lane to run blank in the knock out mice (Fig. 18). Since, OMgp is expressed only CNS, we also do not expect a signal for the liver cells.

We do however expect the expression of MBP in the cells of both types of mice, considering that it is commonly expressed in all CNS cells (Fig. 18).

Method 3: Analysis of Brain Histology
To ensure that the absence of OMgp gene is not affecting the structure and general function of the brain, hematoxylin, eosin, and luxol fast blue staining assays (Kim et al., 2003) will be applied to the cerebral cortex, cerebellum, and corpus callosum sections as explained in aim 1, strategy c, method 1. The expression of β-galactosidase gene will be also tested as explained in aim 1, strategy c, method 1.

Expected Results
We expect that both the wild type and the knockout mice will demonstrate similar brain histology considering that we should not have affected any proteins other than OMgp (Fig. 19). For the cells grown in X-Gal plates, we expect to see blue coloration due to the expression of the b-galactosidase gene (Fig.19).

b) Rotarod Test: The rotarod test was used to compare the functional recovery of locomotor activity following SCI in knockout mice compared to wild type mice, and was performed as explained in aim 1, strategy c., method 2.

Expected Results
Wild type and OMgp-/- mice are expected to behave similarly in the rotarod test (Fig. 20).

Strategy C: *In Vitro* Investigation of young (6-11 weeks) and adult (>11 weeks) OMgp Mice.

OMgp is produced by oligodendrocytes, which are present in myelin. To investigate the different effects of OMgp in younger (6-11 weeks) and older mice (>11 weeks), we will compare the growth of wild type axons in cultures containing myelin derived from young and old $OMgp^{-/-}$ mice. We will use two different assays for this investigation:

Method 1: Neurite Outgrowth Assay
The neurite outgrowth assay will measure the extent to which axons sprout and will be conducted as explained in aim 1, strategy c, method 1.

Expected Results
We expect to see more neurite outgrowth in older $OMgp^{-/-}$ mice compared to young $OMgp^{-/-}$ mice, due to the presence of Nogo-A&B in younger mice (Fig. 21).

Method 2: Growth Cone Collapse Assay
The growth cone collapse assay will measure the extent of axonal branching by specifically detecting the number of growth cones in a culture. The protocol for the assay will be adapted from aim 1, strategy c, method 2.

Expected Results
We expect to see more growth cones in older $OMgp^{-/-}$ mice (>11 weeks) compared to young $OMgp^{-/-}$ mice(6-11 weeks) due to the presence of Nogo-A&B in younger mice (Fig. 22).

Strategy D: *In Vivo* Investigation of the OMgp knockout Mice

The most important part of the third aim is the investigation of the axonal regeneration after SCI in young (6-11 weeks) and old $OMgp^{-/-}$ mice (>11 weeks). This study will be conducted as explained in aim 1, strategy d.

Expected Results
a) Coronal Studies
We expect to see more axonal sprouing in older $OMgp^{-/-}$ mice (6-11 weeks) compared to younger $OMgp^{-/-}$ mice (>11 weeks) due to the presence of Nogo-A&B in younger mice (Fig. 23). We also expect the sprouting to extend ipsilaterally and contralaterally to the CST considering that factors that control the directions of axonal growth are usually absent at the adult stages.

b) Sagittal Studies
We expect to see extensive axonal sprouting rostral and caudal in both contralateral and ipsilateral directions around SCI in older $OMgp^{-/-}$ mice and no sprouting should be observed in younger $OMgp^{-/-}$ and wild type mice due to the presence of Nogo-A and Nogo-B.

Step 2: Nogo-A/Nogo-B/$OMgp^{-/-/-}$ Knockout Mice

Strategy A: Design of the Nogo-A/Nogo-B/$Omgp^{-/-/-}$ Knockout Mice

The removal of OMgp in step 1 of aim 1 should demonstrate OMgp as an axonal growth inhibitor in older mice. Considering the presence of other inhibitors like Nogo-A & B, the next logical step is to investigate the extent of axonal regeneration following SCI in older and younger Nogo-A/Nogo-B/$OMgp^{-/-/-}$ mice. We expect to see extensive axonal sprouting as was seen by Strittmatter et al., 2003 in the younger Nogo-A/Nogo-B/$OMgp^{-/-/-}$ mice and a similar result as seen in step 1 of aim 3 in older Nogo-A/Nogo-B/$Omgp^{-/-/-}$ mice. The Nogo-A/$B^{-/-}$ mice used in the investigation of aim 1 will be used for designing the Nogo-A/Nogo-B/$OMgp^{-/-/-}$ mice. The same fusion gene used in step 1 of aim 3, which consists of the neomycin drug resistant gene and β-geo gene will be put under the promoter of the OMgp gene. The Nogo-A/Nogo-B/$OMgp^{-/-/-}$ mouse will be created from the Nogo-A/B, consisting of the neomycin drug resistant gene and the β-geo gene as explained in aim 1, strategy A.

Strategy B: Verifying the genotype of the Nogo-A/Nogo-B/$Omgp^{-/-/-}$ Knockout Mice *in vitro*

Before proceeding with further investigation on the knockout mice, it is necessary to test its phenotype both *in vitro* and *in vivo*. Therefore, we will conduct Northern and Western blots, and histological and behavioral studies on the Nogo-A/Nogo-B/$OMgp^{-/-/-}$ mice as explained in aim 1, strategy b.

Method 1: Northern Blotting
Total brain RNA will be isolated and hybridized with the OMgp specific probe used in aim 3, Strategy b, method 1 as explained in aim 1, strategy b, method 1. A negative control for the assay will be conducted on cell extracts from the liver and a loading control for testing the efficiency of the assay will be conducted using the β-actin specific probe used in aim 3, strategy b, method 1.

Expected results
Signals for OMgp are expected to be seen in wild type extracts, but not in brain and cell extracts of the Nogo-A/Nogo-B/$OMgp^{-/-/-}$ mice (Fig. 25). Also, transcription of

β-actin mRNA should be observed in both wild type and the triple knockout mice.

Method 2: Western Blotting
A western blot will be performed to test the existence of OMgp protein in Nogo-A/Nogo-B/OMgp$^{-/-}$ mice, which will be conducted on total brain lysate using the OMgp specific antibody used in aim 3, strategy b, method 2. A negative control will be conducted on cell extracts from liver cells, and the loading control for the assay will be conducted by analyzing the MBP protein expression in both wild type and Nogo-A/Nogo-B/OMgp$^{-/-}$ mice using the MBP specific antibody aim 3, strategy b, method 2.

Expected Results
We expect to see signals for OMgp expression n wild type mice, while observing no bands in brain and cell extracts of Nogo-A/Nogo-B/OMgp$^{-/-}$ mice (Fig. 26). We also expect expression of MBP in both wild type and Nogo-A/Nogo-B/OMgp$^{-/-}$ mice for the loading control.

Strategy B: Verifying the genotype of the Nogo-A/Nogo-B/Omgp$^{-/-}$ Knockout Mice *in vivo*

Method 1: General Brain Histology
The histological analysis for testing the structural integrity and functionality of the triple knockout mice will be conducted on the cerebral cortex, cerebellum, and corpus callosum as explained in aim 1, strategy b, method 1. The expression of the β-galactosidase gene in the fusion gene construct will be investigated using X-gal staining as described in aim 1, strategy b, method 1.

Expected Results
We expect to observe expression of OMgp in wild type cells and no expression in Nogo-A/Nogo-B/OMgp$^{-/-}$ mice (Fig. 27). We expect expression of the β-galactosidase protein in all the cells analyzed, which will indicate successful introduction of the gene construct (Fig. 27).

Method 2: Rotarod Test
The rotarod test will be used to assess the functional recovery of locomotor activity of knockout mice compared to the wild type mice, which will be conducted as explained in aim 1, strategy c, method 2.

Expected Results
We expect the rotarod test to demonstrate relatively equal time spend by wild type and knockout mice, which would demonstrate normal behavior of the Nogo-A/Nogo-B/Omgp$^{-/-}$ mice (Fig. 28).

Strategy C: *In Vitro* Investigation of the Nogo-A/Nogo-B/Omgp$^{-/-}$ Knockout Mice.

The *in vitro* studies explained in aim 1, strategy c, method 1 & 2 will be repeated on the triple knockout mouse.

Method 1: Neurite Outgrowth Assay
As explained in prior sections, the neurite outgrowth assay is used to study axonal growth in a culture.

Expected Results
Extensive axonal sprouting is expected to occur in cultures containing myelin derived from both young and old Nogo-A/Nogo-B/Omgp$^{-/-}$ mice, as well as in the control. Considering that these, cultures are devoid of the major axonal growth inhibitors such as Nogo-A, Nogo-B and OMgp.

Method 2: Growth Cone Collapse Assay
The growth cone collapse assay measures the extent to which axons will branch, by assaying the number of growth cones is a culture.

Expected Results
We expect to see several growth cones in both younger (6-11 weeks) and old (>11 weeks) Nogo-A/Nogo-B/OMgp$^{-/-}$ mice.

Strategy D: In Vivo Investigation of the Nogo-A/Nogo-B/Omgp$^{-/-}$ Knockout Mice

In this part of the study, we will investigate axonal regeneration following SCI in younger (6-11 weeks) and older (>11 weeks) Nogo-A/Nogo-B/OMgp$^{-/-}$ mice using BDA injections as explained for the coronal and sagittal studies for young and old Nogo-A/B$^{-/-}$ mice in aim 1, strategy d, method. For rostral and caudal investigations, coronal sections at the T4 and C8 vertebrae of the spinal cord will be observed for rostral and caudal investigations respectively.

Expected Results
a) Coronal Studies
We expect to observe extensive axonal sprouting rostral and caudal in both ipsilateral and contralateral orientations to the SCI. We do not expect any axonal regeneration to occur in wild type mice.

b) Sagittal Studies
The trajectory of axonal sprouting around the SCI is expected to occur both rostral and caudal in both ipsilateral and contralateral directions in both young and old Nogo-A/Nogo-B/OMgp$^{-/-}$ mice. No sprouting is expected to occur for wild type mice.

Aim four: The creation of transgenic mice to overexpress OMgp

Rational: Since OMgp expression is increasing with age in Nogo-A/B$^{-/-}$ mice, we want to determine whether increasing the expression of OMgp in Nogo-A/B$^{-/-}$ mice will decrease the regeneration of axons, and whether this decrease will be dependant on age. It is necessary to create a transgenic mouse with a different promoter for the OMgp gene so that the protein will be over expressed. Once the mice have been created, we will conduct western blot to check the presence of the induced gene construct. The mice will then be tested for axonal regeneration sagittaly and coronally using BDA injections into the cerebral cortex as explained in aim 1, strategies c & d.

Strategy A: Creation of transgenic Nogo-A/B$^{-/-}$ mice with overexpression of OMgp

Method
We will use our Nogo-A/B$^{-/-}$ mice created in aim 1 as the basis for our transgenic study. The transgene will be created using the OMgp gene with an oligodendrocyte specific promoter for the Myelin Basic Protein (MBP) gene (Chen et al, 1999), which will then be put into a vector. An ampicillin resistance gene will also be added, so that the cells containing the transgene (OMgp^{++}) can be selected for on ampicillin containing media. The altered gene will then be introduced into a line of mouse embryonic stem (ES) cells, and the transgene will randomly insert itself into the ES cell genomes through homologous recombination. These ES cells will be injected into a fertilized egg, several of which are then implanted into a pseudopregnant mouse. The resulting offspring will be

tested for the presence of the gene within their germ cells. The mice testing positive will then be used to study, because all of their progeny will also contain the transgene, and 25% will be homozygous.

Expected Results
The mice containing the transgene within their germ-line cells will be bred together to produce offspring. Of these offspring 25% are expected to be homozygous for the transgene based on Mendelian genetics (Fig. 33). The homozygous mice will then be used for further studies.

Strategy B: Verification of transgenic Nogo-A/B$^{-/-}$ mice with over expression of OMgp *in vitro*

Method 1: Western blotting
Western blotting will be used to prove the presence of OMgp is greater in the transgenic mice. The western blots will be preformed according to standard procedure (Amersham Biosciences) using the OMgp specific antibody (Wang et al., 2003). The loading control will be conducted using MBP and MAG antibodies (Kim et al., 2003) and the negative control will be conducted on liver cells using the OMgp antibody.

Expected Results
We expect that the mice we create will not produce any Nogo-A or Nogo-B, and the levels of OMgp within the oligodendrocytes will be significantly higher than the control mice. The western blots should show a much more intense band for OMgp in the experimental mice (Fig. 34). Other proteins common to myelin are myelin associated glycoprotein (MAG) and myelin basic protein (MBP), which are used here to display that increased OMgp production is not affecting the production levels of other proteins within the oligodendrocytes. For the loading control, we expect equal amount of expression in the wild type and experimental mice and we do not expect any signal for OMgp, MBP or MAP in liver cells.

Strategy B: Verification of transgenic Nogo-A/B$^{-/-}$ mice with over expression of Omgp *in vivo*

Method 1: General Brain Histology
Histology will be tested to prove the molecular composition of the mice is the same, and it will be done using hemotoxylin and eosin stains (Kim et al., 2003) to view the neuronal make up of the cerebral cortex and cerebellum. The assays will be conducted as explained in aim I, strategy c, method 1.

Expected Results
The neuronal make up of the brain should be unaffected by the overexpression of OMgp. This will be shown by analysis of general brain histology (Fig. 35). The cellular compositiion of the cerebral cortex does not appear different in WT mice compared to mice expressing normal OMgp levels and mice overexpressing OMgp (Fig. 35 A-C). Parasagittal sections of the cerebellum demonstrate similar results, as no significant difference is seen (35 D-F).

Strategy C: *In Vitro* Investigation of the Transgenic Mice that overexpresses OMgp.

OMgp is produced by oligodendrocytes, which compose the myelin sheath surrounding axons. To investigate the differential effects of the overexpression of OMgp we will compare the growth of wild type axons in cultures containing myelin derived from OMgp^{+} and OMgp^{++}-Nogo-A/B$^{-/-}$ mice to the culture without axonal growth inhibitors. We will use two different assays for this investigation:

Method 1: Neurite Outgrowth Assay
This test is designed to view the sprouting of axons from cell bodies on control media containing no myelin and compare the sprouting to axons grown on myelin derived from OMgp^{+}Nogo-A/B$^{-/-}$ and OMgp^{++}-Nogo-A/B$^{-/-}$ mice. The assay will be conducted as explained in aim 1, strategy c, method 1.

Expected Results
The control mice should show no extensive sprouting because they are not being grown on myelin, and thus are not subjected to any inhibitory factors. Extensive sprouting should also be seen on the plate containing myelin of the genotype OMgp^{+}-Nogo-A/B$^{-/-}$ due to the lack of Nogo to inhibit sprouting (Fig. 36). The plate with myelin of the genotype OMgp^{++}-Nogo-A/B$^{-/-}$ should show very little sprouting due to the overexpression of OMgp inhibiting growth (Fig. 36).

Method 2: Growth Cone Collapse Assay
This test is designed to examine whether the neurons are forming synapses with other neurons by evaluating the number of growth cones visible. The test is performed the same way as the neurite outgrowth assay described above, except that the growth cones are viewed instead of axons sprouting from cell bodies. The assay will be conducted as explained in aim 1, strategy c, method 1.

Expected Results
The cells on the control plates should show a substantial number of growth cones, due to the lack of inhibitory factors. The cells on the OMgp^{+}-Nogo-A/B$^{-/-}$ myelin should also show a significant number of growth cones because of the lack of Nogo-A/B. The cells on the OMgp^{++}-Nogo-A/B$^{-/-}$ myelin should show no growth cones, since OMgp is being overexpressed and replacing Nogo as the major inhibitor of neural regeneration (Fig. 37 D-F).

Strategy D: In Vivo Investigation of the OMgp Knockout Mice

Method
Similar to the in vivo techniques used in aim 1, strategy d, SCI was introduced in T7 and the resulting axonal sprouting was investigated using BDA injections at sites rostral and caudal to the SCI (Kim et al., 2003). For rostral and caudal investigations, coronal sections at the T4 and L2 vertebrae of the spinal cord were observed respectively.

Expected Results
a) Coronal Studies
For the coronal sections rostral to injury site at T4, the BDA dye should mark the corticospinal tract clearly. The WT mice should show no axonal sprouting, the OMgp^{+}-Nogo-A/B$^{-/-}$ mice should show axonal sprouting both ipsilateral and contralateral to the CST (Fig. 38B & E), and the OMgp^{++}-Nogo-A/B$^{-/-}$ mice should show no axonal sprouting, the same as displayed by the WT mice (Fig. 38A-C).

The caudal sections will not show a prominent marking of the vCST. The WT mice will display no axonal sprouting, the OMgp^{+}-Nogo-A/B$^{-/-}$ will show axonal growth both contralateral and ipsilateral to injection site, and the OMgp^{++}-Nogo-A/B$^{-/-}$ mice will show no axonal growth, in a pattern similar to that of the WT mice (Fig. 38D-F)

b) Sagittal Studies

Sagittal sections of the SC are viewed for WT, $OMgp^{+}$-Nogo-$A/B^{-/-}$ and $OMgp^{++}$-Nogo-$A/B^{-/-}$ (Fig. 39). The WT mouse shows an axon labeled by BDA running to just before the injury site at T7. No growth is seen either rostral or caudal to the injury site, which is due to the presence of Nogo. The $OMgp^{+}$-Nogo-$A/B^{-/-}$ shows axonal sprouting both ipsilateral and contralateral, as well as running past the site of injury. The $OMgp^{++}$-Nogo-$A/B^{-/-}$ mouse shows no axonal sprouting, due to the overexpression of OMgp.

Strategy E: Investigation of the Locomotory Activity of the Transgenic Mice

We considered whether the over expression of OMgp inhibits the recovery patterns observed by Strittmatter et al in Nogo-$A/B^{-/-}$ through the BBB test and was conducted as explained in aim 1, strategy e.

Expected Results

The $OMgp^{++}$-Nogo-$A/B^{-/-}$mice are expected to score significantly lower on the BBB test than are the $OMgp^{+}$-Nogo-$A/B^{-/-}$ mice, due to the added inhibition of axonal sprouting by the overexpression of $OMgp^{+}$. However, some recovery, following a similar pattern as the $OMgp^{+}$ mice is expected (Fig. 40). This result is expected because Strittmater et al saw this result when comparing Nogo-$A/B^{-/-}$ mice to WT mice on the same scale. Since the $OMgp^{+}$-Nogo-$A/B^{-/-}$ mice are the same as the Nogo-$A/B^{-/-}$ mice and the $OMgp^{++}$-Nogo-$A/B^{-/-}$ mice display similar regeneration to WT mice, the BBB results should also correspond.

Conclusion

Revolutionary studies by Steven Strittmatter (Kim et al., 2003) and Martin Schwab (Simonen et al., 2003) shows immense potential in developing genetic techniques that could inactivate axonal growth inhibitors of the CNS for initiating axonal regeneration. Nevertheless, the existence of the Nogo-controversy (Clifford, 2003) in the field had generated great suspicion in the consistency of the aforementioned studies. Our study aims to resolves the controversy in the field with in the next six to eight years by hypothesizing that Nogo-A/B and OMgp act as inhibitors of axonal regeneration in younger and older respectively. We expect that each knock out transgenic model system proposed in our study can be established and studied in 12-20 months. The expected findings should reveal the mystery behind modest axonal regeneration in the Schwab`s mice and in the older (>11 weeks) mice of Strittmatter (Kim et al., 2003). Thus, the study has the potential to initiate new lines of research to elucidate the unknown factors that inhibits axonal regeneration in older mammals. However, we think that the application of the expected findings of the proposal and even the experimental findings of Strittmatter (Kim et al., 2003) into therapeutic techniques needs further investigation. Since, the results that we expect and that had been shown by Strittmatter (Kim et al., 2003) do not recover the damaged synaptic connection, but instead resulted in non-directional axonal sprouting.

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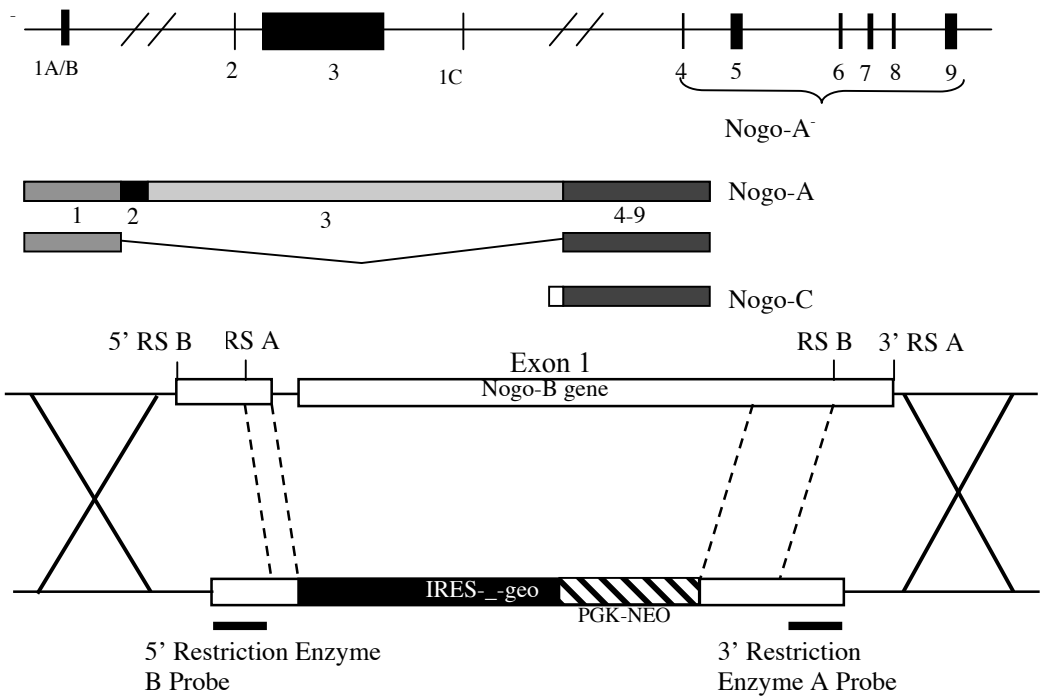


Figure 1. Strategy for creating Nogo-A/B$^{-/-}$ knockout from Nogo-A^{-} knockout

(A) Schematic representation of the Nogo gene and its major protein products, Nogo-A, Nogo-B, and Nogo-C
(B) Schematic representation of the region of the genome covering exon 1 of the Nogo-B-specific portion of the Nogo gene, and the Nogo-A/B$^{-/-}$ knockout construct. This diagram shows the replacement of the Nogo-B gene by the IRES-_-geo and Neomycin-resistant (pGK-NEO) genes. Dotted lines connect homologous sequences of Nogo-B. The various restriction sites (RS) are shown, and restriction enzymes are referred to as letters A and B.

A1	G	G
G	GG	GG
A	GA	GA

A

G-> Wild Type Mice
A-> Mice with cells containing the fusion gene

A2	G	A
G	GA	GA
A	GA	AA

B

Figure 2. Creation of A1 & A2 Generation Mice

Fig. 2A: A1 generation: Cross of Wild type Mice (G) & the offspring of the Pseudo-pregnant mice (A) produces 50% homozygous mice (GA). Fig. 2B: A2 generation: Cross between A1 generation mice (GA) produces 25% homozygous mice (AA).

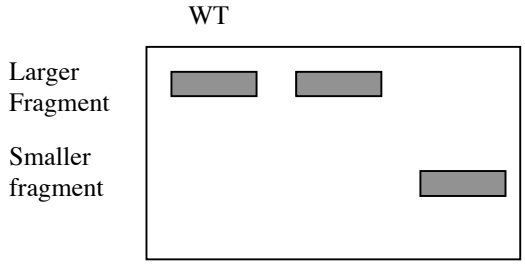


Figure 3. Southern blot analysis of mouse DNA

The 3' probe should identify the larger and smaller bands generated by restriction enzyme A. The wild type (+/+) and control (-/+) mice should only have the larger fragment, while the knock out mouse (-/-) should have only the smaller fragment.

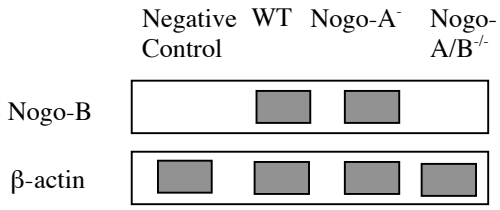


Figure 4. Northern blot analysis of mRNA expression
The WT and Control mice should hybridize with the Nogo-B probe, producing bands for Nogo-B RNA. Negative control and knockout mice should show no bands. Bands should be produced for β-actin in all cell types.

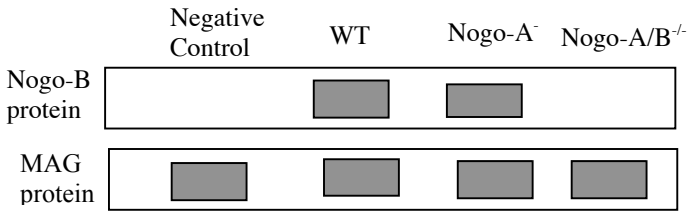


Figure 5. Western blot analysis of mouse strains

The anti-Nogo antibody should only hybridize with proteins of wild type (WT) and Nogo-A- mice only, while the Nogo-A/B-/- lane should run blank to indicate the absence of any Nogo-B protein. The MAG protein should be highlighted in each lane.

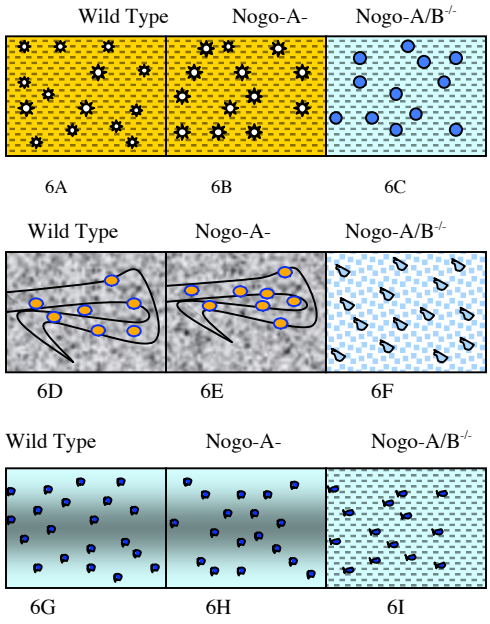


Figure 6. General Brain Histology in Nogo-A/B-/- Mice
(A-C) Coronal sections of the cerebral cortex, stained with hematoxylin and eosin (A and B) or X-gal (C).
(D, E, and G-I) Parasagittal cerebellar sections stained with hematoxylin and eosin (D and E), Luxol fast blue (G and H), or X-gal (I) (F) Coronal section of corpus callosum stained with X-gal.

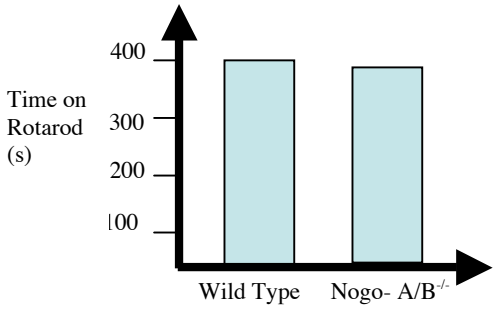


Figure 7. Rotarod Test
Reports the time mice were able to remain on the rotating rod at constant acceleration

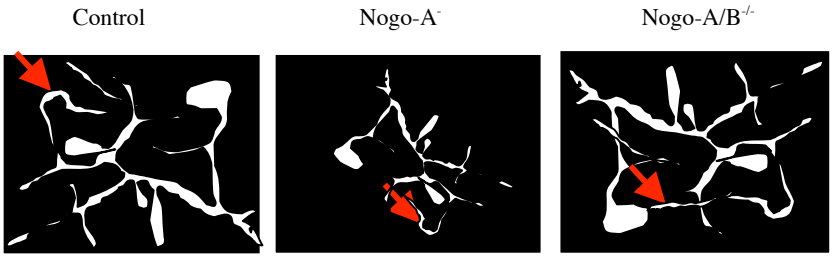


Figure 8: Neurite Outgrowth Assay

Should indicate the presence of more extensive neurite outgrowth in myelin derived from Nogo-A/B$^{-/-}$ (C) and control (A) mice when compared to Nogo-A^{-} (B). Arrows point to extending axons.

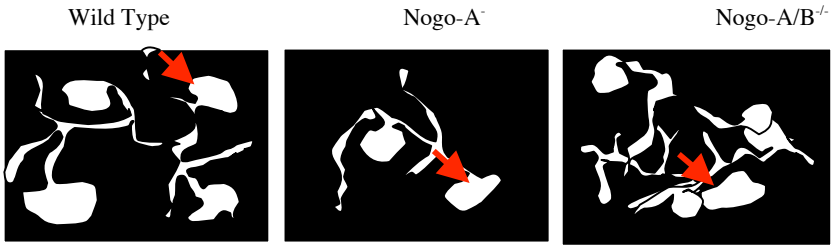


Figure 9. Growth Cone Collapse Assay

Should demonstrate more extensive growth cone collapse in Nogo-A^{-} control mouse axons (B) compared to Nogo-A/B$^{-/-}$ experimental mouse axons (C) and **c**ontrol axons. Arrows point to collapsed growth cones

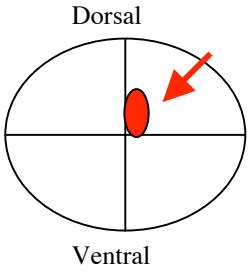
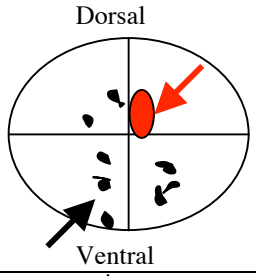
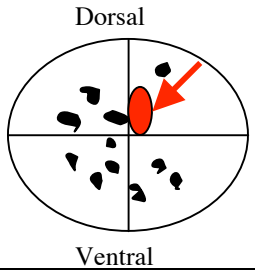
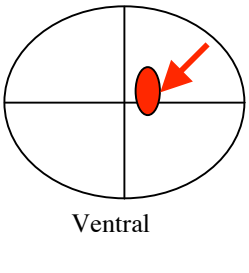
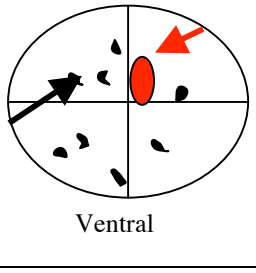
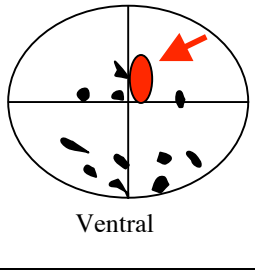
Wild Type Mice	Nogo-A- Mice	Nogo-A/B$^{-/-}$ Mice
Coronal Sections at T4		
		
10A	10B	10C
Coronal Sections at C8		
		
10D	10E	10F

Figure 10. CST Fiber Sprouting Following SCI in Coronal Sections of T4 and C8
More pronounced (larger quantity) and extensive (further projecting) axonal sprouting is expected to occur in coronal sections of the Nogo-A/B$^{-/-}$ mouse T6 and C6 spinal cords (C & F compared to sprouting observed in the Nogo-A^{-} mouse spinal cord (B & E). No sprouting should be observed in Wild Type mice (A, D)

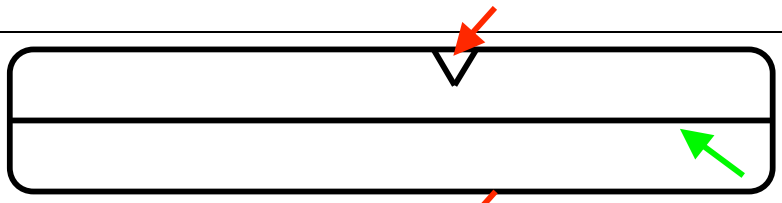
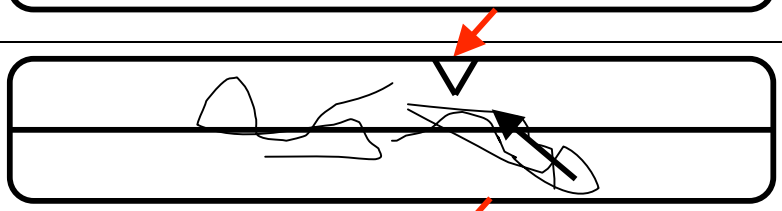
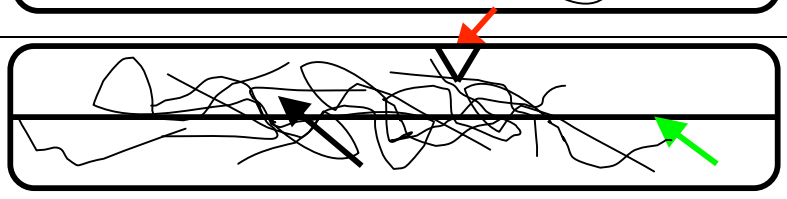
11A	Wild Type	
11 B	Nogo-A^{-}	
11C	Nogo-A/B$^{-/-}$	

Figure 11. Sagittal Sections of the Injury Site
More pronounced and extensive sprouting is expected to occur in Sagittal sections of the Nogo-A/B$^{-/-}$ mouse spinal cord (11C) compared to sprouting observed in the Nogo-A^{-} mouse spinal cord (11B). No sprouting should be observed in Wild Type mice (11A).

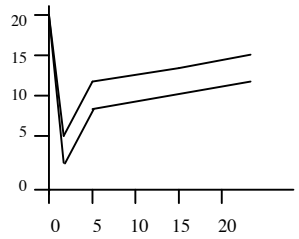


Figure 12. Improved Functional Recovery in Nogo-A/B Knockout Mice
Nogo-A/B$^{-/-}$ mice are expected to regain open-field locomotor performance (hind-limb function) to a much greater extent, at a more accelerated speed, and at an earlier post injury period than Nogo-A^{-} mice.

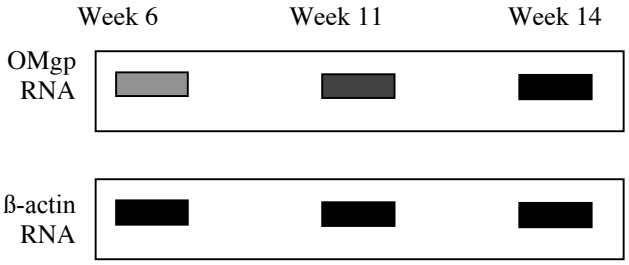


Figure 13. Northern blot analysis of mRNA expression
The probe should hybridize more strongly with increased age to account for age-dependant inhibition seen in Strittmatter's Nogo-A/B$^{-/-}$ mice. The loading control (ß-actin mRNA) should appear consistent in all mouse ages.

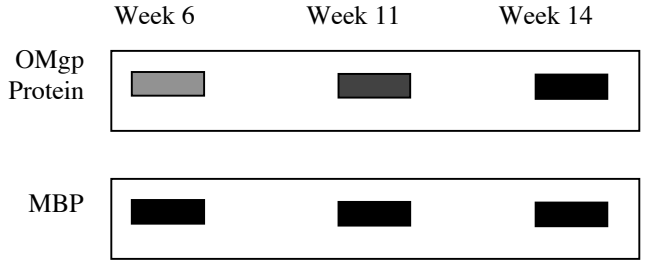


Figure 14. Western blot analysis of protein synthesis
The OMgp antibody should bind more strongly with adult mice compared to younger mice. Myelin Basic Protein should have consistent expression in all mouse ages.

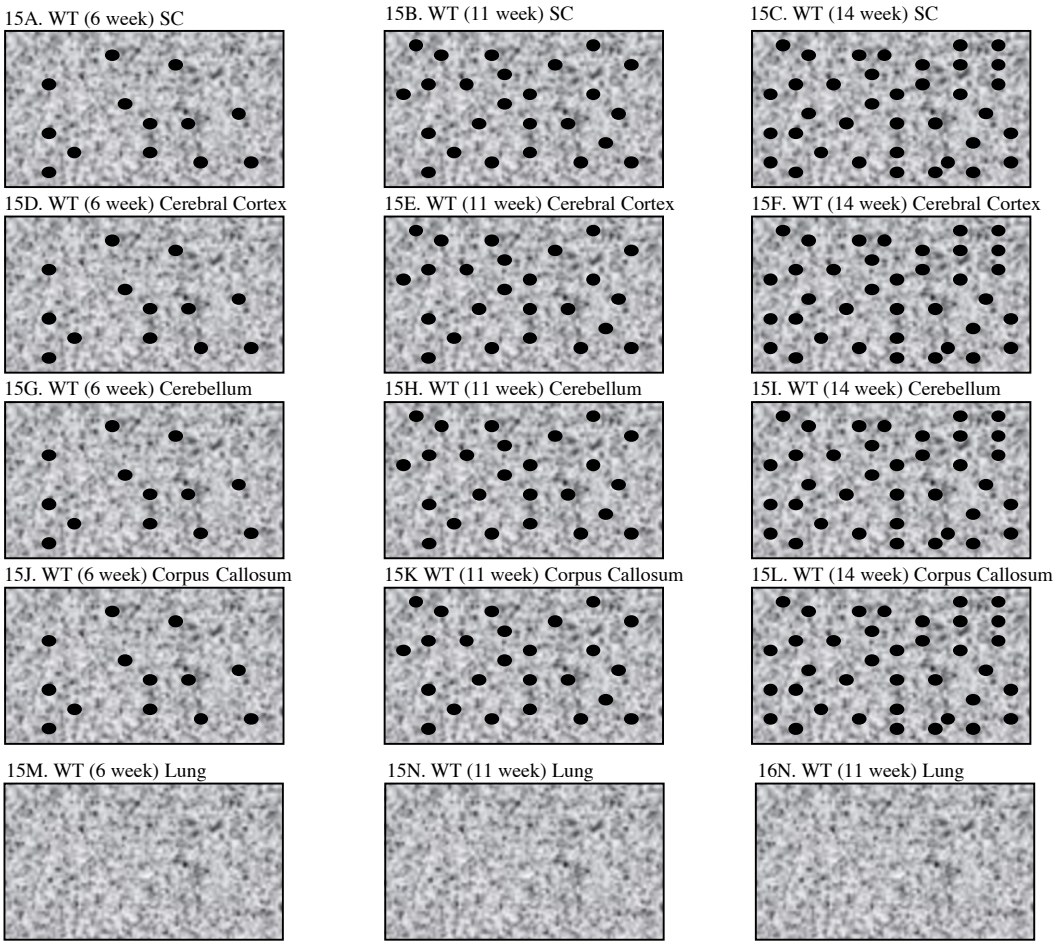


Figure 15. Immunohistochemisty analysis
OMgp protein expression should be evident in the spinal cord (A, B, and C) cerebral cortex (D, E, and F), cerebellum (G, H, and I) and corpus callosum (J, K and L) of all ages of mice. OMgp should immunofluoresce by primary and secondary antibodies in larger quantities as age of mice increases, to account for age-dependant inhibition. OMgp should not immunofluoresce in lung tissue (M, N and O).

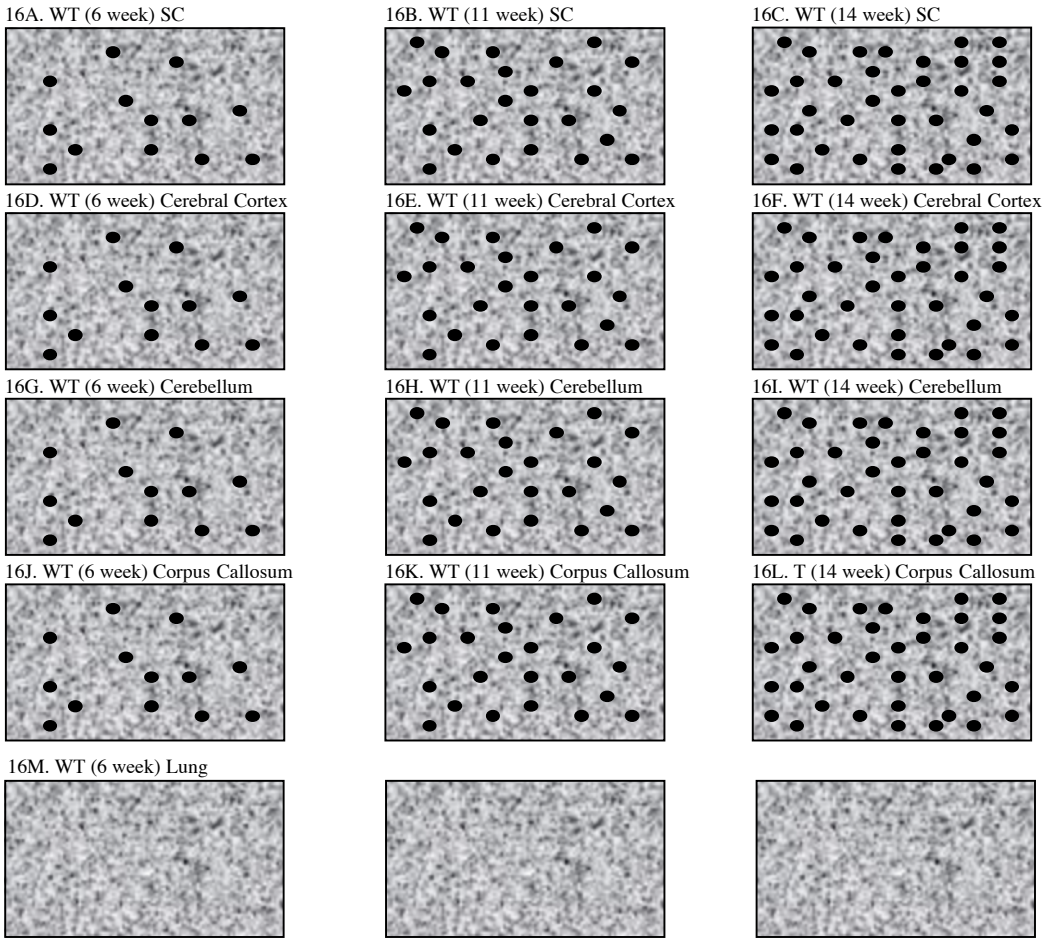


Figure 16. In situ hybridization analysis.
OMgp protein expression should be evident in the spinal cord (A, B and C), cerebral cortex (D, E and F), cerebellum (G, H and I), and corpus callosum (J, K and L) of all ages of mice. OMgp should be increasingly tagged by its probe as age of mice increases to account for age-dependant inhibition. OMgp should not be expressed in lung tissue (M, N and O).

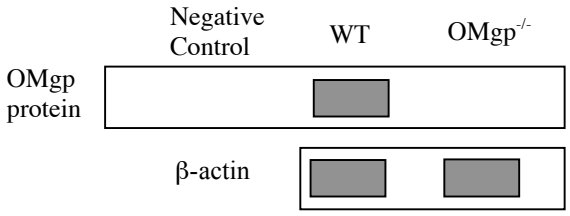


Figure 17A. Northern Blot analysis for OMgp in OMgp knockout mice.
The blot shows the absence of the respective mRNA strand in OMgp-/- mice. β-actin is transcribed in both types of mice.

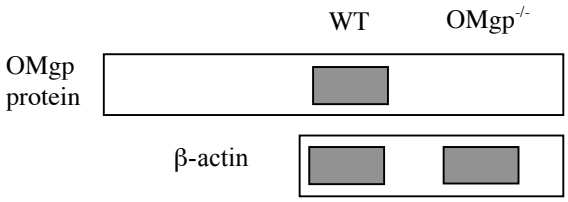


Figure 18: Western Blot Analysis for OMgp Protein.
The blot should show the complete absence of the OMgp protein in brain extracts and liver cells of the knockout mice and the expression of MBP protein in both wild type and knock out mice.

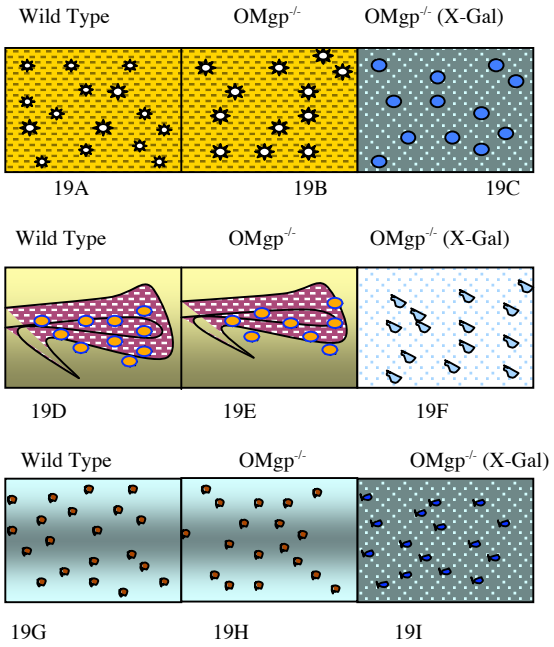


Figure 19. General Brain Histology

Coronal section of cerebral cortex stained with hematoxylin and eosin (A & B) and X-Gal (C). Parasagittal Sections of the cerebellum with hematoxylin and eosin (D andE). Coronal section of cerebellum stained with luxol fast blue (G & H) and X-Gal (I). Coronal section of corpus callosum stained with X-gal (F).

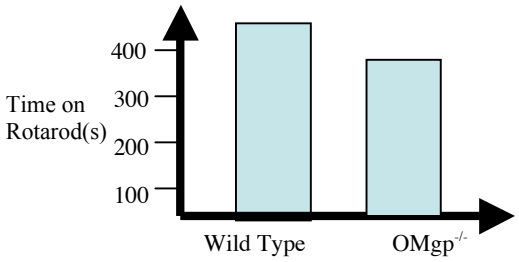


Figure 20. Rotarod testing on Wild type & OMgp Knockout Mice

The test should show that the retention time for the wild type and the knockout mice are nearly the same.

Figure 21: Neurite Outgrowth Assay.

The control (A) should show excessive neurite outgrowth due to the absence of any axonal growth inhibitors. The culture with myelin from OMgp$^{-/-}$ older mice (B) should show extensive branching of axons and very little branching should be observed in the culture with myelin from OMgp$^{-/-}$ younger mice (C).

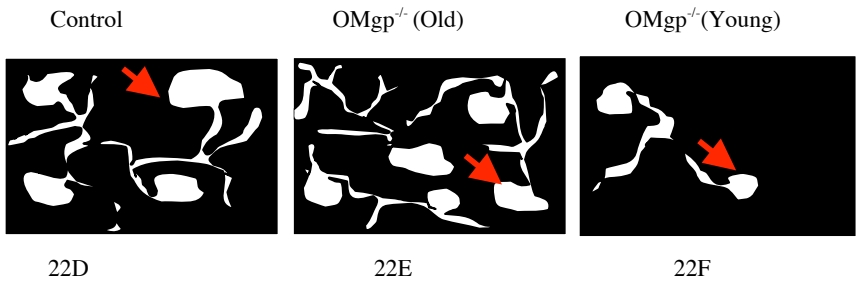


Figure 22. Growth Cone Collapse Assay.
The control (22A) is expected to demonstrate excessive axonal growth. The culture containing myelin from OMgp$^{-/-}$ older mice (22B) is expected to demonstrate extensive branching of axons, with very little branching occurring in the culture with myelin from OMgp$^{-/-}$ younger mice (22C).

Wild Type Mice	OMgp$^{-/-}$ Older Mice	OMgp$^{-/-}$Young Mice
Coronal Sections at T4		

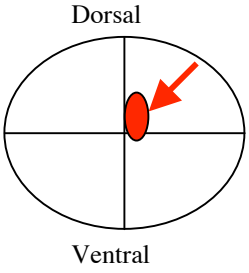
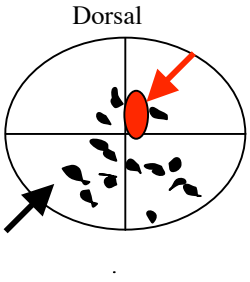
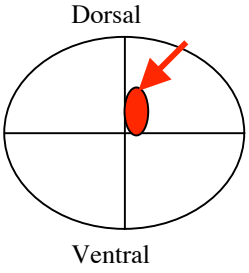

		

Fig. 23A	Fig.23B	Fig.23C
Coronal Sections at C8		

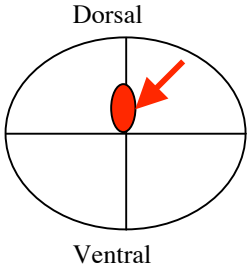
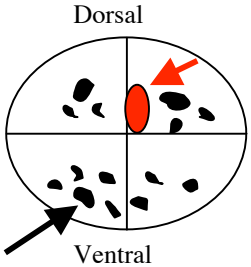
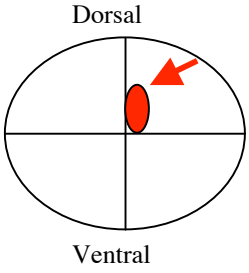

		

Fig. 23D	Fig.23E	Fig.23F

Figure 23. CST Fiber Sprouting Following SCI in Coronal Sections of T4 & C6.
Red arrows indicate corticospinal tract injection with BDA from the cerebrum at the contralateral position of the spinal cord. Extensive axonal sprouting (indicated by black arrows) is expected to be observed in older OMgp$^{-/-}$ mice (B & E) rostral and caudal to SCI, where as none should be observed in younger OMgp$^{-/-}$ and wild type mice (A, C, D, and F). The arrows also indicate that the axonal sprouting is taking place both ipsilateral and contralateral to the site of SCI.

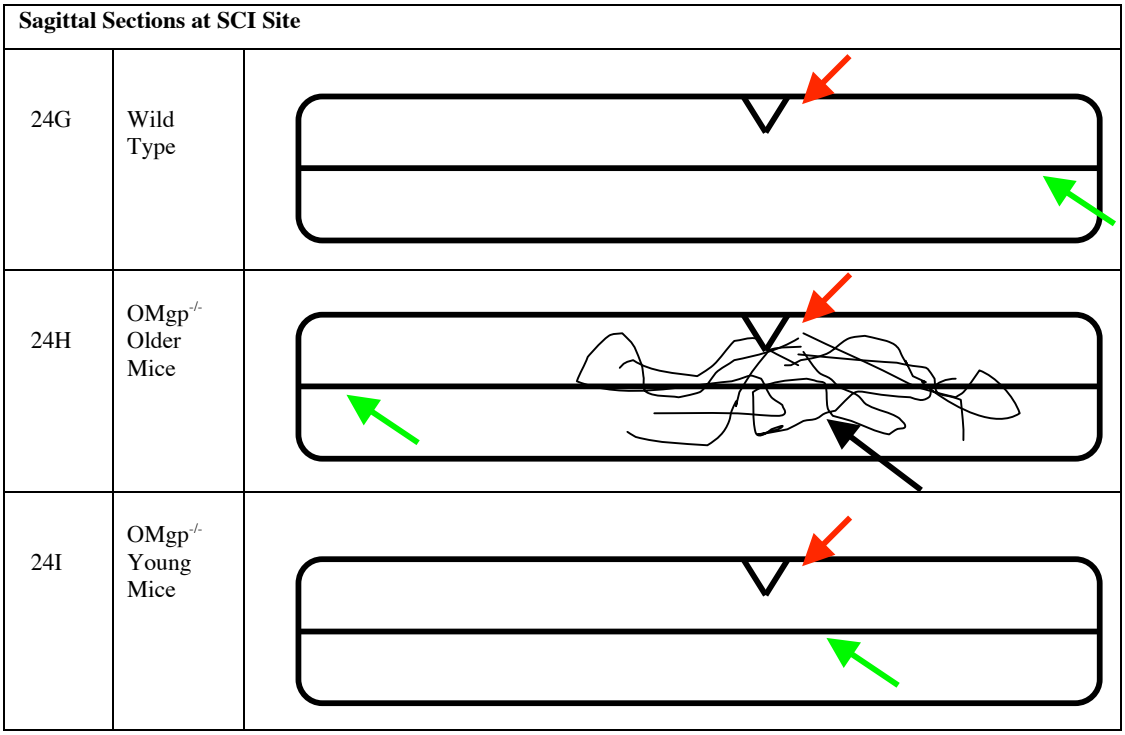


Figure 24.CST Fiber Sprouting Following SCI in Sagittal Sections.
Red arrows and the green arrows indicate the injury site at T6 and the corticospinal tract stained with BDA respectively. The sagittal sections of the older OMgp-/- mice are expected to demonstrate extensive axonal sprouting (indicated by black arrow) rostral and caudal to SCI (H) while no sprouting in younger OMgp-/- mice should occur (I). Fig.24H should also indicate that axonal sprouting is taking place both ipsilateral and contralateral to SCI.

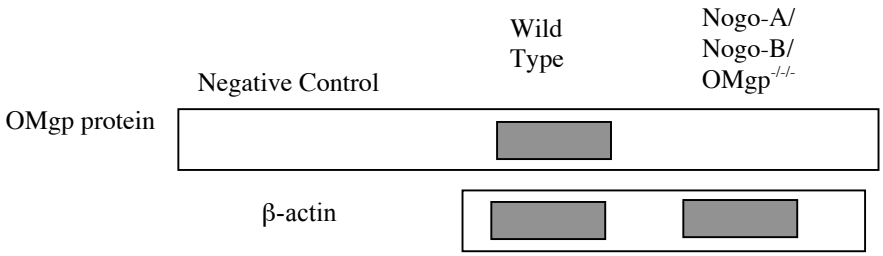


Figure 25: Northern Blot analysis for OMgp in OMgp knockout mice.
The blot should contain signal for OMgp mRNA in wild type mice but not Nogo-A/Nogo-B/OMgp$^{-/-/-}$ mice. Signals for β-actin protein should be observed in both the wild type and triple mutant mice.

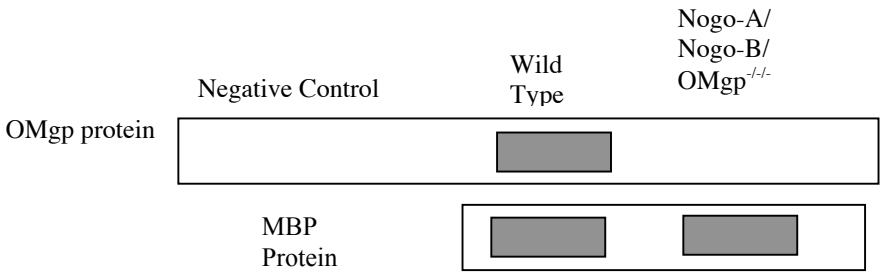


Figure 26: Western Blot Analysis for OMgp Protein.
The blot should indicate the presence of signals for OMgp in wild type mice and absence of signals for Nogo-A/Nogo-B/OMgp$^{-/-/-}$ mice. The blot should also display the presence of the loading control protein MBP in both wild type and Nogo-A/Nogo-B/OMgp$^{-/-/-}$ mice.

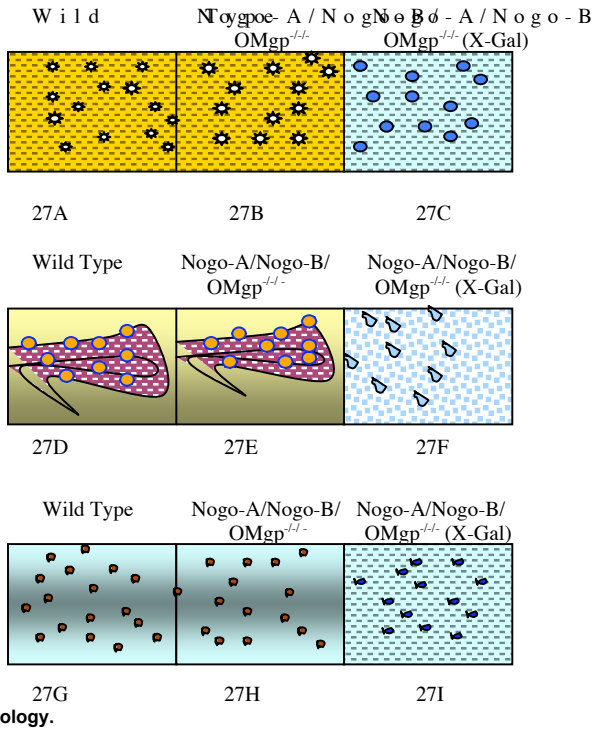


Figure 27: General Brain Histology.
Coronal sections of cerebral cortex, stained with hematoxylin (A), eosin (B), and X-Gal (C). Parasagittal Sections of the Cerebellum are stained with hematoxylin (D), eosin (E), and with Luxol fast blue (G & 27 H). Coronal section of Corpus Callosum with hematoxylin (G), eosin (H), and X-Gal (I).

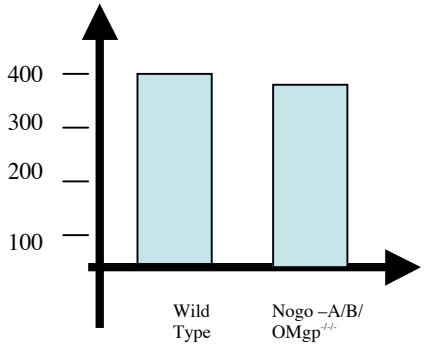


Figure 28. Rotarod test
The plot is expected to demonstrate that equal amounts of time are spent on the rotarod by wild type and Nogo-A/Nogo-B/OMgp$^{-/-}$ mice.

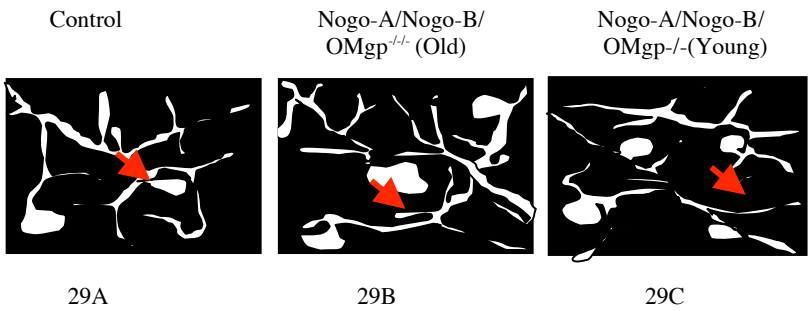


Figure 29. Neurite Outgrowth Assay
The assay should show that both old and young Nogo-A/Nogo-B/OMgp$^{-/-}$ mice (B& C) allows extensive axonal regeneration (indicated by red arrows).

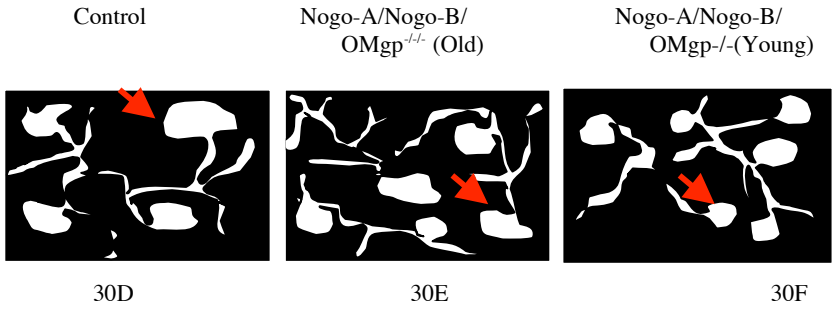


Figure 30. Growth Cone Collapse Assay
The assay is expected to demonstrate extensive branching of axons (indicated by red arrows) in cultures with myelin derived from both older and younger Nogo-A/Nogo-B/OMgp$^{-/-}$ mice (E & F), in addition to the control culture (D).

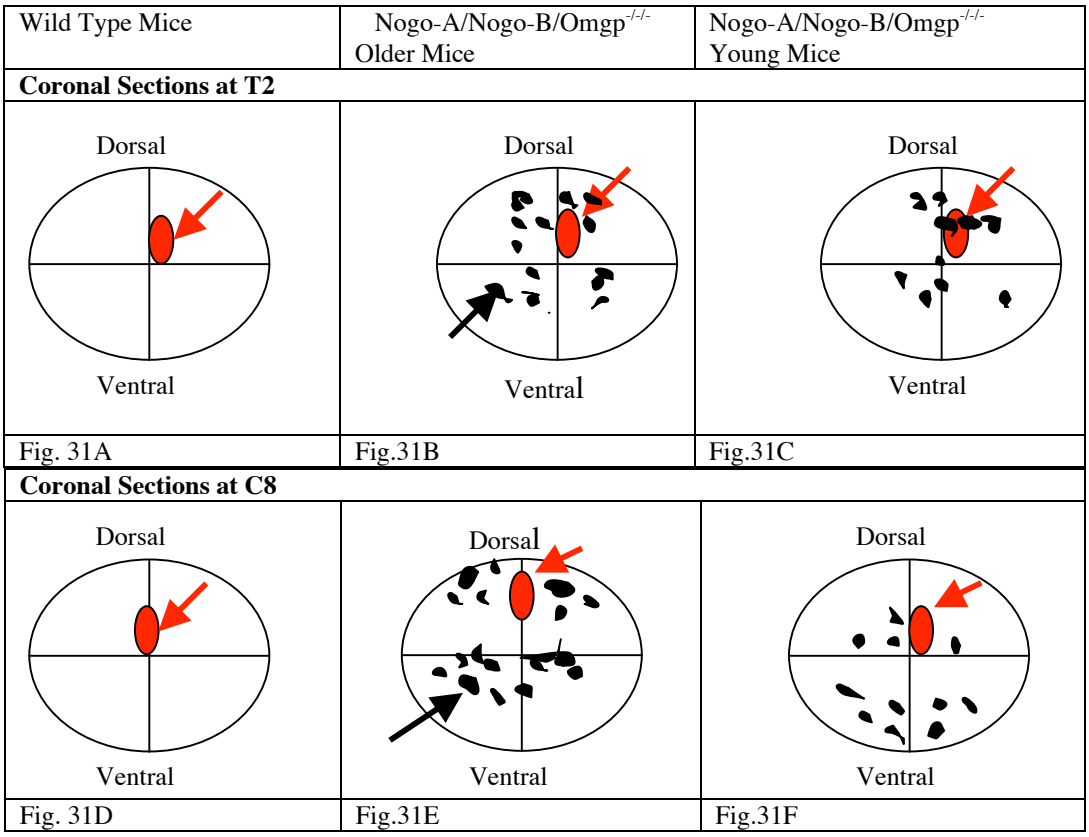


Figure 31. Coronal Sections of T2 & C6
Red arrows indicate corticospinal tract injection with BDA from the cerebrum at the contralateral position of the spinal cord. Extensive axonal sprouting (indicated by black arrows) should be observed in older and younger Nogo-A/Nogo-B/OMgp$^{-/-}$ mice (Fig.31B, 31C, 31E& 31F) rostral and caudal to SCI. The arrows also indicate that the axonal sprouting is taking place both ipsilateral and contralateral to the SCI site.

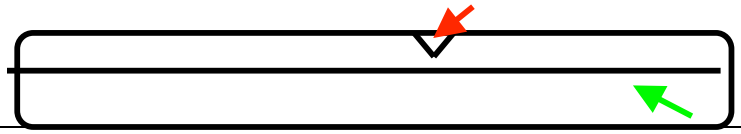
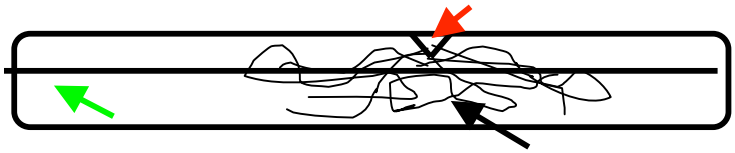
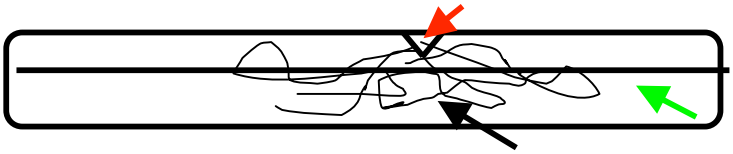
Sagittal Sections at SCI Site		
Fig. 32G	Wild Type	
Fig. 32H	Nogo-A/ Nogo-B/ OMgp$^{-/-/-}$ Older Mice	
Fig. 32I	Nogo-A/ Nogo-B/ OMgp$^{-/-/-}$ Young Mice	

Figure 32. Sagittal Sections of the injury site.
Red arrows and green arrows indicate the injury site at T6 and the corticospinal tract stained with BDA respectively. The sagittal sections of the older and younger Nogo-A/Nogo-B/Omgp$^{-/-}$ mice are expected to demonstrate extensive axonal sprouting (indicated by black arrow) rostral and caudal to SCI (H & I), while no sprouting should occur in wild type mice (G). Fig.32H should also indicate that axonal sprouting takes place at both and contralateral and ipsilateral levels

	O	o
O	OO	Oo
O	Oo	oo

Figure 33. Crossing of two mice heterozygous for the OMgp^{++} transgene
A capital O represents a WT allele, and a lower case o represents an allele containing the transgene. The 25% of the progeny represented in the lower right cell of the table are the homozygous mice to be studied.

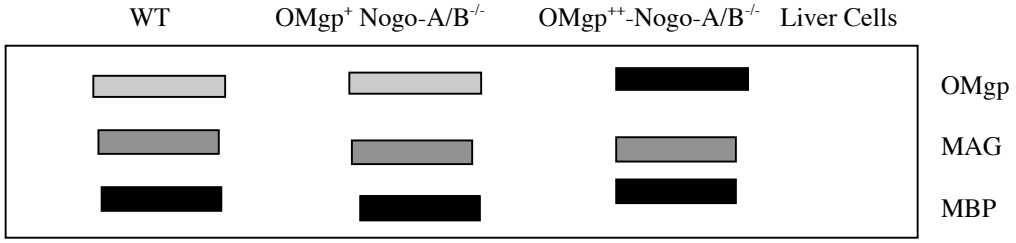


Figure 34. Western Blot analysis of OMgp levels in transgenic and control mice.
The wild type mouse should produce the same level of OMgp as the Nogo-A/B$^{-/-}$ mouse, but the level of OMgp in the OMgp$^{+/+}$Nogo-A/B$^{-/-}$ should be significantly higher. The level of MAG should remain constant in all three mice, as should the level of MBP.

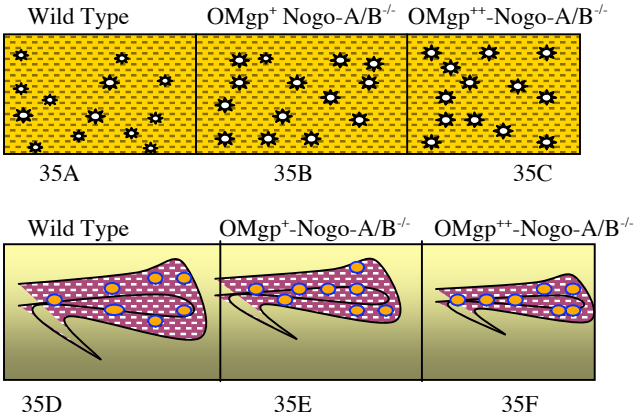


Figure 35. General Brain Histology
General Brain histology should demonstrate no defects in the structural integrity of the brain. A-C: Coronal sections through the cerebral cortex stained with hematoxylin and eosin. The neuronal makeup should be the same. D-F: Parasagittal sections through the cerebellum, stained with hematoxylin and eosin. Again, the similar cellular makeup will show that there is no difference in the neuronal composition of the brains despite the varying expression levels of OMgp.

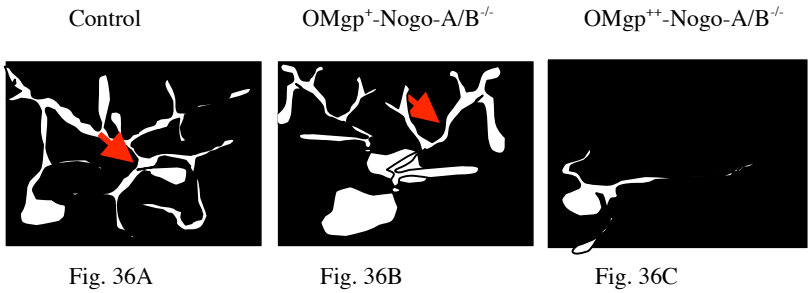


Figure 36 A-C. A: Cells on the control plate should show extensive axonal growth from the cell bodies, indicated by arrow. B: Cells on the OMgp^{+}-Nogo-A/B$^{-/-}$ myelin should show axonal growth due to the lack of inhibitory factors, indicated by arrow. C: Cells on OMgp^{++}-Nogo-A/B$^{-/-}$ myelin are expected to display axonal growth due to the overexpression of OMgp. The protocol for the assay will be adapted from Strittmatter et al (Kim et al., 2003).

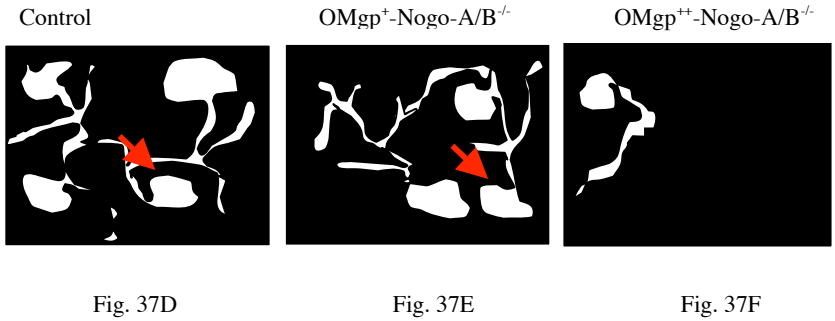


Figure 37 D-F. Growth Cone Collapse Assay D: The cells on the control plates should show the presence of growth cones, indicated by arrow. E: The cells on OMgp^{+}-Nogo-A/B$^{-/-}$ myelin should show a large number of visible growth cones similar to the control, indicated by arrow. F: The cells on OMgp^{++}-Nogo-A/B$^{-/-}$ are expected to show low numbers of growth cones compared to the control and the OMgp^{+}-Nogo-A/B$^{-/-}$.

Wild Type Mice	OMgp^{+}-Nogo-A/B$^{-/-}$	OMgp^{++}-Nogo-A/B$^{-/-}$
Coronal Sections at T4		

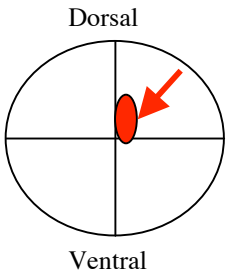
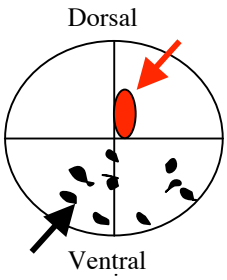
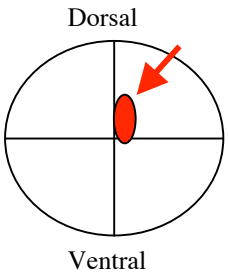

		

Fig. 38A	Fig.38B	Fig.38C
Coronal Sections at L2		

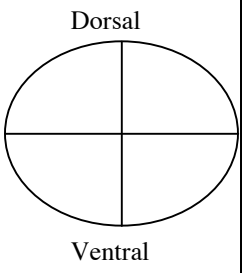
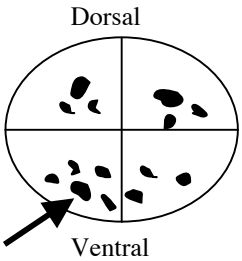
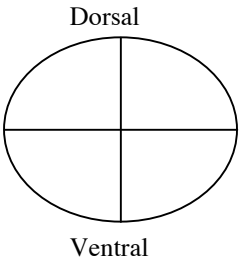

		

Fig. 38D	Fig.38E	Fig.38F

Figure 38. Coronal Sections at T4 and L2.
A: Section at T4 of WT mouse, no axonal sprouting should be seen. B: Section at T4 of OMgp^{+}-Nogo-A/B$^{-/-}$ mouse, axonal sprouting is expected to be seen both contralateral and ipsilateral to injury site. C: Section at T4 of OMgp^{++}-Nogo-A/B$^{-/-}$ mouse, no axonal sprouting ought to be seen, which is the same as the WT result. D: Section at L2 of WT mouse, no axonal is supposed to be seen. E: Section at L2 of OMgp^{+}-Nogo-A/B$^{-/-}$ mouse, where axonal sprouting is seen both contralateral and ipsilateral to the injection site. F: Section at L2 of OMgp^{++}-Nogo-A/B$^{-/-}$

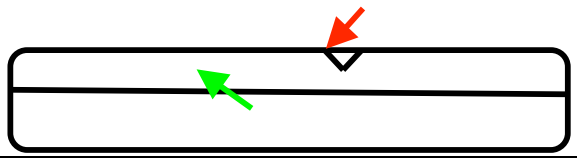
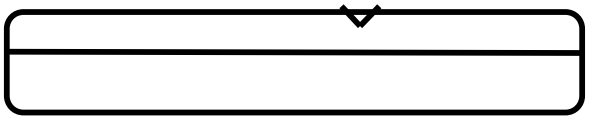
Sagittal Sections at SCI Site		
39G	Wild Type	
39H	OMgp^{+}-Nogo-A/B$^{-/-}$	
39I	OMgp^{++}-Nogo-A/B$^{-/-}$	

Figure 39: Sagittal Sections of the injury site.
The injury (red arrow) is marked by a small triangle on the dorsal side of the spinal cord. The axons of the CST (green arrow) are seen running along the dorsal half of the spinal cord and are marked by arrows. G: The WT mice are expected to show no axonal growth leading up to or past the injury site. H: The OMgp^{+}-Nogo-A/B$^{-/-}$ mice should show axonal sprouting (black arrow) rostral to the injury site and running more than 5mm caudal to it. Also, sprouting is seen both on the ipsilateral and contralateral side. I: The OMgp^{++}-Nogo-A/B$^{-/-}$ mice ought to show no axonal sprouting either rostral or caudal to the injury site, much like the WT mice.

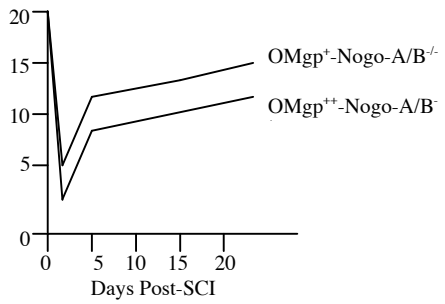


Figure 40. Quantification of BBB.
Score (x-axis) in relationship to days post injury. All mice were between 7-9 weeks of age at the time of SCI, and their gain of functionality was monitored daily. The $OMgp^{++}$ mice are supposed to regain function in a pattern similar to that of the $OMgp^{+}$ mice, but do not should not recover as fully.