ENHANCEMENT OF OLIGODENDROCYTE REMYELINATION THROUGH THE ABLATION OF NON-MUSCLE MYOSIN II B

By

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Abstract

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The myelinating cell of the central nervous system, the oligodendrocyte (OL), undergoes a dramatic change in the organization of its cytoskeleton as it differentiates from an oligodendrocyte precursor cell to a myelin forming OL. This change involves an increase in branching, which is required for the OLs ability to myelinate multiple axons.

Our laboratory has shown that levels of non-muscle myosin II (NMII), a regulator of cytoskeletal contractility, decrease as a function of differentiation and that inhibition of NMII activity increases branching and myelination of OL in coculture with neurons. It was also found that mixed glial cultures derived from NMIIB knockout mice display an increase in the number of mature myelin basic protein expressing OL compared to wild type cultures.

These studies have now been extended to investigate the role of NMIIB ablation in myelin repair following focal demyelination by lysolecithin. To this end, we employed an OL-specific inducible knockout model using a PLP driven promoter in combination with a temporally activated CRE-ER fusion protein. The data indicate that conditional ablation of NMII

in adult mouse brain, promotes faster lesion resolution and remyelination when compared to that observed in control brains.

Although several pathways have been implicated in oligodendrocyte morphogenesis, their specific contribution to the regulation of NMII activity has not been directly examined. We tested the hypothesis that the activity of NMII in OPC is controlled by Fyn kinase via downregulation of RhoA-ROCK-NMII phosphorylation. The resulting data confirm the function of NMII as a negative regulator of OL maturation and demonstrate that Fyn kinase downregulates NMII activity thus promoting oligodendrocyte morphological differentiation. Furthermore this study provides a novel target for promoting myelin formation and repair in the adult brain.

Acknowledgments

I owe so much for the support that my family has always provided me with, and one would wish that a dedication in a dissertation were all it took to square the deal. I am also extremely grateful for the support and guidance that my mentor Carmen Melendez-Vasquez has given me during my time in her lab. One of the most rewarding things in my life has been the experience of learning how to do science, and the people at the department of biology at Hunter College have made that experience all that it was and more. Special thanks go out to Ben Ortiz and Patricia Rockwell, who guided me since my days as an undergrad. My rotations in the labs of Mitch Goldfarb and Maria Figueiredo-Pereira were a pleasure and made my decision to work on myelin all the more difficult. I am thankful to Peter Canoll and Patrizia Casaccia for being on my committee. I would also like to thank Cedric Raine and Gareth John, for spending time with me on the microscope and showing me what the brain really looks like. Conversations with Mitch Goldfarb, Paul Feinstein and Diana Bratu I could have not done without. I had a lot of help from Jennifer Nam and Evangelos Damanakis, for which I am thankful. Finally, I want to thank Rivka Rudner and Mélissa Hilaire, for giving me peace during the writing of this dissertation.

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List of Abbreviations

CA constitutively active
cKO conditional knock-out
CNP 2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS central nervous system
DN dominant negative
DPI days post injection
DRG dorsal root ganglion
EAE experimental allergic encephalomyelitis
EGFP enhanced green fluorescent protein
GFAP glial fibrillary acidic protein
MBP myelin basic protein
MHC myosin heavy chain
MLC myosin light chain
MLCK myosin light chain kinase
MS multiple sclerosis
MYPT myosin phosphatase
NG2 neuron-glial antigen 2
NMHC non-muscle MHC
NMII non-muscle myosin II
OL oligodendrocyte
OPC oligodendrocyte progenitor cell
PDGF platelet derived growth factor
PLP proteo-lipid protein
PNS peripheral nervous system
ROCK rho kinase
SC schwann cell
SVZ subventricular zone
T3 thyroid hormone
WT wild type

Chapter 1: Introduction

Myelin

The vertebrate nervous system relies on a vast network of neuronal connections, which span distances many times greater than the length of the individual cell bodies. These connections are used to propagate the signals that are thought to be the basis of all cognition and behavior of an organism. The neuronal action potential, which travels down the axon is therefore a point of evolutionary pressure, as an organism that can send faster signals to its limbs for instance will have an advantage over its competition. Thus evolved the process of myelination in vertebrates, which envelops and insulates neuronal axons in a specialized multi-layered proteo-lipid rich membrane, permitting a much faster conduction of action potential down the myelinated axon along gaps in the myelin called nodes of Ranvier where sodium channels are concentrated (Hartline and Colman, 2007). While myelin is not necessary for neuronal communication at short distances, it does become vital when connecting processes of greater length, such as those between the two hemispheres of the brain or along the length of the spinal cord. Aside from its electrical insulator properties, myelin also plays a role in axonal maintenance [reviewed in (Nave, 2010)]. Loss of myelin in diseases such as multiple sclerosis (MS) results in conduction failure, and underlies many of the clinical deficits (weakness, paralysis and blindness) associated with this disorder. Myelination in the central nervous system (CNS) is a drawn out process, that in humans starts during the second half of fetal life in the spinal cord, peaks during the first year of life outside the womb and can continue right up to twenty years of age in some parts of the brain (Baumann, N. & Pham-Dinh, 2001).

Oligodendrocytes

Myelin in the CNS is produced by the oligodendrocyte (OL), a cell arising from ventral regions of the neural tube, early in embryogenesis and later in the gestational and early postnatal mammalian brain, from the subventricular zone (SVZ). Maturation of oligodendrocytes in the mammalian CNS is an event of early postnatal life. Oligodendrocyte precursors arise from multiple foci distributed along the neural tube from where they migrate as oligodendrocyte progenitor cells (OPCs) into the future white matter of the brain (reviewed in Colognato *et al*., 2004). OPCs are bipolar cells, which can be characterized by the surface antigens A2B5 as well as the chondroitin sulfate proteoglycan NG2 (Baumann and Pham-Dinh 2001). These cells actively proliferate and respond to the platelet-derived growth factor (PDGF) through the PDGFα receptor (PDGFαR), which is another characteristic surface protein (Richardson *et al.* 1988). Following migration to and along neuronal fiber tracts of the CNS, OPCs differentiate into pre-oligodendrocytes, which accompanies a dramatic change in their morphology. Pre-oligodendrocytes then lose expression of PDGFαR and begin expressing 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Reynolds *et al.* 1988). They also form a large network of branched processes, which make contact with and myelinate as many as 40 different axons (Simons and Trotter, 2007). This final step of OL differentiation is characterized by the expression of myelin membrane proteins such as proteolipid protein (PLP) and myelin basic protein (MBP) (Monge *et al.* 1986). Oligodendrocytes in culture differentiate progressively through a sequence of stages characterized by distinct morphologies and expression of specific markers much the same as they do *in vivo* (Pfeiffer *et al*., 1993; also visualized in Figure I 1). As OPCs mature and form lamella, the complexity and branching of their cytoskeleton increases (Behar 2001).

The Cytoskeleton

The defining characteristic of an oligodendrocyte is its multitude of elaborate cytoskeletal processes, which terminate in an even more complicated structure, the myelin segment. Early studies on the nature of the cytoskeleton in OL of varying maturity revealed that immature OL processes are rich in filamentous actin as well as microtubules (Kachar *et al.* 1986). The mature OL studied *in vitro* extends MBP positive sheets, which contain microtubule veins and much fewer microfilaments (Wilson and Brophy, 1989). The primary processes of OL are at the base composed of microtubules, formed by α- and β-tubulin (Richter-Landsberg, 2001). Secondary and tertiary branches however, which make up the bulk of a developing OL, are primarily composed of F-actin which is distributed ahead of microtubules in growing processes and branches (Song *et al*. 2001). Thus, a lot of studies have focused on the various actin interacting proteins, which have an effect on OL branching and morphological differentiation. Juxtanodin for example which contains a well characterized actin-binding domain promotes arborization of cultured OL. Juxtanodin with a shortened actin-binding domain did not have this effect (Zhang *et al.* 2005). Another actin binding protein Ermin, has been found to be specific to OL. Expression of Ermin, but not a mutant lacking its actin binding properties, can induce branching in Cos-7 cells, and has been found localized along branch points in OL (Brockschnieder *et al*. 2006). Actin polymerization has also been found to be important, as WAVE1 knockout mice, lacking the ability to scaffold monomeric actin, show hypomyelination in the corpus callosum (Kim *et al*. 2006). It has also been found that mutations in myosin Va result in abnormal myelination. Loss of the myosin Va cytoskeletal motor results in OL with decreased process number, length and branching (Sloane and Vartanian 2006). The cellular mechanism whereby OL, extend

multiple cell processes that wrap around CNS axons is still poorly defined but it clearly involves active remodeling of the cytoskeleton [reviewed in (Bauer *et al.* 2009)]. It is also of note that most of the morphological changes associated with cytoskeletal perturbations have been linked to differentiation and myelin formation both *in vivo* and *in vitro*.

Non-Muscle Myosin II

When referring to actin, it is usual to also speak about myosin, and in the case of the OL it is no different. The myosin super family of actin-based molecular motors consists of at least 25 different classes. The myosin II subfamily, which includes skeletal, cardiac and smooth muscle myosins, as well as non-muscle myosin II (NMII), has the most members. In vertebrates, there are over 15 different myosin II isoforms, each of which contains a different myosin II heavy chain (MHC). This MHC diversity is generated by multiple genes as well as by alternative splicing of the pre-mRNA that encodes them (Eddinger 2007).

In mammals, three different genes (Myh9, Myh10 and Myh14) encode three different non-muscle MHCs (NMHCs), commonly referred to as NMHC IIA, NMHC IIB and NMHC IIC (Conti *et al*., 2008). The complete myosin molecule is a hexamer, consisting of two heavy chains and four myosin light chains (MLC). The MLCs, which are very tightly but noncovalently bound to the MHCs (230 kDa), play a role in stabilizing (MLC17) and regulating (MLC20) myosin structure and activity. MLC20 is a substrate for a number of kinases, including Ca2+-calmodulin-dependent MLC kinase (MLCK) and Rho kinase (ROCK). These kinases phosphorylate MLC20 primarily on Ser19 (and to a lesser extent on Thr18), which increases actin-activated MgATPase activity, filament formation and contractile activity *in vitro* and *in vivo*. Dephosphorylation leads to decreased contractile activity and is catalyzed by a single class

of myosin phosphatase (MYPT) that can also be regulated by a number of kinases, including Rho kinase (Ito *et al*., 2004). Phosphorylation of MYPT by Rho kinase is inhibitory and thereby enhances NMII activity by increasing MLC20 phosphorylation (Zhao and Manser, 2005; Matsumura, 2005).

The role of Non-muscle myosin II in the cell

The contractile properties of NMII have been shown to be important in establishing epithelial cell polarity, cell-cell adhesion and gastrulation in *Drosophila* (Conti and Adelstein, 2008). Mammalian cells also employ NMII for cell polarity and cell-cell adhesion, additionally NMII was identified to be important for cell migration, as depletion of NMII in a breast tumor cell line impaired the process (Betapudi *et al.* 2006). More recently, and with the use of better *in vitro* measurement and imaging techniques, investigators have been asking how NMII interacts with the cytoskeleton to carry out its functions. Using TIRF microscopy and optical tweezers on purified NMII proteins, James R. Sellers and his team have worked out the kinetics in single molecule motility assays. This work defined NMII as a molecular motor with an intermediate duty ratio compared to skeletal muscle myosin or myosin V, which are low and high duty respectively. Skeletal muscle myosin spends only a small fraction of its time bound to actin but works in concert with hundreds of other myosins in thick filaments. Myosin V has a high duty ratio and has the ability to move cargo along actin as a single molecule. Meaning NMII would only be capable of moving along actin in a bundle of no less than 50 molecules, thus excluding its function in vesicular trafficking (Nagy *et al.* 2013). Cortical tension has emerged as the strongest mechanistic mode of action for NMII and several studies have shown this to be the case. Cortical tension stabilizes the plasma membrane and the inhibition of NMII causes some

cell types to generate filipodial extensions (Straight *et al.* 2003, Shin *et al.* 2011). Another important study has shown that NMII localized at the cell cortex is partially released under conditions promoting branching. Moreover, branch initiation follows depletion of cortical NMII and this can be demonstrated by local inhibition of NMII by using micropipette injection of NMII inhibitor blebbistatin, which leads to new branch initiation (Fischer *et al.* 2009).

Non-muscle myosin II in oligodendrocytes

Our laboratory has demonstrated that NMII is a negative regulator of OL morphogenesis and myelination (see model in Figure I 2). We have found that differentiation of OLs is promoted in the absence of NMII activity. So that when NMII is inhibited or downregulated, the percentage of OL expressing MBP, the complexity of OL branching, and the amount of myelin segments formed in culture by individual cells are all increased (Wang *et al*., 2008). Our group has also demonstrated accelerated maturation of OL purified from NMIIB null mice (Wang *et al.* 2012). This indicates that NMII activity modulates the morphological status of OL, which in turn is vital for its role as a CNS myelinating cell. Moreover, others and we have found that there is an inverse correlation between the levels of MBP, a major protein component of myelin, and NMII expression and activity during OL development (Cahoy *et al*., 2008). This indicates that downregulation of NMII may be an intrinsic part of the differentiation program of OL. The three NMII isoforms, are differentially localized and regulated in many cell types, and appear to have different functions during cell migration and spreading (Conti *et al*., 2008). We have found that OL express all three isoforms, although in cultures NMIIB predominates over IIA and IIC. NMIIB is detected with actin at the leading edge of growing OL processes (Song *et al*., 2001). This pattern of expression makes NMIIB a good candidate to mediate the effects of total NMII

inhibition on glial cell branching and differentiation. In agreement with this idea, we have found that siRNA knockdown of either NMIIB or IIA, but not IIC, mimics the effects of total NMII inhibition on OL branching (Wang *et al.* 2012), presumably allowing increased actin protrusion at the leading edge of OL processes and/or at new branch points along processes.

Although the direct role of NMII in actin cytoskeleton dynamics is still not well understood; phosphorylation of NMII by ROCK has been identified as a negative regulator of F-actin polymerization in developing growth cones (Medeiros *et al*., 2006) providing a mechanism for the regulation of OL cell branching via the RhoA-ROCK-NMII pathway. Although several pathways have been implicated in OL morphogenesis, their specific contribution to regulation of NMII activity and expression in OL has not being directly examined.

The tyrosine-protein kinase Fyn

The src family tyrosine kinase Fyn has been shown to be an important regulator of OL development, as mice lacking it have a fifty percent decrease in myelination (Umemori *et al*., 1994). This reduction is due to a decrease in both the total number of mature OL as well as their morphological complexity (Sperber and McMorris, 2001). It was then demonstrated that p190 RhoGAP, a GTPase activating factor, is a substrate of Fyn and that its activity increases during the differentiation of OL (Wolf *et al*., 2001). In agreement with this, it has been shown that Fyn signaling decreases active RhoA levels and that this can cause changes in the morphological differentiation of OL in culture (Liang *et al*., 2004). We have shown that levels of NMII decrease during OL development and that inhibition of NMII activity increases branching and myelination of OL (Wang *et al*., 2008). NMII is a downstream target of RhoA through Rho-kinase (ROCK),

which phosphorylates the regulatory myosin light chain (MLC) (Somlyo *et al*., 2000). This leads to the hypothesis that the activity of NMII in OL is negatively regulated by Fyn (Figure I 3).

The Cyclin-dependent kinase 5

Another possible upstream regulator of NMII is Cyclin-dependent kinase 5 (Cdk5), a unique member of the Cdk family (Lew *et al*., 1992), which plays no apparent role in cell cycle regulation and requires an activating protein (either p35 or p39) that is not a member of the cyclin family (Smith *et al*., 2001). Cdk5 seems to be an important regulator of cytoskeletal dynamics, cell adhesion, transport, and membrane trafficking in both neuronal and non-neuronal cell types, and more importantly it has been linked to NMII through its activator p39 binding to myosin light chain (Ledee *et al*., 2007), the regulatory subunit of myosin which activates it when phosphorylated at serine 19 (Adelstein and Conti, 1975). Cdk5 is a proline directed serine/threonine kinase enriched in neuronal tissues (Dhariwala *et al*., 2008). Cdk5 is important for cortical neuron migration (Ohshima *et al*., 2007) and proper axon guidance (Gilmore *et al*., 1998). More recently its activity has been shown to be important for OL differentiation and branching (Miyamoto *et al*., 2007). The involvement of Cdk5 in cytoskeletal dynamics, is supported by the finding that the Cdk5 regulator p39 binds MLC (Ledee *et al*., 2007). Moreover, evidence of NMIIB splice variants containing an extra 10 amino acids with the Cdk5 consensus phosphorylation sequence motif (Ma *et al*., 2006) suggests that NMIIB1 could be a target of Cdk5, which has been shown by *in vitro* phosphorylation (Pato *et al.* 1996). MLCK, a Ca2+/ calmodulin dependent kinase that can activate MLC by phosphorylation of ser19 (Matsumura *et al.* 1998), contains a consensus motif for Cdk5 phosphorylation. Phosphorylation of MLCK by Cdk5 has not been demonstrated in any studies, however MLCK is negatively regulated through

phosphorylation by PAK (Goeckeler *et al.,* 2000). Taken together, these observations lead us to hypothesize that Cdk5 is a potential negative regulator of NMII activity in OL (Figure I 4).

Disease of the central nervous system

Multiple sclerosis (MS) is the most common form of demyelinating disease in the CNS. MS is believed to be an autoimmune disorder of the human CNS, with inflammatory cells invariably present in demyelinating lesions, but the primary cause of lesion formation and whether it originates from a problem with myelin itself or some environmental insult, is not well understood. Recovery of neural function accompanies remyelination, which restores nerve conduction and re-establishes the normal molecular organization of the myelinated axon (Salzer, 2003). Remyelination is mediated by several sources of oligodendrocyte precursor cells (OPC), some of which migrate from the sub-ventricular zone, while others such as NG2+ multipotential progenitors are found throughout the CNS [reviewed in (Levine *et al.* 2001)]. Although remyelination in MS is initially associated with OL proliferation, over time however, as lesions expand and become chronic, this repair process becomes less efficient, as there is little that can be done to limit the progressive burden on the reservoir of OPC resulting from recurrent demyelination.

Understanding why oligodendrocytes must "loosen up"

The ultimate goal of our research is to understand the intrinsic mechanisms that regulate OL cytoskeleton remodeling and its impact on terminal differentiation. Since the process of remyelination essentially recapitulates events taking place during normal OL development (Fancy *et al.* 2011), we have tested the hypothesis that conditional ablation of NMIIB in adult

brain may promote myelin repair via acceleration of OL differentiation. Using the lysolecithin model of demyelination, we show in chapter 2 that targeted deletion of NMIIB from OL expressing proteolipid protein (Plp) accelerated lesion resolution and increased the number of mature OL found inside the remyelinating lesion. Collectively, our results provide a novel strategy to enhanced myelin repair by promoting OL maturation, and identify NMII as a promising therapeutic target for diseases such as MS, a conclusion that deserves follow-up and confirmation in other MS disease models such as EAE.

While exploiting our findings for possible therapeutic uses is exciting, it is also important to remember that understanding the mechanisms behind our observations is something equally important. Chapter 3 deals with bridging the gap in our understanding of how OLs differentiate and how signaling to down-regulate NMII activity plays an important role in their morphological development. Our work bridges all the results generated by Umemori et al. 1994 and Liang et al. 2004 to implicate Integrin/Fyn kinase signaling in OL differentiation with NMII and the cytoskeleton. As we show that Fyn effected down regulation of active RhoGTP affects downstream phosphorylation status of a key cytoskeletal regulator of NMII, MLC.

Figure I 1. Morphogenetic changes during oligodendrocyte differentiation.

Primary Cultures of rat OPC immunostained for actin, tubulin, MBP and nuclear stain DAPI, which display striking changes in cytoskeletal organization as the cells differentiate *in vitro.*

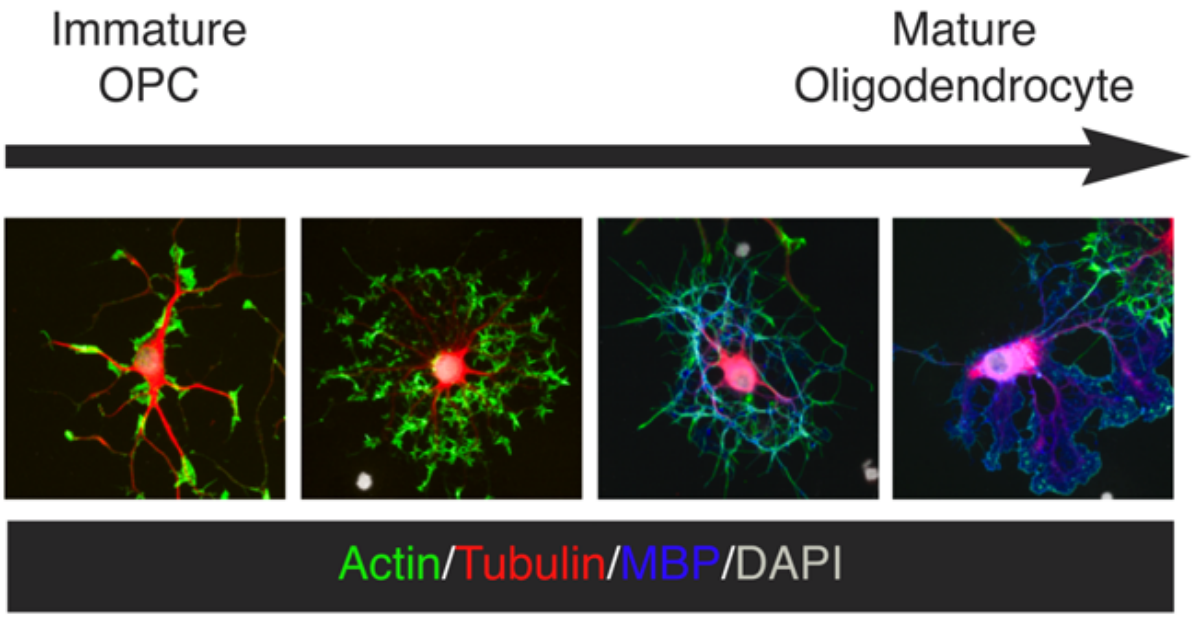


Figure I 2. Model of NMII control of OL differentiation.

Downregulation of NMII activity acts to promote OL morphological differentiation and myelination. (Modified from Wang *et al.* 2008)

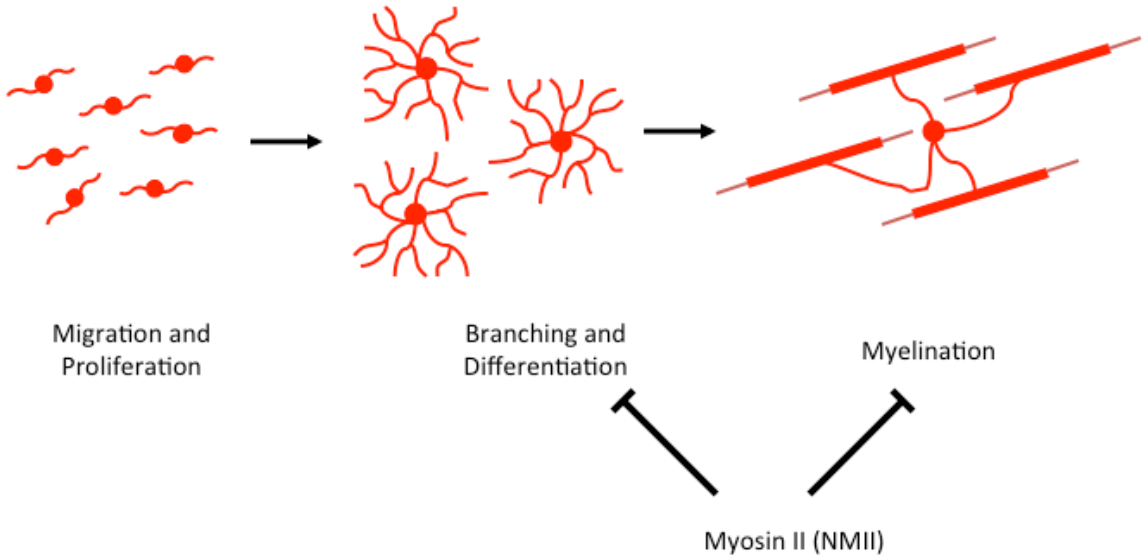


Figure I 3. Model of Fyn-RhoA signaling and myosin II activation.

Engagement of integrins by their extracellular matrix ligands activates Fyn, a member of the Src family of tyrosine kinases. Fyn phosphorylates p190RhoGAP, a GTPase activating factor thus reducing the levels of active RhoA (RhoGTP). Activation of ROCK downstream of RhoGTP promotes phosphorylation of MLC and activation of myosin II mediated contraction. Activation of RhoA also inhibits MLC phosphatase (MLCP). Thus, inhibition of RhoA activity and myosin II contraction downstream of Fyn signaling might promote process extension.

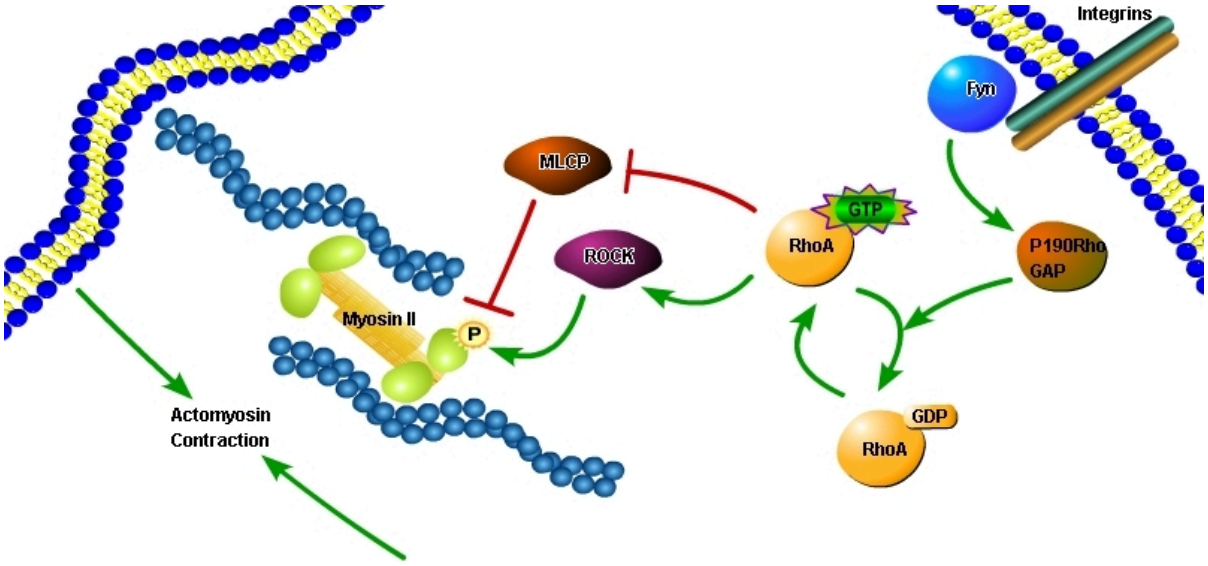
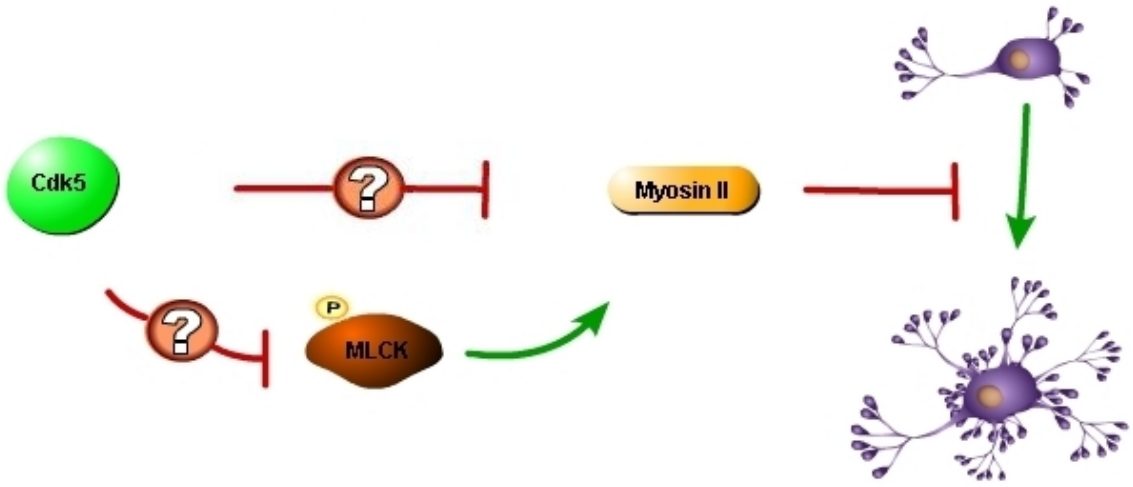


Figure I 4. Model of NMII regulation by Cdk5.

The heavy chain of NMII isoform NMIIB1 contains a consensus motif for Cdk5 (SPK) at ser214 near the ATP binding region of its head domain. The long isoform (220kD) of MLCK contains the same motif at ser758 in an immunoglogulin domain at the N-term. We propose that Cdk5 inhibits myosin II either directly or through inhibition of MLCK, which is required for myosin II activation.



Chapter 2: Accelerated repair of demyelinated CNS lesions in the absence of non-muscle myosin IIB

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Summary

The experiments described in this chapter demonstrated that ablation of NMIIB in OL (cKO) before the induction of a demyelinating lesion results in accelerated repair. We found that the volume of the remyelinating lesions in cKO mice is almost half that of control animals at the intermediate time point during the remyelination paradigm, and that this difference is due to an increase in total mature OL present at the sites of remyelination. Furthermore, using fluorescent reporter tagging of the PLP+ OL lineage we were able to demonstrate the presence of NMIIB-ablated PLP+ OL in the remyelinated area. These data suggest that the myelinogenic potential of a small pool of local PLP+ adult progenitors is potentiated to accelerate repair in the absence of NMIIB. Our results excluded any difference in the immune response, reactive astrocytosis and recruitment of NG2+ progenitors as the cause for the observed differences in lesion resolution

Materials and Methods

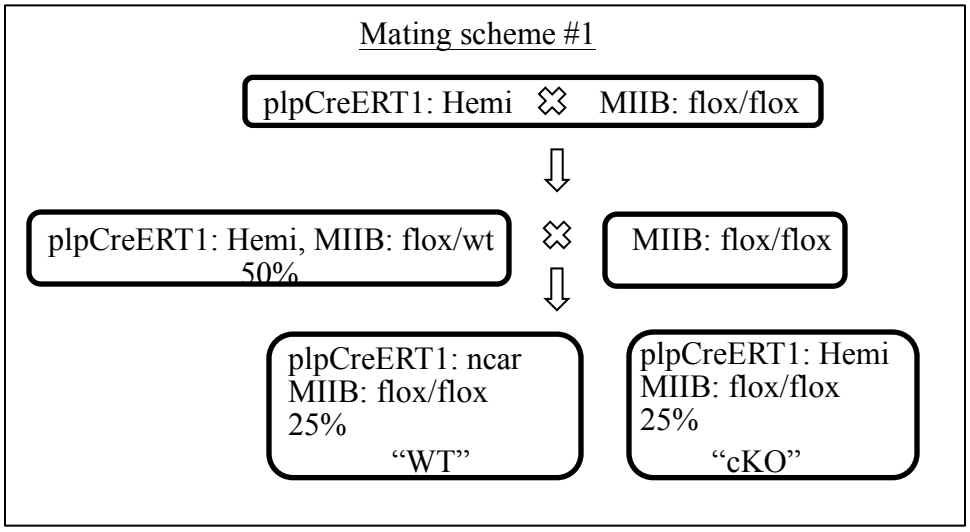
Mice

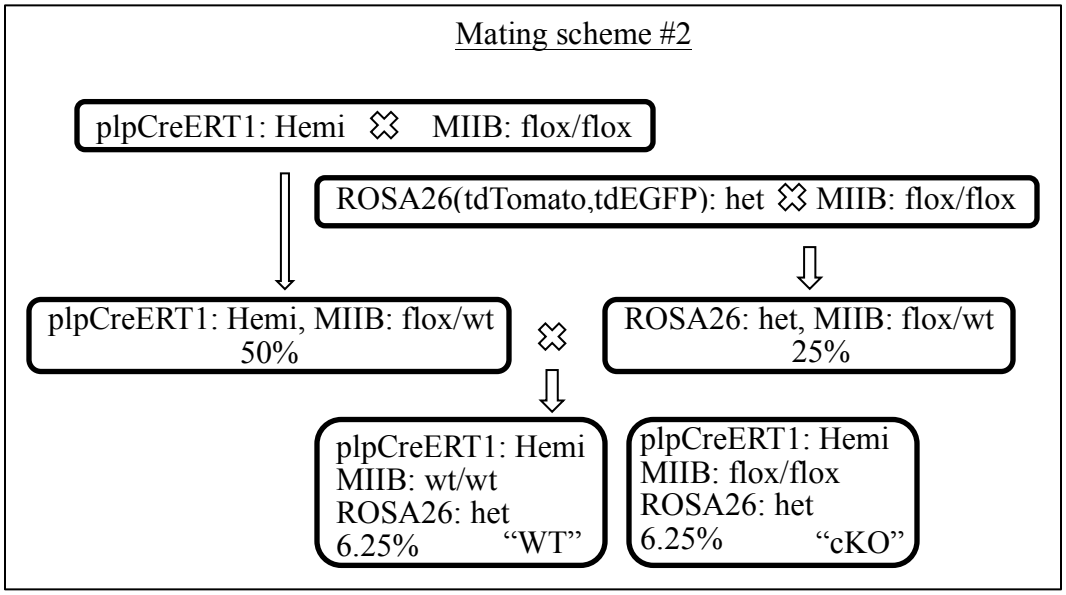
NMIIB $^{fl/fl}$ mice (Ma *et al*, 2009) were kindly provided by Mary Anne Conti and Robert Adelstein (Laboratory of Molecular Cardiology, NHLBI). *PlpCre/ESR1* mice (Doerflinger *et al*, 2003) were purchased from the Jackson Laboratory (Stock # 005975). Gt(ROSA)26Sortm4*(ACTB-tdTomato,-EGFP)Luo*/J (Rosa26-mT/mG) mice (Jackson Laboratory stock # 007576) were kindly provided by Paul Feinstein (Hunter College CUNY). *PlpCre/ESR1* hemizygous mice were crossed with Rosa26-mT/mG heterozygotes to check for recombination efficiency. *PlpCre/ESR1* males were crossed with *NMIIB* $^{fl/fl}$ females and the F1 *PlpCre/ESR1*:*NMIIB* $^{fl/+}$ males were then backcrossed to *NMIIB* $^{fl/fl}$ females to generate *PlpCre/ESR1*:*NMIIB* $^{fl/fl}$ (cKO) and NonCre:*NMIIB* $^{fl/fl}$ (Control) mice for remyelination analysis. *PlpCre/ESR1*:*NMIIB* $^{fl/+}$ animals were also crossed to the offspring (F1) from a Rosa26-mT/mG x *NMIIB* $^{fl/fl}$ cross to generate *PlpCre/ESR1*:Rosa26-mT/mG:*NMIIB* $^{fl/fl}$ (mT/mG; cKO) and *PlpCre/ESR1*:Rosa26-mT/mG:*NMIIB* $^{+/+}$ (mT/mG; Control). All procedures were carried out in accordance with the National Institutes of Health guidelines and were approved by Hunter College Institutional Animal Care and Use Committee.

Genotyping and PCR

Genotyping for *PlpCre/ESR1* was performed with the following PCR (Transgene allele: 100bp, internal positive control: 324bp; primers transgene fwd GCG GTC TGG CAG TAA AAA CTA TC, transgene rws GTG AAA CAG CAT TGC TGT CAC TT, internal positive control fwd CTA GGC CAC AGA ATT GAA AGA TCT, internal positive control rws GTA

GGT GGA AAT TCT AGC ATC ATC C). *NMIIB* $^{fl/fl}$ using (wildtype allele: 250bp, loxP allele: 350bp; primers IIB fwd GAC CGC TAC TAT TCA GGA CTT ATC, and IIB rws CAG AGA AAC GAT GGG AAA GAA AGC) Gt(ROSA)26Sortm4*(ACTB-tdTomato,-EGFP)Luo*/J with (mutant allele: 250bp, wildtype allele: 330bp; primer wildtype fwd CTC TGC TGC CTC CTG GCT TCT, wildtype rws CGA GGC GGA TCA CAA GCA ATA, mutant rws TCA ATG GGC GGG GGT CGT T)





Reagents and antibodies

Tamoxifen citrate (T9262**)** and L-α-lysolecithin (L4129) were obtained from Sigma-Aldrich (St.Louis, MO). The following primary antibodies were used: rbαNG2 (AB5320), moαCC1 (OP80) and rbαOlig2 (AB9610) (EMD Millipore Corporation, Billerica, MA); moαGFAP (#3670) (Cell Signaling Technology Inc, Danvers, MA); chαNF (PCK-593P) and moαMBP (SMI-94R) (Covance Inc, Princeton, NJ); rbαIba1 (#019-19741) (Wako, Osaka, Japan). FITC, Rho, or Cy5- conjugated donkey secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). FlouroMyelin Green (F34651), Topro3 iodide (T3604) and Prolong Gold (P36930) were purchased from Invitrogen (Life Technologies Corporation, Grand Island, NY). Paraformaldehyde, gluteraldehyde, osmium tetroxide and Epoxy resin components were all obtained from Electron Microscopy Sciences (Hatfield, PA).

Tamoxifen injections

Eight-week-old male and female mice were injected intraperitoneally (i.p) with 49mg/kg tamoxifen for 5 consecutive days. Tamoxifen was dissolved at 45°C in ethanol and then evaporated into corn oil for injection.

Lysolecithin injections

Twelve-week-old mice were anesthetized by inhalation of isoflourane 2.5%, positioned in a stereotactic frame (Kopf, Tujunga, CA) and injected unilaterally using a Hamilton syringe into the corpus callosum (5.5 mm anterior to lambda, 1mm lateral to bregma, 2mm deep) with 1.5 μL

of a solution of 1% lysolecithin in PBS (Nait-Oumesmar *et al.* 1999). Animals were sacrificed 7, 14 or 28 days later (Figure II S1). These time points correspond to well-characterized phases of active demyelination, proliferation and remyelination of lysolecythin-induced lesions (Zhang *et al.* 2009).

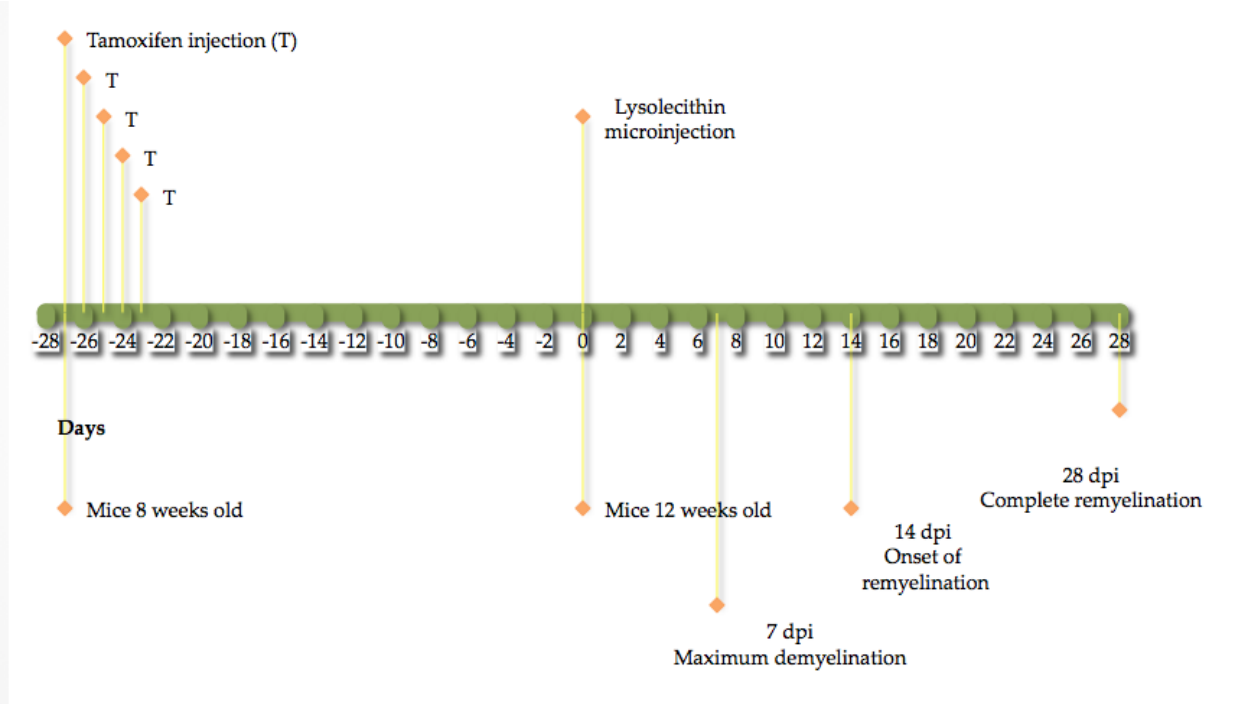


Figure II S1. Timeline of induction and lysolecithin injections.

Immunocytochemistry

Mice were given a lethal dose of ketamine/acepromazine and perfused transcardially with 4% paraformaldehyde. Brain tissue was extracted and 30µm cryostat sections in OCT were collected over a 1mm distance centered on the needle track. Frozen sections were permeabilized with 0.5% Triton X-100 and blocked with PBS containing 5% bovine serum albumin, 2%

donkey serum and 0.2% Triton X-100 for 1 h followed by primary antibody overnight in blocking solution, washed with PBS 0.2% Triton X-100 and incubated with secondary antibodies for 1 hr at RT. When used, FlouroMyelin or Topro3 were incubated in PBS for 1 hour. Slides were sealed with ProLong Gold anti-fade reagent and imaged using a Zeiss LSM 510 confocal microscope.

Image Analysis

Image analysis was performed using ImageJ 1.46m and Adobe Photoshop CS5. Adjustment of image brightness or contrast was performed in some cases but without misrepresenting data. Lesion area was measured using the ImageJ freehand selection tool inside FlouroMyelin negative areas of the corpus callosum. Subsequent size and volume calculations were based on the number of sections collected for each lesion multiplied by their thickness and their average lesion area. Mean gray values of fluorescent intensity of antibody staining were performed using the ImageJ measure tool restricted to lesion areas selected with the freehand tool. All measurements were normalized by the background mean gray value obtained from a section of normal appearing white matter adjacent to the lesion. When calculating remyelination by $EGFP^+$ oligodendrocytes at 28 dpi, the ImageJ particle analysis tool was used to measure the total cell area (including cell body and processes) within the lesion shadow ($EGFP^-$, MBP^+) in the corpus callosum. This value was then divided by the total shadow area to obtain the percentage covered by $EGFP^+$ oligodendrocytes. Statistical tests were performed using Graph Pad Prism software.

Histopathology

Mice were perfused with 2.5% gluteraldehyde and brain tissue was trimmed and postfixed in 1.5% osmium tetroxide followed by dehydration in 30-100% ethanol and embedding in Epon resin. Semithin sections (1μm) were cut and stained with toluidine blue and imaged using a Zeiss Axioplan microscope. Semithin sections were cut and stained with uranyl acetate and lead citrate and were imaged using TEM (JEOL JEM-100C/CX). G-ratios from electron micrographs taken at 13000x magnification of corpus callosum of 16 week-old mice were obtained using ImageJ measurements of axon diameter divided by whole fiber diameter.

Cell Extracts and Western Blotting

Brain tissue was homogenized with a tissue grinder in lysis buffer [150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO)], subjected to SDS-PAGE, and blotted onto nitrocellulose. Appropriate regions of the blots were cut and incubated with specific antibodies and developed using chemiluminescent substrate (Pierce, Rockford, IL). For estimation of changes in protein levels, films from three independent experiments were scanned, and the relative intensity of each protein band was calculated in Image J by dividing the absolute intensity of each protein band (the area of the band by the number of pixels contained in that area) by the absolute intensity of the corresponding actin band.

Results

Ablation of NMII in adult oligodendrocytes does not affect myelin morphology

Previous data from our laboratory has showed that inhibition of NMII enhances OL differentiation and CNS myelination *in vitro* (Wang *et al.* 2008) and that OPC isolated from embryonic brains of NMIIB KO mice exhibit accelerated maturation (Wang *et al.* 2012). To validate the functional relevance of our *in vitro* findings to myelin formation and repair *in vivo* we generated tamoxifen-inducible; glial cell-specific NMIIB knockouts (cKO), by mating *PlpCre/ESR1* mice (Doerflinger *et al.* 2003) to floxed NMIIB animals (*NMIIB* $^{fl/fl}$) (Ma *et al.* 2009). *PlpCre/ESR1:NMIIB* $^{fl/fl}$ (cKO) mice are viable, phenotypically normal and born at the expected mendelian ratios.

PlpCre/ESR1 system has been extensively used to generate tamoxifen-induced, Cre-mediated recombination under the control of the myelin preoteolipid protein (*Plp1*) promoter (Doerflinger *et al.* 2003, Leone *et al.* 2003, Murtie *et al.* 2007, Forrest *et al.* 2009, Pillai *et al.* 2009) and previous studies have shown that upon treatment with tamoxifen efficient recombination occurs in mature myelinating OL and in undifferentiated NG2+ OPC throughout the adult CNS as well as developing and mature SC in the peripheral nerve (Doerflinger *et al.* 2003, Leone *et al.* 2003).

We carried out our own assessment of *PlpCre/ESR1* mediated-recombination using the reporter line Rosa26-mT/mG (Muzumdar *et al.* 2007) and confirmed extensive and efficient recombination in OL throughout the corpus callosum 4 weeks after tamoxifen treatment (Fig. II 1). Approximately 87% of the MBP^+ white matter area in the corpus callosum contains $EGFP^+$ cells indicating extensive recombination.

To evaluate whether conditional ablation of NMIIB in adult mice *per se* has an effect on myelin maintenance and/or organization, we examined uninjured brain from cKO (*PlpCre/ESR1:NMIIB* $^{fl/fl}$) and control (NonCre*:NMIIB* $^{fl/fl}$) mice by light and electron microscopy and confirmed the presence of normal appearing white matter in cKO brain (Fig. II 2). Detailed morphometric analysis carried out in 16 week-old mice (the end-point of our remyelination studies) showed no differences in g-ratio, myelin thickness and/or periodicity between uninjured control and cKO brains. These results are consistent with the observation that during CNS development the levels of NMIIB protein are reduced (Wang *et al.* 2008, Cahoy *et al.* 2008, Dugas *et al.* 2007) and it is therefore unlikely that NMIIB has a role in myelin maintenance.

Remyelination is accelerated in the absence of NMII

To test if ablation of NMII in adult brain potentiates CNS remyelination, we induced focal demyelination in 12 week-old cKO (*PlpCre/ESR1:NMIIB* $^{fl/fl}$) and control (NonCre*:NMIIB* $^{fl/fl}$) mice, via stereotactic injection of lysolecithin into the corpus callosum. Analysis of lesions 14 days after lysolecithin injection (14 dpi), showed the presence of significantly smaller lesions (fluoromyelin negative area) in the corpus callosum of cKO mice compared to controls (Figure II 3A,B). Quantitation of total lesion volume at 14 dpi (Figure II 3D) showed that lesions in the cKO were on average 50% smaller than those found in control animals (47.4±11.2 vs. 88±40.6 $x10^6$ μm^3; mean±SD; p=0.007, t-test). This reduction in lesion size in cKO mice was not due to differences in the initial extent of demyelination, as the average lesion volume measured at 7 dpi was comparable to that of controls (338.8±227.1 vs. 461±181.9 $x10^6$ μm^3). Similarly, analysis of lesion volume at 28 dpi, the end point of the experiment, indicated that there were no differences

in the extent of repair between cKO and control mice (33.3±12.6 vs. 19.9±11.7 x10^6 μm^3). We also observed a significant increase in the number of mature $CC1^+$ oligodendrocytes (Figure II 3B,C) present at the lesion at 14 dpi in cKO mice compared to controls (10.1±3 vs. 6.8±2.2 x10^4 cells/μm^2; mean±SD; p=0.002, t-test). These data suggest that myelin repair was accelerated in cKO mice. Morphological analysis by light and electron microscopy (Figure II 4) further confirmed our immunofluorescence results, showing that at 14 dpi, lesions in the cKO brain were smaller (Figure II 4A) and contained more cells with the morphology of oligodendrocytes as well as many thinly myelinated axons (Figure II 4B), a landmark of remyelination (Figure II 4C). Of note, remyelinated axons were observed as early as 7dpi in cKO (Figure II 4E), a very rare event in control animals (Figure II 4D). Additionally, no differences in cell proliferation (Ki67 staining), and/or apoptosis (Caspase 3 staining), were observed between control and cKO lesions (Table 1). Similarly, no differences in the total number of CC1+ cells in normal appearing white matter were found between the cKO and controls mice. These results are consistent with the hypothesis that similar to its effects during myelin formation *in vitro*, ablation NMIIB potentiates the maturation of oligodendrocytes and promotes myelin repair *in vivo*.

Axon preservation and recruitment of NG2+ progenitors is not altered by the absence of NMIIB.

Although primary demyelination is the main effect following focal injection of lysolecithin in the white matter, minimal axonal damage can be detected within the lesion (Woodruff *et al.* 1999). Since remyelination has been associated with axonal preservation and neuroprotection (Raine *et al.* 1989, Kornek *et al.* 2000), we examined the extent of neurofilament staining in both cKO and control lesions to establish if axon preservation was better in the

absence of NMIIB. Staining intensity for neurofilament at the center of the lesion was slightly reduced in controls compared to cKO but this difference was not statistically significant (Figure II 5A,B). Similarly staining intensity for $NG2^+$ progenitors, cells that mediate CNS repair and remyelination (Kucharova *et al.* 2011, Watanabe *et al.* 2002), although slightly increased in cKO mice lesions was not statistically significant to that of controls (Figure II 5A,B). Collectively these results suggest that improved remyelination in cKO mice is not due to better axonal preservation and/or enhanced recruitment of $NG2^+$ progenitors.

Inflammation and reactive astrogliosis response at lesion site is not affected by NMII ablation

Inflammation and astrogliosis are two anticipated responses to local damage, critical for myelin debris clearance and repair (Skripuletz *et al.* 2012). We investigated if ablation of NMIIB affected these responses by staining lesions at 14 dpi with antibodies to Iba1, to identify cells of the phagocyte/monocyte lineage, and GFAP to identify astrocytes. Results shown in Figure II 5A,B indicated that there were no significant differences in the extent of inflammation and astrogliosis between cKO and control animals. Therefore accelerated repair in the cKO mice cannot be attributed to these factors.

Oligodendrocytes lacking NMIIB are present in remyelinated areas

To investigate if accelerated myelin repair in cKO mice is directly mediated by OPC and oligodendrocytes lacking NMIIB, we generated double transgenic *PlpCre/ESR1*:Rosa26-mT/mG mice carrying wild type (Control) or floxed (cKO) NMIIB alleles. Focal demyelination with lysolecithin was induced at 12 weeks as before and lesion resolution was examined at 28 dpi.

The results shown in Figure II 6 demonstrate that $EGFP^+$ myelinating oligodendrocytes were more frequently found within the remyelinated area of cKO mice (Figure II 6A). Thus, the percentage of the remyelinated area covered by $EGFP^+$ cells (Figure II 6B) was significantly larger in cKO mice than controls (4.3% ±2.6 vs. 1.06% ±1.1, mean±SD; p=0.0004, t-test) further confirming our hypothesis that lack of NMIIB enhances oligodendrocyte differentiation and CNS myelin formation.

Myosin IIB levels are slightly elevated in demyelinated lesions

In order to evaluate at the biochemical level the efficiency of NMIIB knockout in OL at the site of myelin injury, we performed western blot on tissue from 7dpi lysolecithin lesions and uninjured-normal appearing white matter (NAWM) in the contralateral corpus callosum. Comparison of the levels of NMIIB in NAWM shows that cKO animals show, as expected, a slight decrease of NMIIB levels compared to control mice (Figure II 7). Since Cre-mediated recombination only targets a sub-population of cells in the brain, namely the OL, detection of significant changes in overall protein levels is unlikely. Interestingly, we found that NMIIB levels were slightly elevated in the lesioned corpus callosum as compared to the contralateral side in both controls and cKO animals. Although this trend was not statistically significant (Mann Whitney t-test p=0.1, N=3 mice), it indicates that NMII signaling could have a role in demyelination.

Figure II 1. Analysis of tamoxifen-driven *PlpCre/ESR1* recombination.

Rosa26-mT/mG mice have loxP sites on either side of a membrane-targeted tdTomato cassette and express strong red fluorescence in all tissues. When bred to *PlpCre/ESR1* mice, the resulting offspring have the mT cassette deleted in the Cre expressing cells, allowing expression of the membrane-targeted EGFP cassette. *NMIIB* $^{fl/fl}$ mice carrying both the *PlpCre/ESR1* transgene and the Rosa26-mT/mG allele (PLP-Cre^{+}) or Rosa26-mT/mG allele alone (NonCre) were injected with tamoxifen for 5 days at 8 weeks of age. The animals were sacrificed at 12 weeks and their tissue was processed for immunofluorescence with antibodies for CC1 and MBP. **A.** Sections of the corpus callosum (CC) show high EGFP expression in OL in the white matter tract as well as in the cerebral cortex (CTX) and striatum (Str). EGFP label colocalizes with mature OL marker CC1 (Scale bar = 100 μ m.) **B.** Detail of CTX shows EGFP colocalizing with MBP (Colocalization visualized in white using ImageJ) (Scale bar = 50 μ m)

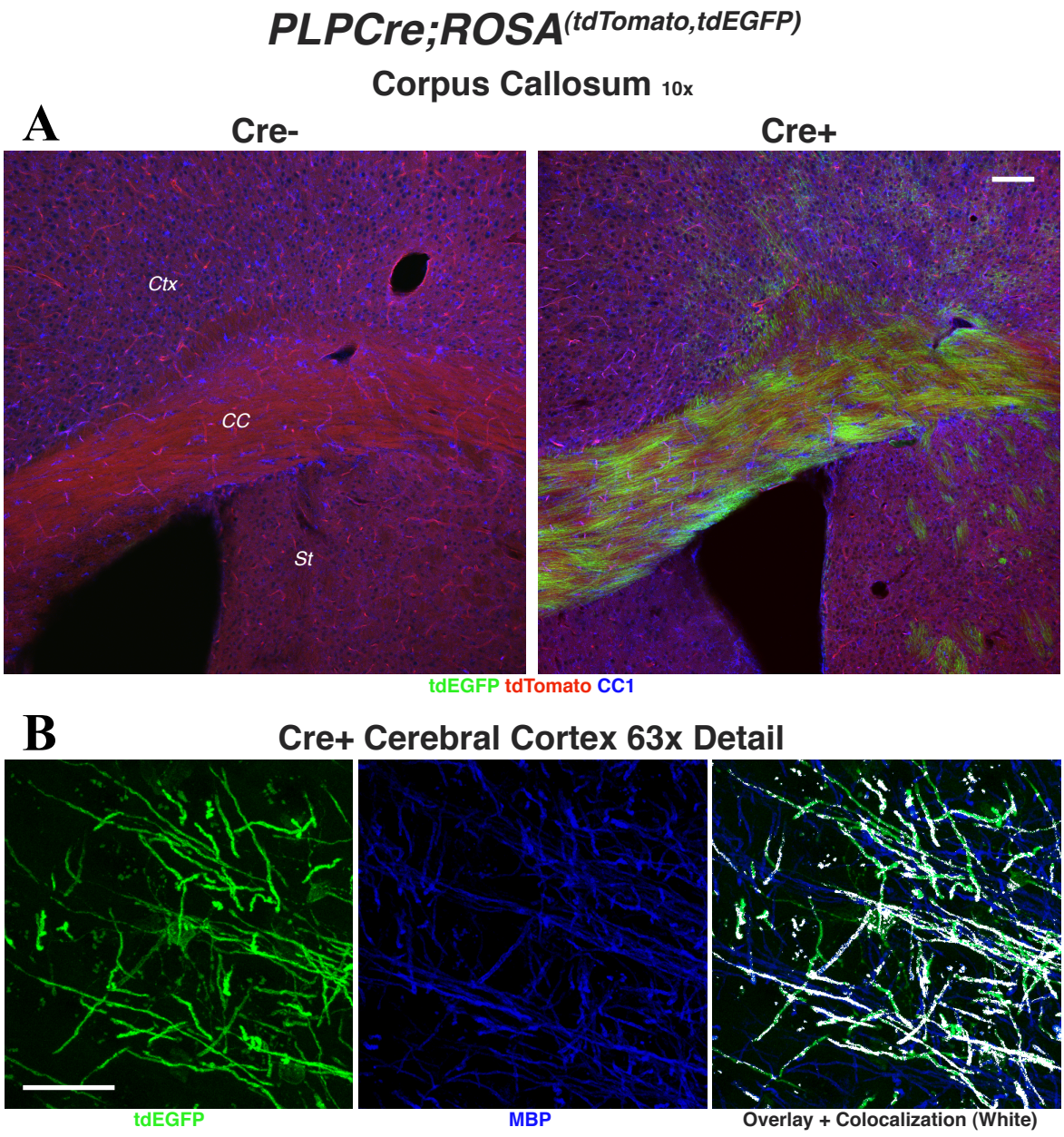

PLPCre;ROSA(tdTomato,tdEGFP)
Corpus Callosum 10x
A
Cre-
Cre+
Ctx
CC
St
tdEGFP tdTomato CC1
B
Cre+ Cerebral Cortex 63x Detail
tdEGFP
MBP
Overlay + Colocalization (White)

Figure II 1

Figure II 2. Normal appearing white matter.

A. Electron micrographs from uninjured corpus callosum of control and cKO of 16 week-old mice reveal normal myelin morphology. (Scale bar = 500 nm) **B**. G-ratio and axon diameter values were obtained from 3 animals per genotype, for a total of 183 axons measured.

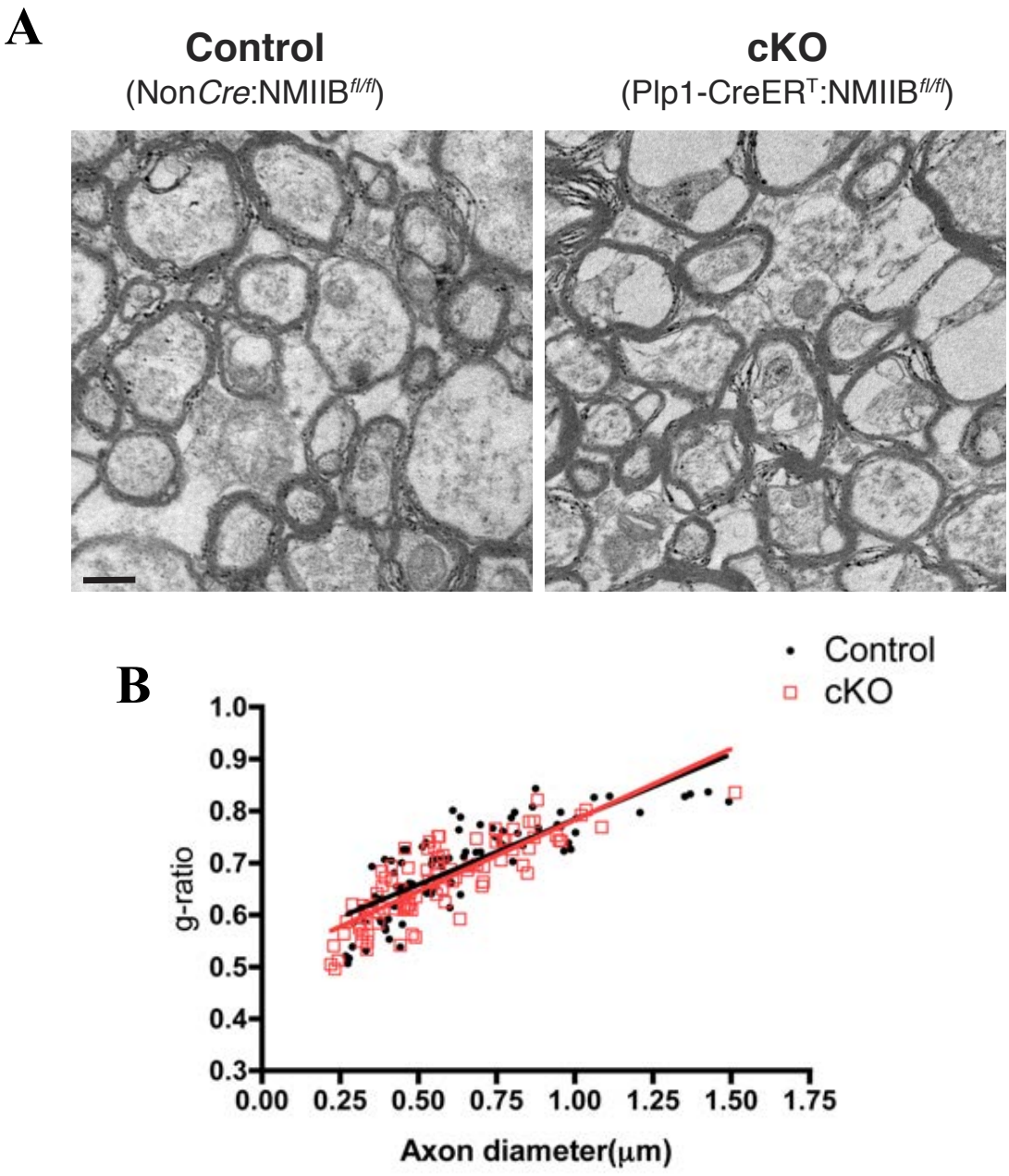


Figure II 3. Lesion volume is reduced in mice with conditional ablation of NMIIB.

A. Caudal-rostral (left to right) projection of 30μm brain sections stained with flouromyelin (green) and CC1 antibodies (blue) showing a significantly smaller lesion span in cKO animals compared to controls at 14dpi. **B**. Images of maximum lesion area at 14 dpi showing an increased number of $CC1^+$ OL (blue) in cKO animals compared to controls. Scale bars 100⌠m. **C**. Quantitation of $CC1^+$ OL at 14 dpi (Mann Whitney t-test **p<0.01). **D**. Graph comparing average lesion volume at 7, 14 and 28 dpi for cKO and control mice, showing that both the initial extent of demyelination (7 dpi) and lesion resolution (28 dpi) were comparable in both genotypes. Results were obtained from 5-9 animals per genotype, per time point, three different fields per animal.

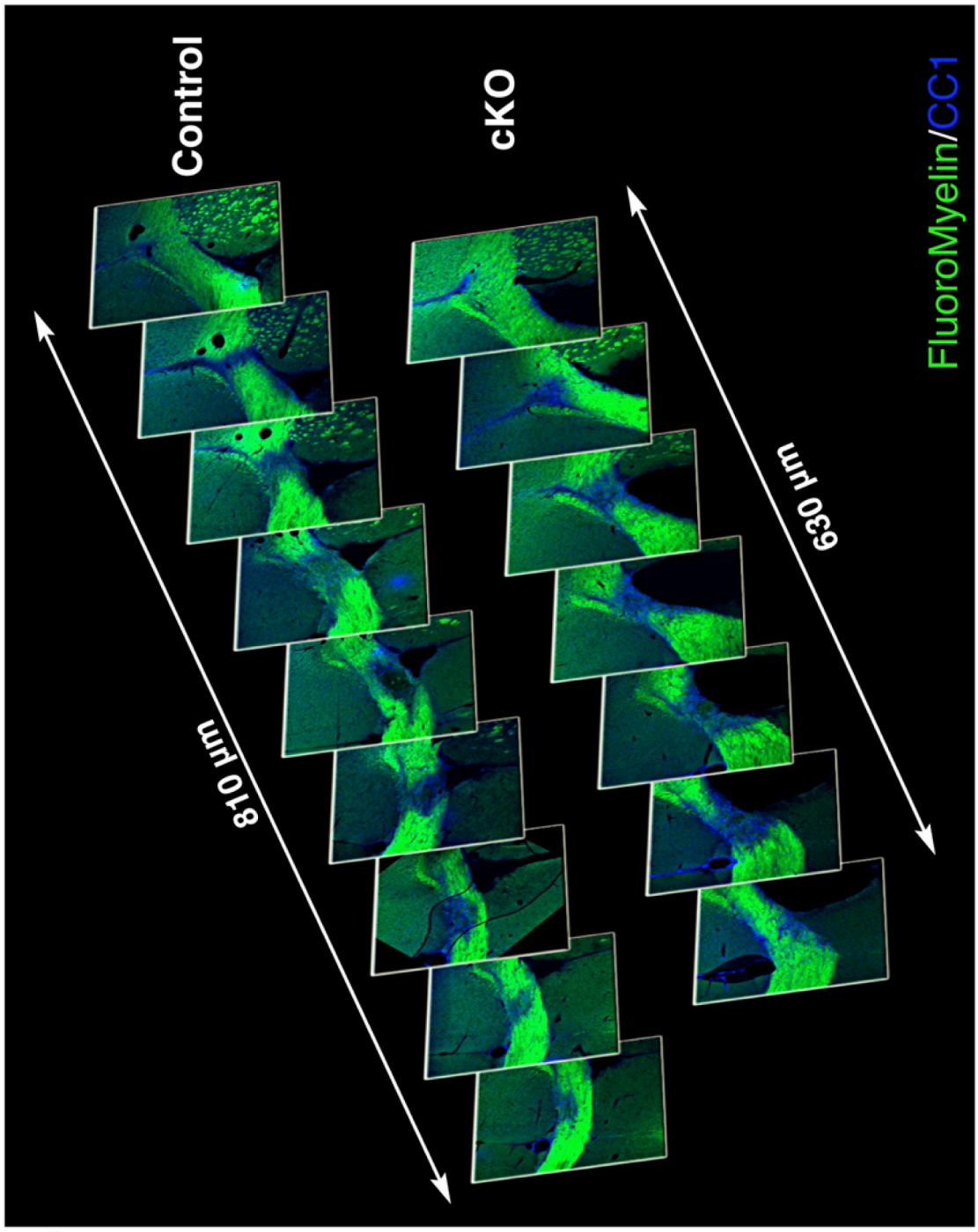


Figure II 3

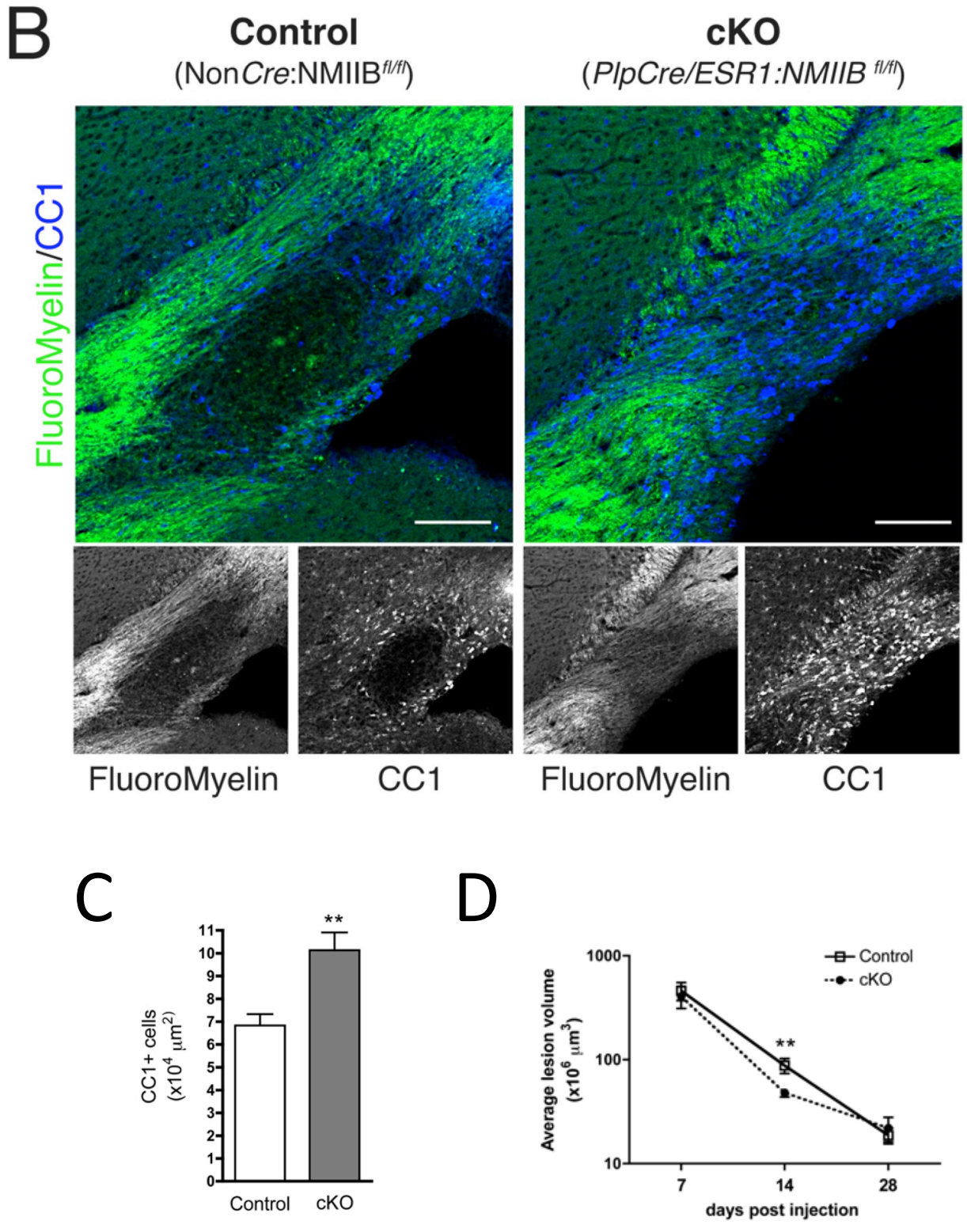


Figure II 3 cont.

Figure II 4. Accelerated remyelination in mice with conditional ablation of NMIIB.

A-B. Light microscopy of resin sections stained with toluidine blue showing representative pathology from 14 dpi lysolecithin lesions in control and cKO animals. Areas highlighted in panel A are shown in detail in panel **B**. The lesion center in both genotypes contains reactive astrocytes (labeled A in panel **B**), lipid filled mononuclear monocytes (labeled M in panel B) and demyelinated axons; but is smaller in cKO animals. The lesion border contains a mix of axons surrounded by abnormally thin myelin, a characteristic of remyelinated axons (arrowheads in **B**, and left panel in **C**) and normal myelinated axons (right panel in **C**). Remyelinated axons (detail in panel **C**) are more commonly observed in the cKO. Normal appearing white matter (NAWM) is also shown. Many cells with a morphology consistent with oligodendrocyte lineage (O) are observed in the border of the cKO lesion (lower panel **A**, detail in **B**). A remyelinated axon in transverse section (*) is also shown. **C**. Examples of normal myelinated axons (left) and remyelinated axons (right). **D-G.** Examples of pathology are illustrated using electron microscopy of serial grids from control (**D**) and cKO (**F-G**) lesions. **D**. A mononuclear monocyte (M) is seen adjacent to a demyelinated axon (arrowheads) at 7dpi. **E**. Details of multiple remyelinated axons (*) at 7dpi. **F**. A lipid-filled mononuclear monocyte (M) at14 dpi. **G**. An oligodendroglial cell (O) is seen at the border of the lesion containing multiple remyelinated axons (*) at 14 dpi.

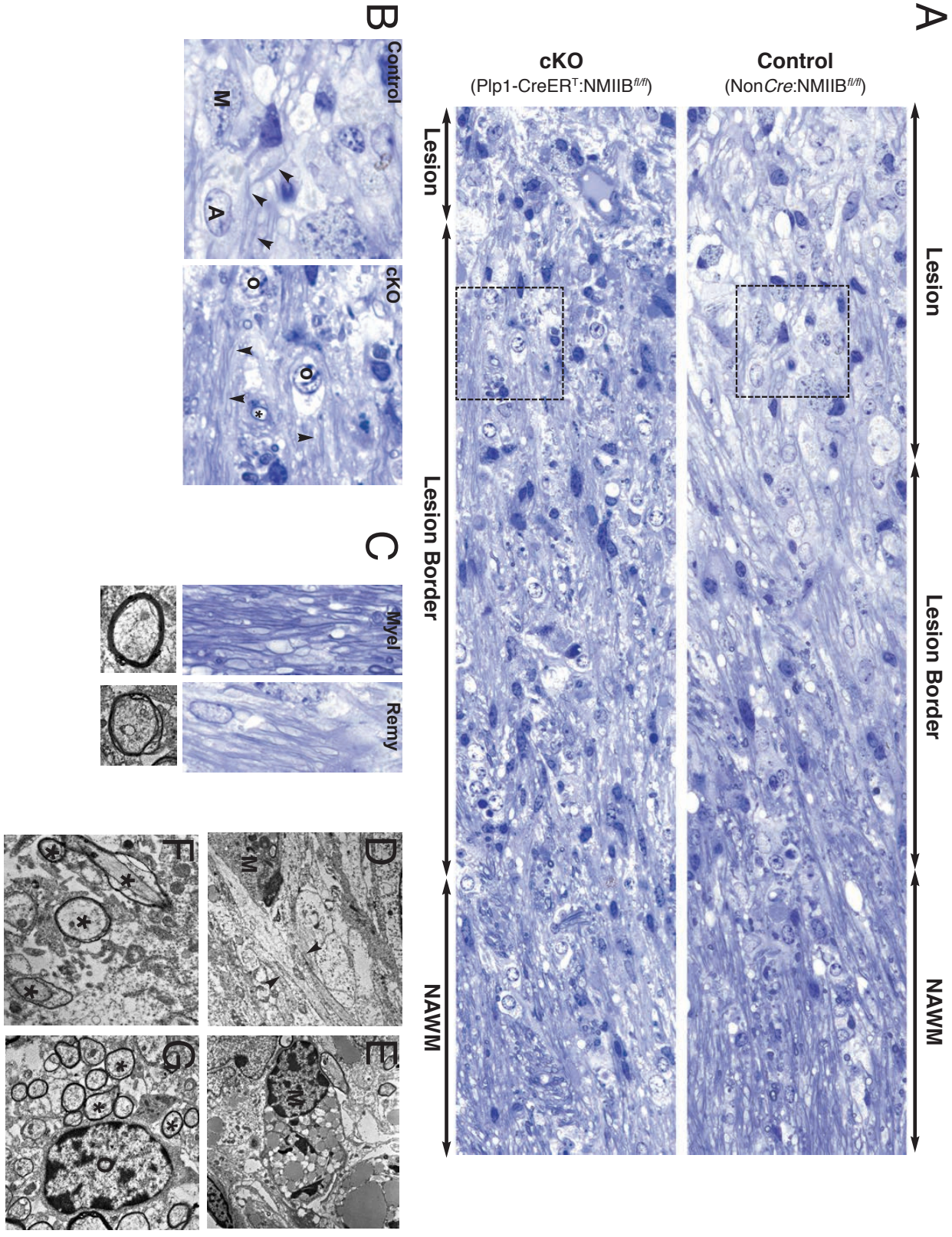

A
Control
(NonCre:NMIIB fl/fl)
cKO
(Plp1-CreER T :NMIIB fl/fl)
Lesion
Lesion Border
NAWM
B
Control
cKO
M
A
o
C
Myel
Remy
D
E
F
G

Figure II 4

Figure II 5. Axonal preservation, progenitor recruitment, inflammation and reactive astrogliosis are not affected by NMII conditional ablation.

A. Sections from control and cKO corpus callosum (14 dpi) immunostained with neurofilament (NF), NG2, Iba1 and GFAP antibodies to evaluate respectively: axonal preservation, the presence of progenitors and the extent of inflammation and reactive astrogliosis at the lesion center. **B**. Quantitation of fluorescence intensity by normalized mean gray value (see Material and Methods) shows that all parameters were comparable between cKO and control mice, and the small differences in fluorescence intensity were not statistically significant. Data collected from 6-9 animals per genotype, three fields per animal. Scale bars 50µm.

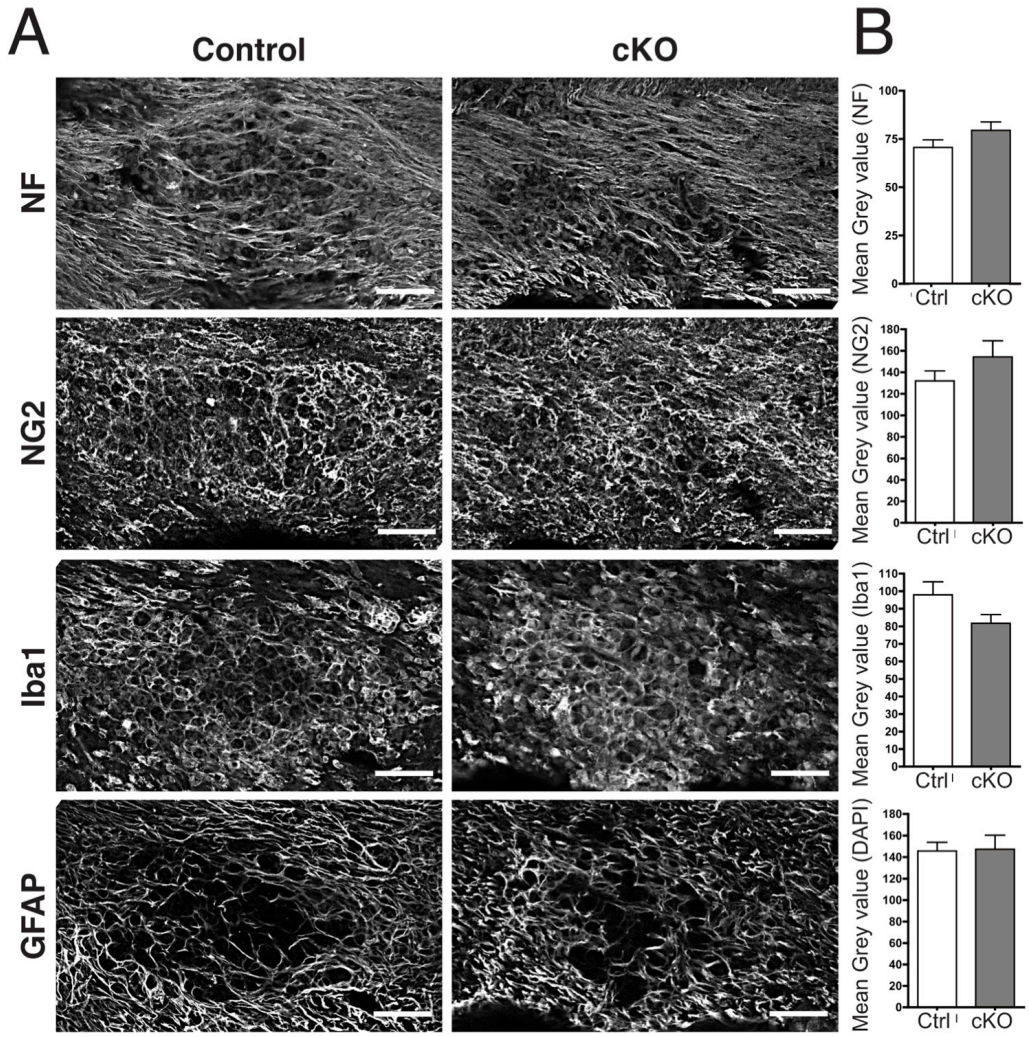


Figure II 6. Myelinating oligodendrocytes lacking NMIIB are present in remyelinated areas.

A. Representative images from 28 dpi lysolecithin lesions from transgenic *PlpCre/ESR1*:Rosa26-mT/mG mice carrying wild type (Control) or floxed (cKO) *NMIIB* alleles. The presence of $EGFP^+$ myelinating cells within the remyelinated MBP^+ area (blue) was significantly more frequent in cKO animals lacking NMIIB in their oligodendrocytes. Scale bar 50µm. **B.** Quantitation of percentage area remyelinated by $EGFP^+$ oligodendrocytes in control and cKO animals (Mann Whitney t-test ***p<0.001). Data collected from 3 animals per genotype, 3-5 fields per animal. Scale bars 50µm.

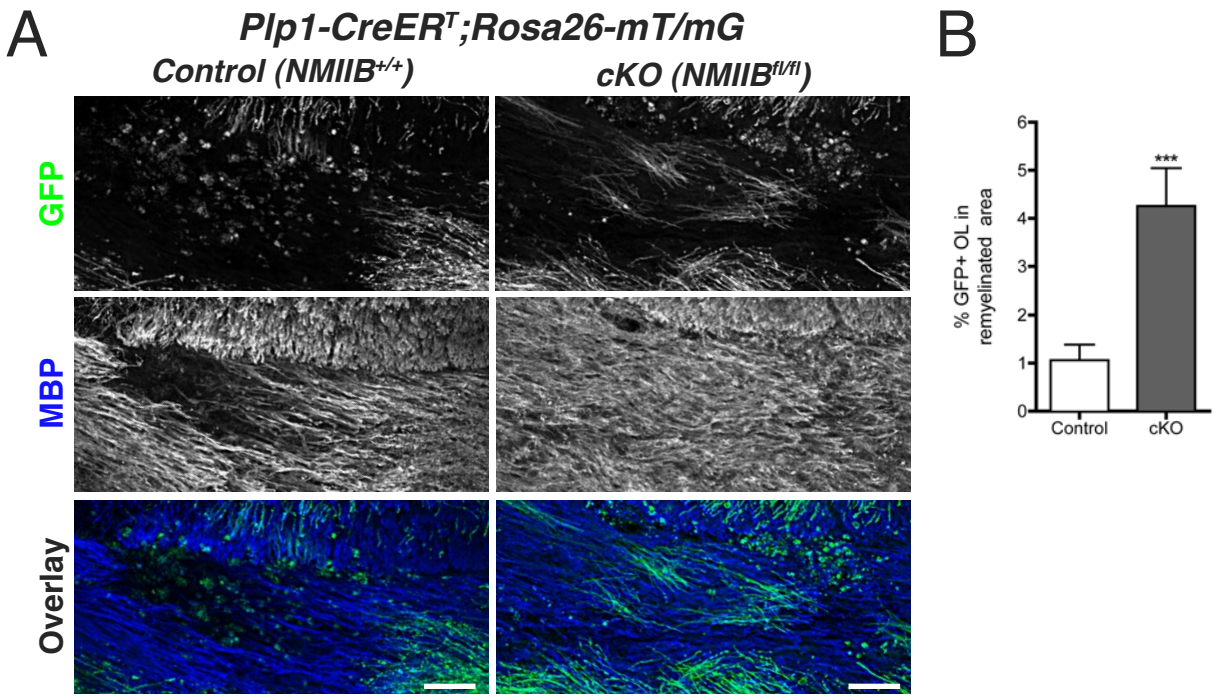


Figure II 7. Myosin IIB levels are elevated in demyelinated lesions.

Fresh tissue was dissected out of 7dpi lysolecithin lesions as well as uninjured contralateral corpus callosum and processed for western blot. **A.** Immunobotting for NMIIB shows an increase in protein levels in lesioned tissue compared to the contralateral side of the corpus callosum. **B.** Graph of densitometry quantitation using ImageJ of NMIIB bands adjusted to actin levels and averaged together based on location and genotype (Mann Whitney t-test p=0.1, N=3 mice).

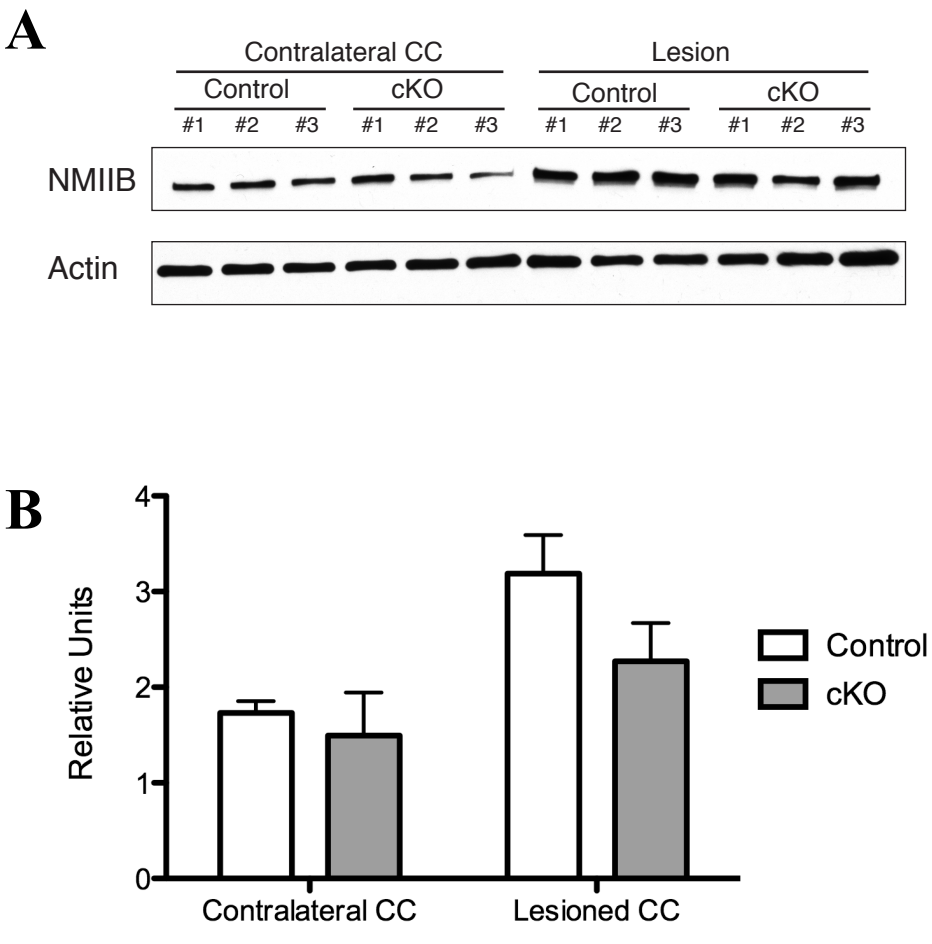


Table 1. Cell proliferation and apoptosis in lysolecithin lesions at 7 dpi.

	Control	cKO
Lesion:		
Total DAPI+ cells/area	10.24 ± 0.38 x10^3 μm^2	11.17 ± 0.24 x10^3 μm^2
% Ki67$^+$ cells	1.31 ± 0.73%	1.78 ± 0.99%
% Caspase 3$^+$cells	0.28 ± 0.05%	0.29 ± 0.06%
NAWM:		
Total DAPI+ cells/area	6.09 ± 0.41 x10^3 μm^2	6.23 ± 0.31 x10^3 μm^2
% CC1$^+$ cells	34.01 ± 4.80%	41.55 ± 3.11%

Data in table represents mean ± SEM calculated from 3 mice per genotype (3 fields per mice).

Chapter 3: Signaling pathways upstream of myosin II

Excerpts from:

"Myosin II is a negative regulator of oligodendrocyte morphological differentiation"

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Summary

The study detailed in this chapter provides a direct link between the process of Fyn-Rho signaling in OL and the control of OL morphology and differentiation by MLC phosphorylation and the levels of NMII. We showed that inhibition of NMII contractility downstream of Fyn promotes OL process extension and that inhibition of Fyn or overexpression of its downstream target Rho resulted in increased levels of phosphorylated MLC, which negatively affected OL branching, but could be reversed by the pharmacological inhibition of upstream targets ROCK or NMII.

The link between Cdk5 and NMII is less clear. Although preliminary studies using the cyclin-dependent kinase inhibitor roscovitine showed promising data to link Cdk5 to NMII, as those experiments showed NMII dependent decreases in OL complexity and differentiation of OL treated with roscovitine. Our subsequent experiments with specific knockdown of Cdk5 did not show any defects in OL morphology. This result would suggest that the effect on OL branching and differentiation seen after treatment with roscovitine was most likely caused by inhibition of the other known targets of the drug, namely Cdc2 or Cdk2.

Materials and Methods

Glial cell purification

Methods for purification of primary rat OPC have been previously described (Wang *et al*., 2008). Briefly, primary OPC from P2 rat cortices were purified by immunopanning (Ran2-, A2B5+) and maintained in Sato media (DMEM, bovine insulin (5 μg/ml), human transferrin (50 μg/ml), BSA V (100 μg/ml), progesterone (6.2 ng/ml), putrescine (16 μg/ml), sodium selenite (5 ng/ml), supplemented with PDGF (10 ng/ ml) and bFGF (10 ng/ ml). OPC were induced to differentiate by removing growth factors from the media and adding T3 hormone (30 ng/ml) for 2-3 days. Mouse cortical OPC were purified by immunopanning from mixed cortical cells of postnatal day 5-9 mouse cerebral cortices as previously described (Wang *et al*., 2001). Briefly, from mixed cortices cells, astrocytes were removed by Thy1.2 antibody panning, mature OL were removed with galactocerebroside antibody and OPC were purified by O4 antibody panning.

Transfection

For transfection of primary OPC, cells were resuspended in the nucleofection solution to a final concentration of 1-5x106 cell/100 μl. The cell suspension was mixed with 1-2.5 μg of DNA or RNA, placed in the nucleofection cuvette, and transfected using the O-17 nucleofection program.

Small interfering RNA (siRNA)

Pools of siRNA duplex targeting rat FYN (NC_005119.3), rat myosin IIB M2B (NM_031520) and rat Cdk5 (140908) were obtained from Dharmacon (siGENOME ON-

TARGETplus SMARTpool duplex). A non-targeting duplex pool (ON-TARGETplus siCONTROL Non-Targeting Pool D-001810-10-05) was used a control.

RT-PCR

RNA was isolated from rat OL (2d T3, 2d PDGF), adult rat optic nerve, adult mouse liver, cerebellum, cortex, and spinal cord tissues using "Absolutely RNA Miniprep Kit" (Stratagene). RT-PCR was used to create cDNA from isolated RNA using "Accuscript High Fidelity RT-PCR System"(Stratagene). Flanking Primers P1 -5` TGC CGG GAA GAC AGA AAA TA 3`- and P2 – 5`CAT GGC TTC CAT GGT CTC CT 3`- were used to amplify exon 9 of *myh10* that might contain the 30bp insert, and PCR was used to determine the expression of NMIIB versus NMIIB1 using (Sigma) JumpStart Taq DNA polymerase and products were electroporated on a 2% agarose TAE gel.

Immunofluorescence and imaging

Cultures were fixed in 4% PFA and processed for immunocytochemistry as previously described (Wang *et al*., 2008), and examined with LSM 510 Zeiss confocal microscope and LSM software. Image processing and quantitation analysis was performed using ImageJ 1.38v. and Volocity software.

Antibodies

For these studies the following antibodies were used: myosin IIA and IIB, tubulin (Covance), MLC2, phosphorylated MLC2, (Cell Signaling Technology); MBP (SMI-94; Sternberger Monoclonals); mouse A2B5, 04, and Ran-2 hybridomas (ATCC); CNPase/Rip

(DSHB); phalloidin-FITC, phalloidin-TRICT (Sigma), Secondary antibodies conjugated to rhodamine, fluorescein, coumarin, or cyanin 5 (Jackson Laboratories).

Plasmids and Inhibitors

pcDNA3-EGFP-RhoA WT, pcDNA3-EGFP-RhoA-Q63L CA, pcDNA3-EGFP-RhoA-T19N DN (Addgene); Blebbistatin and Y27632 (Calbiochem).

Cell Extracts and Western Blotting

OPCs on 6cm dishes were rinsed with PBS and harvested by gentle scraping into lysis buffer [150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO)], subjected to SDS-PAGE, and blotted onto nitrocellulose. Appropriate regions of the blots were cut and incubated with specific antibodies and developed using chemiluminescent substrate (Pierce, Rockford, IL). For estimation of changes in protein levels, films from three independent experiments were scanned, and the relative intensity of each protein band was calculated in Image J by dividing the absolute intensity of each protein band (the area of the band by the number of pixels contained in that area) by the absolute intensity of the corresponding actin band.

Results

Inhibition of Fyn increases MLC phosphorylation.

To test if the inhibition of Fyn will cause an increase in NMII activity we used PP2, a specific inhibitor of Fyn, in purified cultures of primary rat OPCs that are either in proliferation media (PDGF) or differentiation media (T3). Cell lysates were evaluated after 1 day in culture by western blot for the amount of active MLC, using antibodies specific for both total and ser19 phosphorylated MLC. We found that levels of phosphorylated MLC, and hence active myosin II, are downregulated as OL are switched from PDGF to T3 (Figure III 1A). Furthermore, the downregulation of pMLC in T3 is abolished by the inhibition of Fyn. Treatment with PP2 at low doses (1-2.5μM) resulted in a heightened level of phosphorylated MLC compared to that of control (DMSO). These levels were comparable to those seen in proliferating conditions (PDGF). Increased levels of PP2 (>5μM) resulted in cell death (data not shown) and did not affect pMLC relative to controls.

To address the possibility that pharmacological inhibition of Fyn through PP2 could be non-specifically targeting other src-family kinases, we transfected OPCs with siRNA targeting specifically Fyn or scrambled non-targeting siRNA for control. Figure III 1B shows that OPCs induced to differentiate for 1 day in T3 upregulated the expression of Fyn as seen in immunoblotting, thus confirming work done by Liang *et al.* in 2004. We can reduce Fyn levels with siRNA transfection and confirm our previous results using the Fyn inhibitor PP2. Although the increase in pMLC is not as robust as using PP2, it could be as a result of incomplete knockdown of Fyn, which could still act to activate p190rhoGAP thus reducing pMLC through reduction in active RhoGTP.

Inhibition of NMII rescues branching defects from RhoA overexpression.

It has been previously shown that overexpression of RhoA inhibits process extension by OL (Liang *et al*., 2004). We repeated these experiments by transfecting rat OPCs with control GFP expressing plasmids or the same constructs used in the aforementioned work, namely RhoA wild type (WT), RhoA constitutively active (CA) or RhoA dominant negative (DN) mutants and confirmed the effects of RhoA overexpression on process extension. RhoA WT and CA both inhibit process formation with the later more so than the former but not RhoA DN or the control GFP expressing cells. Additionally we found that this effect is completely reversed by the use of blebbistatin, a specific inhibitor of NMII (Limouze *et al.,* 2004) (Figure III 2A). Thus OL overexpressing WT and CA RhoA showed a reduction in process length of 15% and 36% respectively, compared to cells overexpressing GFP alone. By contrast, overexpression of a dominant negative (DN) RhoA construct caused an increase in process length of about 22% (As quantitated in Figure III 2B). Treatment with blebbistatin increased process length significantly in all but the DN RhoA expressing cells. Taken together these results indicate that inactivation of myosin II is downstream of Fyn-Rho signaling and mediates the morphological changes resulting from changes in Rho expression.

Overexpression of CA RhoA increases MLC phosphorylation.

Next we investigated if there is a direct link between active RhoA and NMII activity in OL. Although it is well established that Fyn signaling regulates the activity of Rho, the role of NMII as the downstream effector of the morphological events induced by Rho overexpression has not been directly investigated. We found that overexpression of constitutively active (CA)

RhoA increased phospho-MLC levels in OPCs (Figure III 3) compared to control cells transfected with GFP expressing plasmid or cells transfected with wild type (WT) RhoA. Furthermore, this increase in activity is dependent on ROCK, as treatment of CA RhoA transfected cells with the specific ROCK inhibitor Y-27632, abolishes the increase in phospho-MLC caused by CA RhoA. The overall levels of overexpression between GFP control and RhoA-GFP constructs was similar thus controlling for any effect that GFP overexpression itself might be causing (data not shown).

Roscovitine inhibits OL morphological complexity via NMII

In order to determine the effect of Cdk5 inhibition, we used roscovitine on OPCs and evaluated the cells after 3 days in differentiating media (T3) by immunofluorescent microscopy for markers of immature OPC (A2B5), OL differentiation (Rip) and cell nuclei (DAPI) (Figure III 4). We confirmed effective and lasting knockdown of NMIIB by WB for up 3 days (Fig III 7D). Roscovitine treatment of cultures transfected with non-targeting siRNA (siCtrl), showed a decrease in the percentage of more complex OL morphology (Type III 19%→13% and IV 25%→0% $p = 0.0001$) and an increase in the percentage of type I cells (36%→60%) (Figures III 5). These results confirmed previous observations implicating Cdk5 in OL morphological differentiation (Miyamoto *et al*., 2007). To test if the effects of roscovitine in OL morphology are mediated by NMII, we transfected OPCs with siRNA targeting NMIIB and treated those cultures with roscovitine. Consistent with this hypothesis we found that roscovitine effects in OL morphology were partially rescued by downregulation of NMIIB in OL. Thus, decreasing NMIIB levels in OL using siRNA caused an decrease in the percentage of type I cells compared

to controls when treated with roscovitine (60%→22%, p = 0.0003), and an increase in type III cells (13%→34%, p = 0.0005).

Roscovitine inhibits OL differentiation

Next, we examined the effects of Cdk5 inhibition on OL differentiation by examining the percentage of Rip+ cells. Similar to the effects observed on OL morphology (Figure III 5), decreasing the levels of NMII using siRNA partially rescued the differentiation defect caused by inhibition of Cdk5. Thus, more RIP positive cells where detected in roscovitine treated cultures transfected with siRNA against NMII, than in those transfected with control siRNA (Figure III 7A) (8.1% vs. 19.7% respectively, p = 0.0022). Of note, we did not observe changes in either the number of immature A2B5+ OPC present in these cultures (Figure III 7B) or the total number of cells (DAPI) (Figure III 7D), suggesting that these differences are not due to toxicity.

siRNA knockdown of Cdk5 does not affect OL morphology

To test the specificity of the effect roscovitine had on OL morphological differentiation we used siRNA against Cdk5. OPCs were transfected with either scrambled non-targeting siRNA or one specific for Cdk5. Cells were also co-transfected with a EGFP expressing plasmid to visualize transfected cells vs. non-transfected. Cells were plated on PDL coated coverslips and treated with T3 to stimulate differentiation. Knockdown of Cdk5 did not cause any obvious morphological changes at 1 or 3 days post transfection (Figure III 8A). We confirmed knockdown of Cdk5 by western blot at 1 and 3 days post transfection showing a large reduction in Cdk5 protein levels (Figure III 8B).

OL do not express the NMIIB1 alternative splice isoform

One possible way that Cdk5 could regulate NMII activity is through a direct phosphorylation of the heavy chain of a NMII isoform NMIIB1. Using PCR, we tested for the presence of this isoform in OPC grown in proliferating or differentiating conditions. We designed flanking primers to the site of the splice insert of a small 30bp exon and expected to observe two bands after PCR. As seen in Figure II 9, NMIIB1 isoform was present in mouse cerebellum and spinal cord, however we did not detect its expression in purified rat OPCs.

Figure III 1. Inhibition of Fyn causes an increase in pMLC levels.

Western blots of OPC cultures maintained in proliferating media (PDGF) or differentiation media (T3) for 1 day. Pharmacological inhibition of Fyn (+PP2) **A**, or silencing with siRNA (syFyn) **B**, resulted in increased levels of phosphorylated MLC measured by relative ration of pMLC over total MLC and standardized to actin (N=3 experiments, $^{*}p<0.05$, t-test). Expression of MBP was not significantly affected by inhibition/silencing of Fyn.

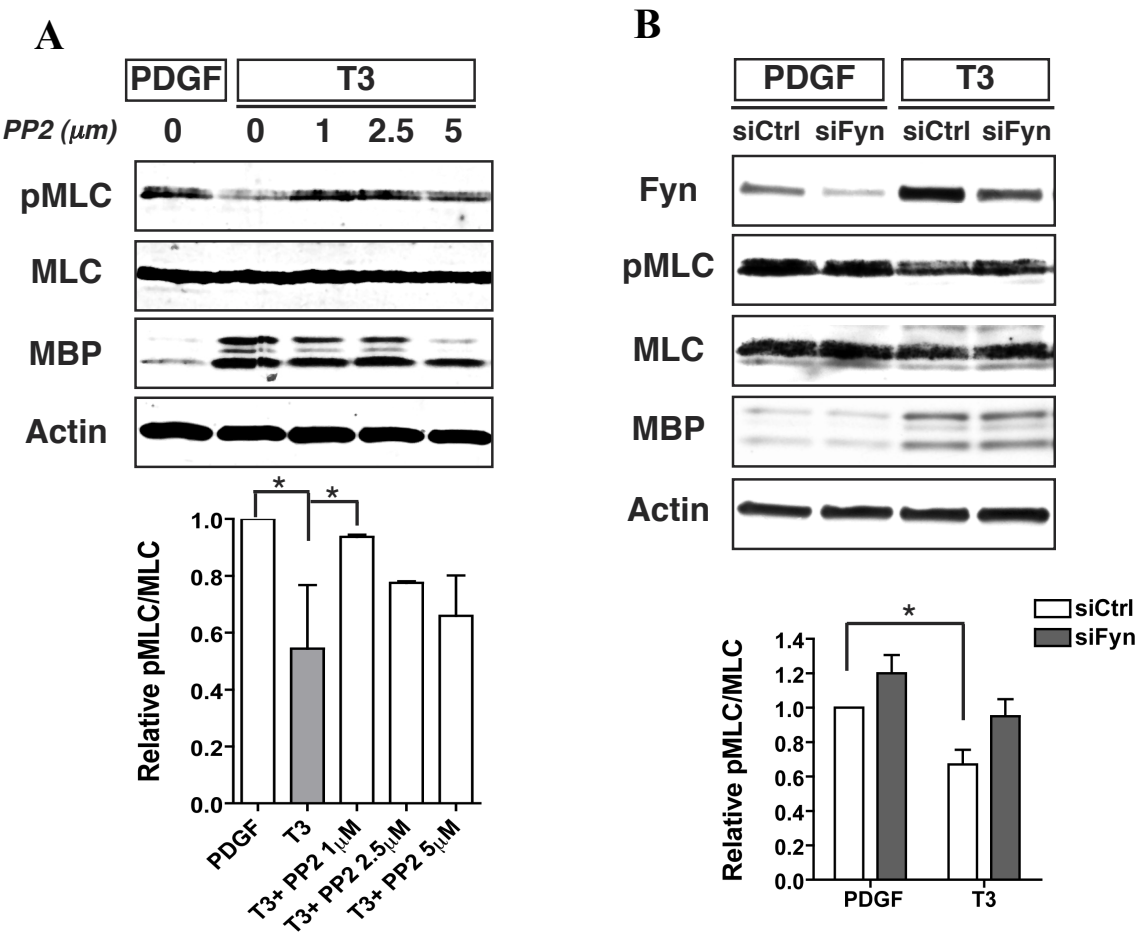
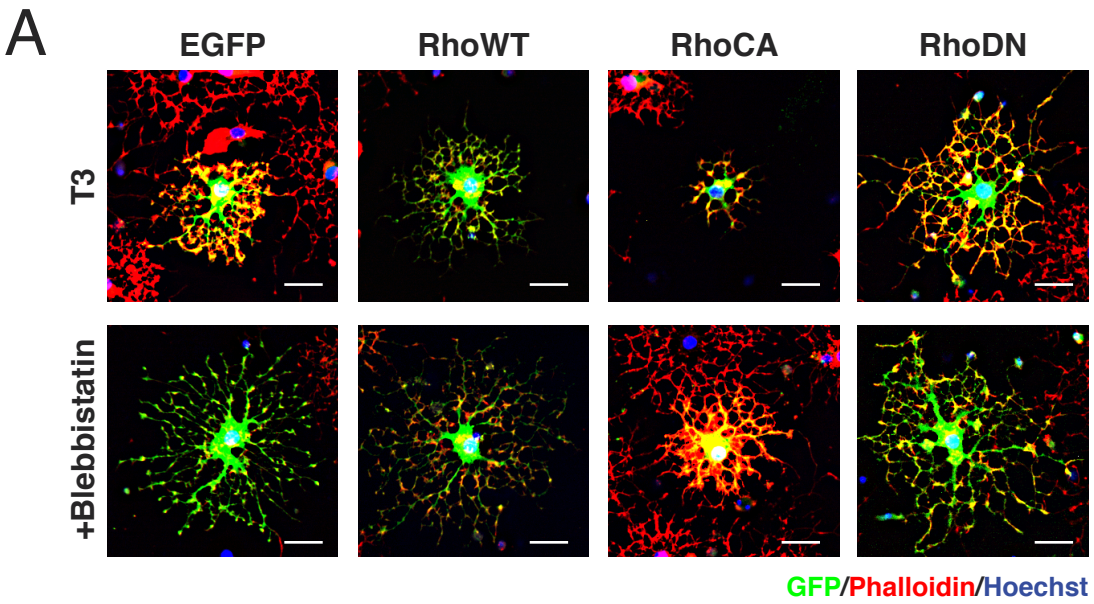


Figure III 2. Inhibition of NMII activity rescues process length defects cause by CA RhoA.

A. Representative images of OPC transfected with control plasmid (EGFP) or plasmids expressing GFP-tagged wild type (WT), constitutively active (CA) or dominant negative (DN) RhoA. Overexpression of WT or CA RhoA resulted in decreased process extension ($^{***}p<0.0001$, t-test). This effect can be reversed by inhibition of NMII with blebbistatin. Overexpression of DN Rho also increases oligodendrocyte branching. Scale bar 25μm. **B**. Quantitation of process length by transfected OPC with or without blebbistatin. Cultures were stained with phalloidin and process length of transfected GFP^+ was measured using Image-J from the cell body to the end of the actin network (EGFP n=192 cells; EGFP+ Bleb n=155 cells; RhoWT n=162 cells; RhoWT+Bleb n=132 cells; RhoCA n=117 cells; RhoCA+Bleb n=102 cells; RhoDN n=50 cells; RhoDN+Bleb n=39 cells).


A
EGFP
RhoWT
RhoCA
RhoDN
T3
+Blebbistatin
GFP/Phalloidin/Hoechst

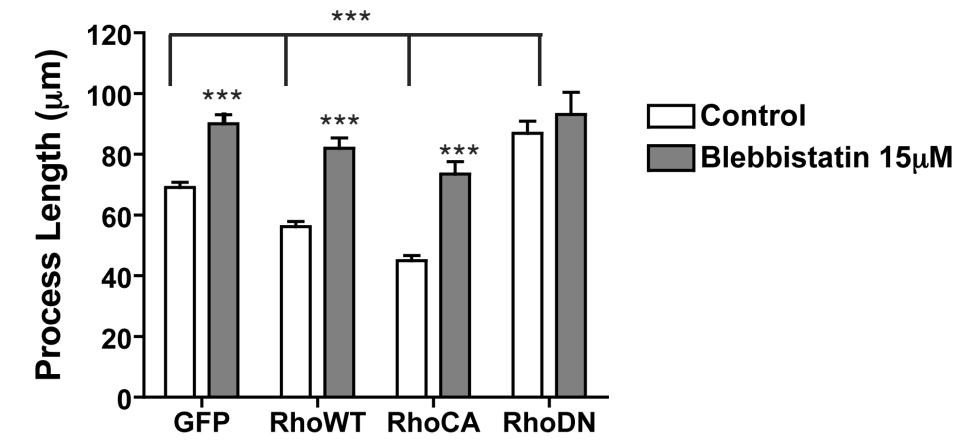

B
Process Length (μm)
120
100
80
60
40
20
0
***
***
***
***
GFP
RhoWT
RhoCA
RhoDN
Control
Blebbistatin 15μM

Figure III 2

Figure III 3. Rho overexpression in OL causes an increase in active NMII.

Western blot of OPC cultures transfected with EGFP, RhoWT or RhoCA and kept in differentiation media (T3) for 1 day. Increased levels of phosphorylated MLC were observed in cultures overexpressing RhoA ($^{**}p<0.01$, t-test). This increase was reversed by treatment with the ROCK inhibitor Y27632.

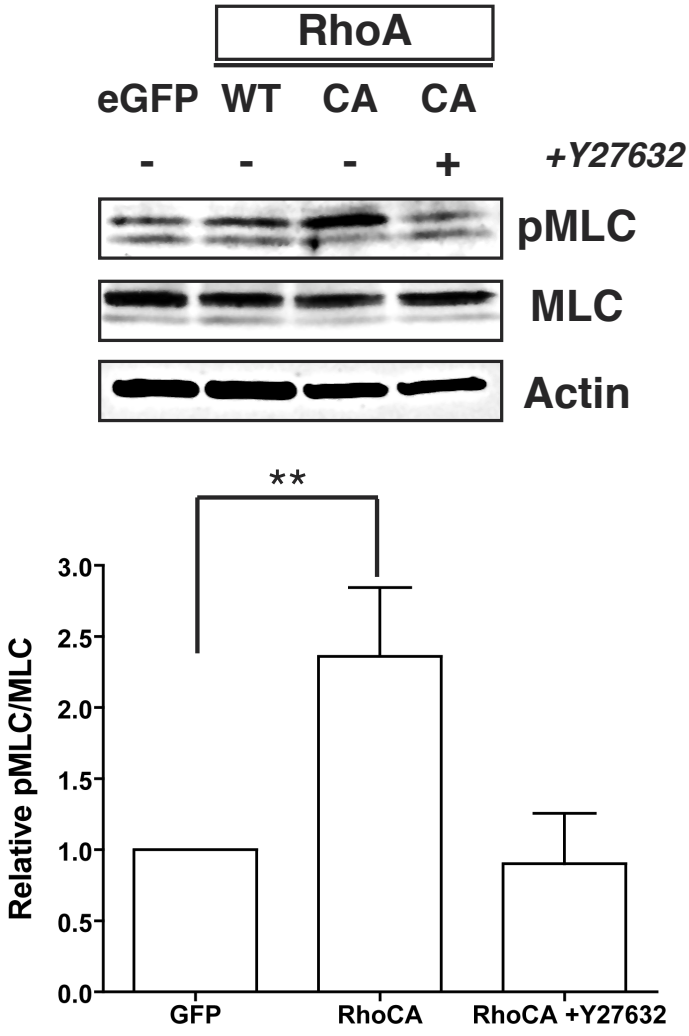


Figure III 4. Effects of Roscovitine treatment on OL morphology and differentiation are reversed by knockdown of NMII.

Rat OPCs transfected with siRNA for NMIIB or non-targeting siRNA and then cultured on glass coverslips for 3 days in T3 with either roscovitine (15μM) or DMSO as a control. Cells were fixed and stained with antibodies for RIP and A2B5, which appear in the FITC and rhodamime channels respectively. Cells were also stained with DAPI to visualize the nucleus. Scale bar = 50 μm. Decreases in OL branching and Rip+ immunoreactivity caused by roscovitine are prevented by NMIIB downregulation.

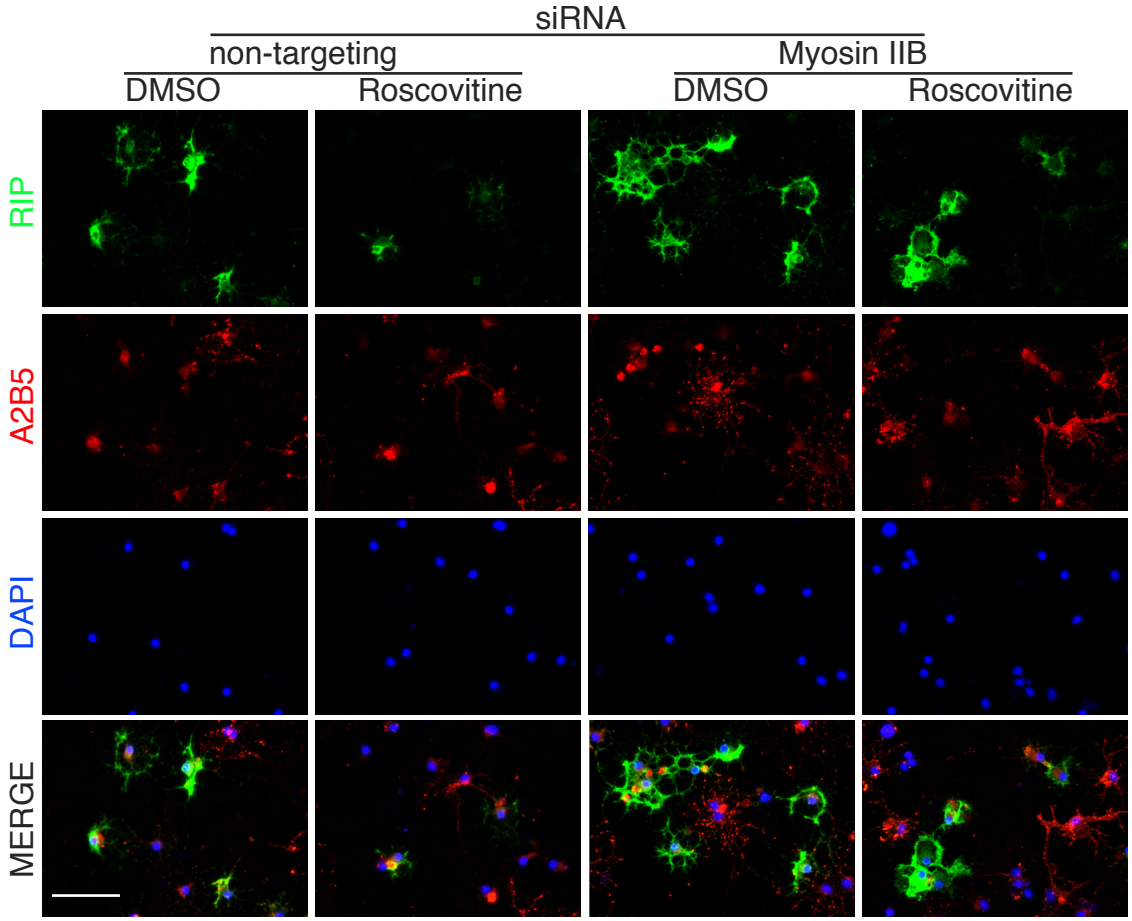


Figure III 5. Quantitation of morphology in NMIIB knockdown treated with roscovitine.

A. Images from experiments represented in Figure 4 were evaluated based on a breakdown of morphology represented in Figure 6. A decrease in Type IV OL was observed when siCtrl transfected cells were treated with roscovitine 15μM ($^{****}p<0.0001$, t-test). There was a reduction in less mature Type I OL in siNMIIB transfected cells treated with roscovitine($^{***}p<0.0003$, t-test), as well as an increase in more mature type III OL when compared with siCtrl transfected cells treated with roscovitine ($^{***}p<0.0005$, t-test).

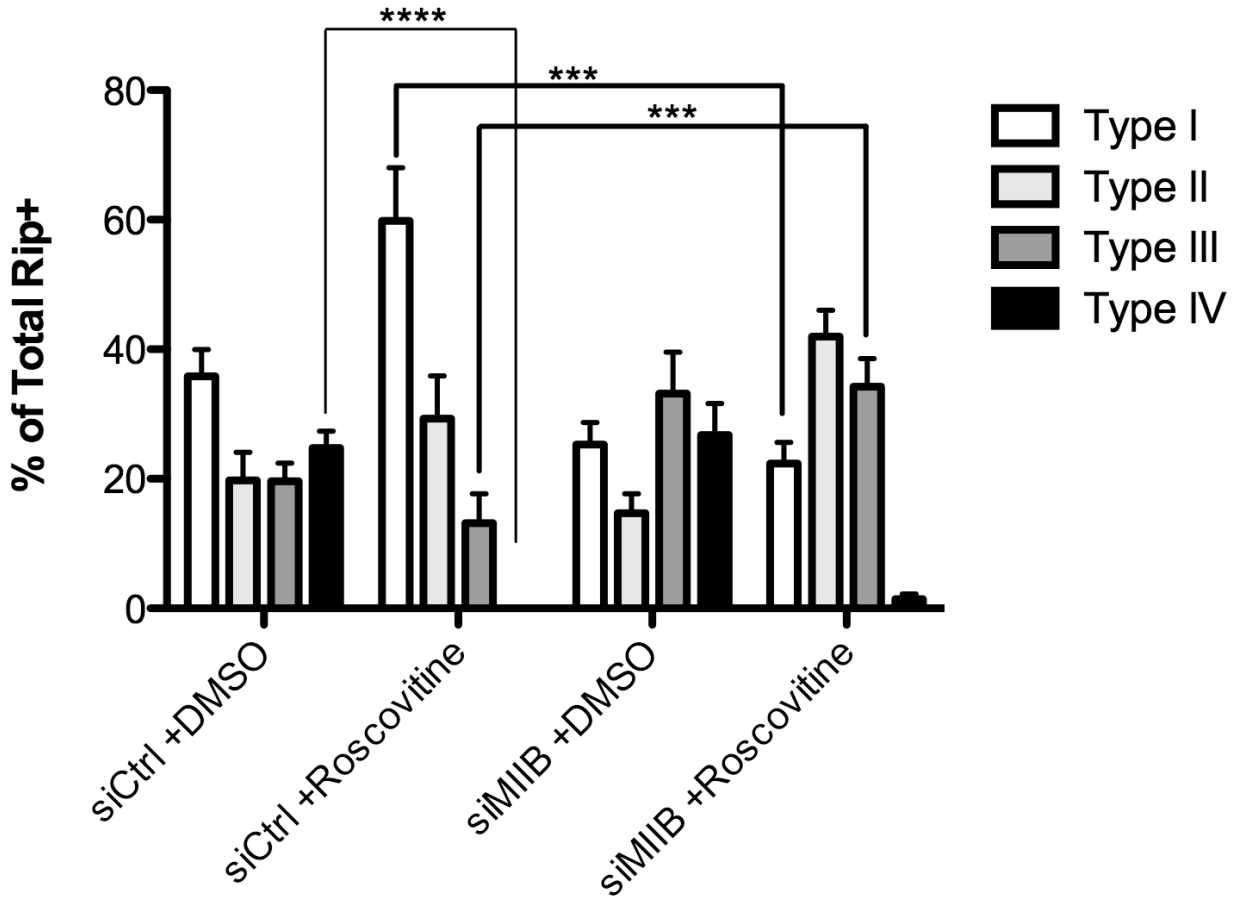


Figure III 6. Classification of OL morphology types.

OL stained with RIP antigen can be grouped into several classes, as seen below from I to IV. The least mature class is characterized by light RIP staining of several small processes. In class II the RIP staining is intensified and complexity increases. Class III is characterized by the beginning of dissolution of the complex process network into flat lamella sheets. Finally class IV encompasses OL that have fully developed lamellar structures, which can be stained MBP positive and are believed to be *in vitro* equivalent of myelin sheaths.

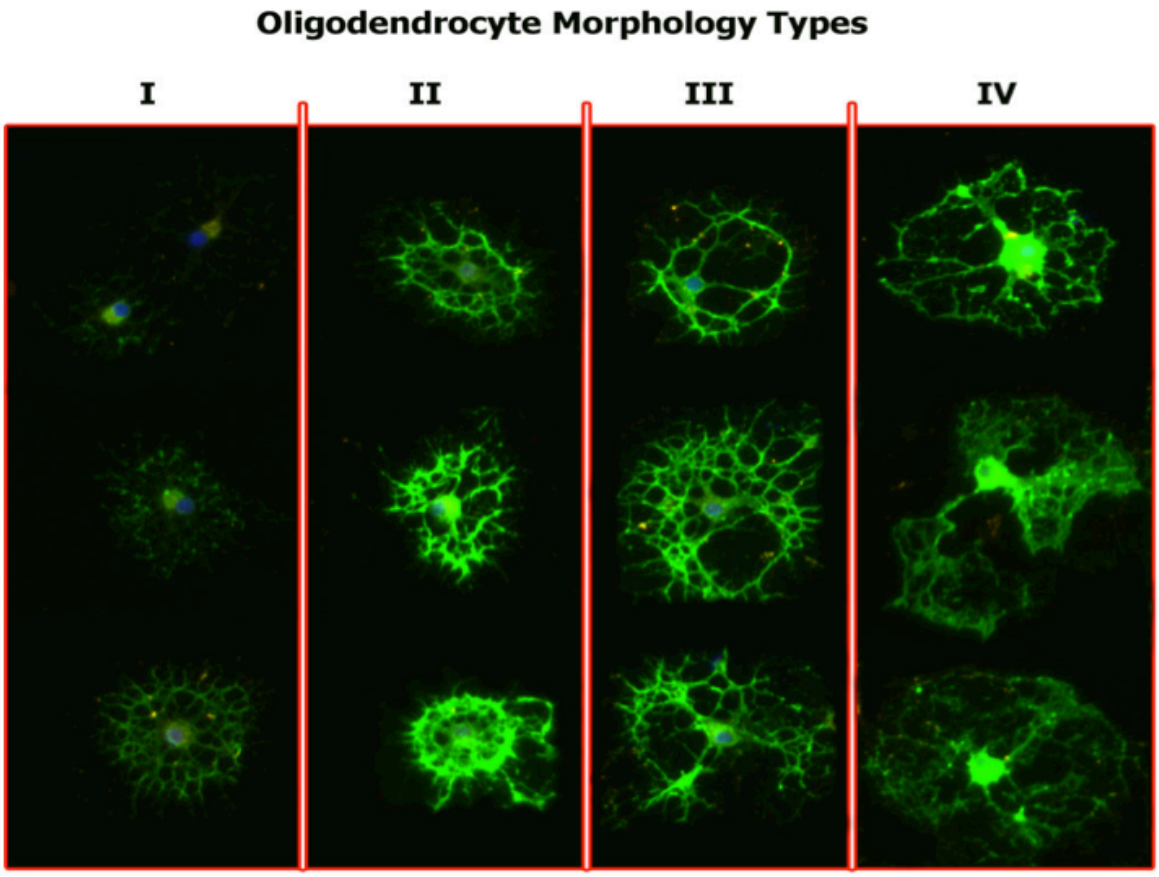


Figure III 7. Quantitation of OL differentiation in NMIIB knockdown treated with roscovitine.

A Data collected using 20x images that were counted for RIP positive immunostaining indicate that knockdown of NMIIB partially rescues the decrease in the numbers of Rip+ cells in cultures treated with roscovitine (** p value = 0.0022). **B** A2B5 positive staining shows that roscovitine does not affect the numbers of immature OPCs. **C**. Counts of total DAPI per field are shown to indicate that the density of cultures was equivalent across all conditions. **D.** The knockdown of NMIIB evaluated by western blot shows a decrease in protein levels at the time of roscovitine treatment.

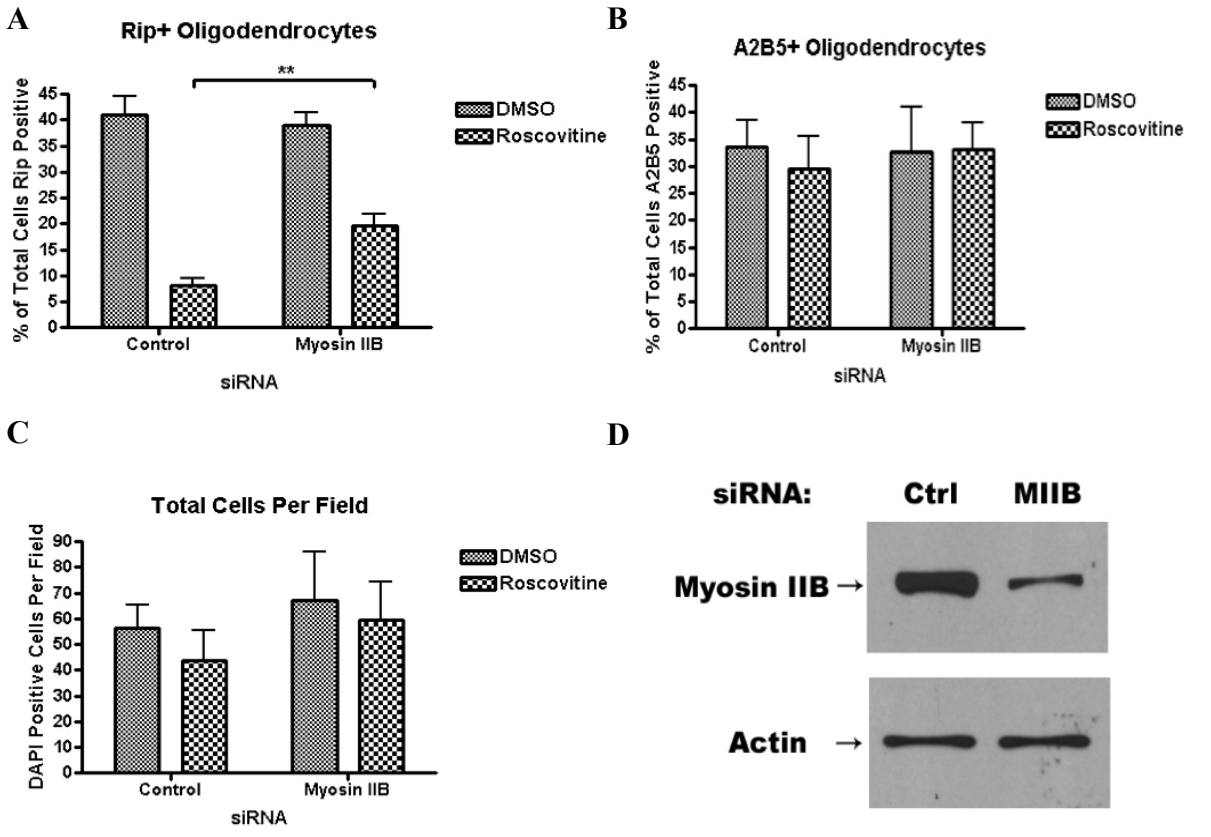
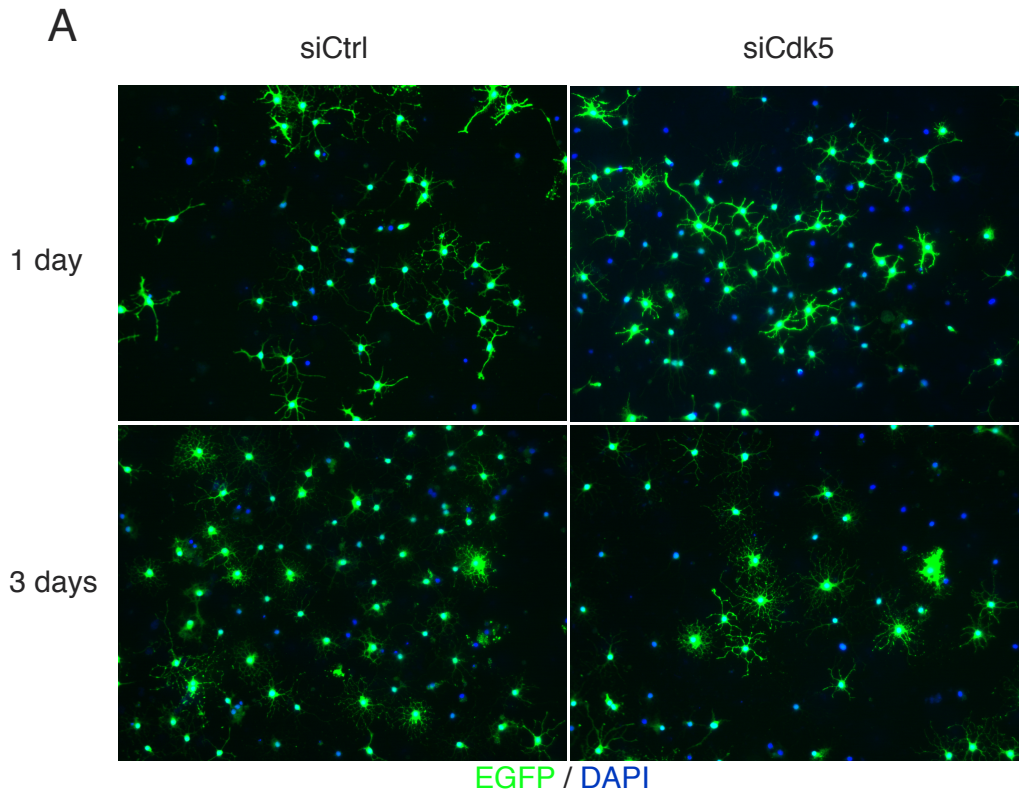


Figure III 8. Specific knockdown of Cdk5 does not cause changes in OL morphology.

A. OL were transfected with scrambled siRNA control (siCtrl) or one targeting Cdk5 (siCdk5). Cells were co-transfected with an EGFP expressing plasmid to visualize transfected cells and plated on PDL coated coverslips with media containing T3 to stimulate differentiation and morphological changes. **B.** Western blot of OL plated on 6 cm dishes after transfection with siCtrl and siCdk5. Cdk5 band shows at 35kd. Actin is shown as a loading control. While the WB shows effective knockdown of Cdk5 there is no observed reduction in morphological complexity in siCdk5 transfected OL compared to those transfected with non-targeting siRNA.



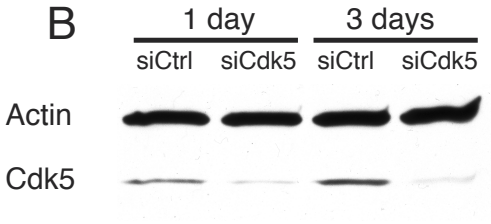
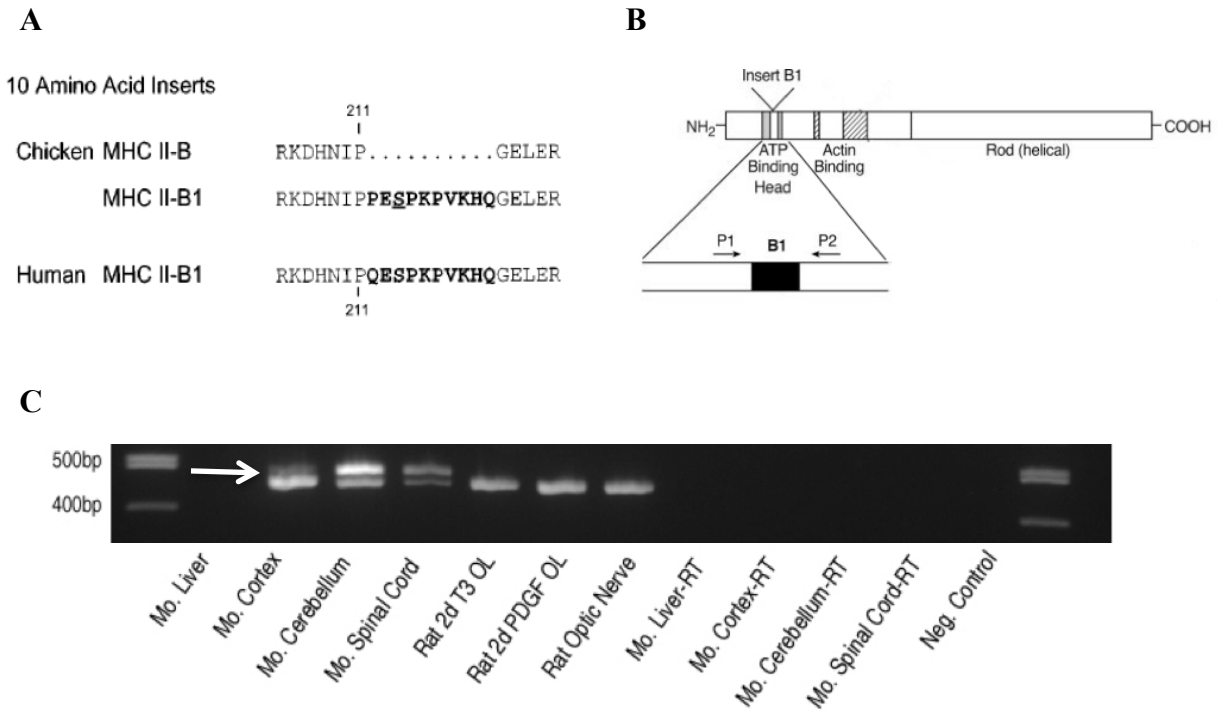


Figure III 9. OL do not express B1 NMII isoform that would permit direct interaction with Cdk5.

A. Diagram representing the alternatively spliced isoform NMIIB1 containing the Cdk5 phosphorylation motif (taken from Pato *et al*,.1996). **B.** Diagram used to illustrate location of primers designed for RT-PCR analysis. (adapted from Ma *et al*,. 2006). **C.** Agarose gel electrophoresis of PCR products from a reaction designed to amplify the region flanking the location of the insert indicated in B, using cDNA isolated from mouse tissues as well as from primary rat OPCs. The white arrow indicates the presence of the 30bp insert band in mouse cortex and cerebellum as well as spinal cord. However rat OL in proliferating (PDGF) or differentiating (T3) conditions, as well as the rat optic nerve show only the lower band indicating that the 30 bp insert is not present in their respective cDNAs.



Chapter 4: Discussion

Downregulation of NMIIB as a strategy to promote remyelination

The present study significantly extends our previous findings that downregulation of NMIIB in OL potentiates differentiation and myelin formation (Wang *et al.* 2008, Wang *et al.* 2012) by demonstrating their relevance to myelin repair *in vivo*. Here we have carried out studies in the context of a toxic insult to myelin, namely lysolecithin-induced demyelination, in the adult mouse corpus callosum. This model permits detailed experimental analysis of the timing and extent of remyelination, a question central to multiple sclerosis (Mallon *et al.* 2002). By specifically targeting OPC and OL using the inducible Cre/lox system driven by the *Plp* promoter, we have been able to demonstrate that mice lacking NMIIB before receiving a demyelinating lesion, which initially exhibits the same size and a similar inflammatory/reactive cell response as those of controls, show accelerated remyelination. This result is consistent with our hypothesis that NMIIB acts as negative regulator of OL development (Wang *et al.* 2008, Wang *et al.* 2012), and that downregulation of cytoskeletal contractility promotes OL morphological differentiation, myelin formation and repair.

Previous studies have established the participation of SVZ-derived progenitors (Murtie *et al.* 2007), as well as of NG2+ progenitors (Frohman *et al.* 2006), to CNS remyelination after lysolecithin. Despite the presence of large pools of heterogeneous progenitors (Bruce *et al.* 2010) and the existence of recent studies demonstrating the expression of Plp in a small subpopulation of NG2+ cells in adult brain (Kimura *et al.* 1996), we observed a significant increase in remyelination by a relatively low number of NG2-, Plp+ OPC and OL lacking NMIIB, meaning these cells are contributing more than they would normally. Thus, by employing the

Rosa26(mT/mG) reporter we have demonstrated the direct involvement of adult Plp-expressing OPC and OL to remyelination. The contribution of this cell population to myelin repair, which are most likely recruited from local sites of myelin injury, has long been debated [reviewed in (Hou *et al.* 2012)]. Some studies have shown limited remyelinating capacity of post-mitotic adult OL (Crang *et al.* 1998), and while we observe similar results in control mice, the mice lacking NMIIB in Plp+ OL display an increase in remyelinating capacity.

Since MS targets white matter throughout the CNS (Mallon *et al.* 2010), affecting regions such as the optic nerve and spinal cord (which are not readily accessible to SVZ-derived progenitors) these disease foci might benefit from strategies that promote remyelination by resident OL. Of note, studies have shown that inhibition of ROCK, a known regulator of NMII activity (Huang *et al.* 2011), ameliorates disease progression in mice with EAE (Yu *et al.* 2010, Kippert *et al.* 2007, Zhang *et al.* 2009). While the conclusions of these studies implicate modulation of both the inflammatory response and blood-brain barrier permeability as the main cause underlying these beneficial effects, it is not implausible that enhanced OL-mediated remyelination could also account for improved clinical recovery.

In support of this interpretation, we and others have found that inhibition of the Rho-ROCK pathway in OL promotes branching, plasma membrane condensation and differentiation (Wang *et al.* 2012, Hilderbrand and Hahn 1978), effects that might translate into enhanced myelinogenic potential of individual cells (Gensert and Goldman 2001, Chong and Chan 2010). Indeed there are both cell intrinsic as well as extrinsic factors regulating the maintenance of unmyelinating cells in the adult brain, such as timer mechanisms and inhibitory cues from

myelin itself, thus by knocking out NMIIB we could be causing a shift in a population of progenitor cells towards remyelination.

Since failure of remyelination may result in axonal atrophy and neurodegeneration, changes that are largely responsible for the progressive functional decline in patients with chronic demyelination, the promotion of endogenous repair by the acceleration of remyelination by resident OPC and OL, might offer the potential to prevent long-lasting damage and clinical disability in MS.

Regulation of NMII activity in oligodendrocytes

NMII is a downstream target of RhoA through ROCK, which phosphorylates the regulatory protein MLC and activates NMII (Somlyo and Somlyo 2000). In agreement with this model we found that overexpression of CA RhoA in OPCs significantly increased the levels of phosphorylated MLC compared to control cells. This increase in activity was dependent on ROCK, as treatment of OPC overexpressing CA RhoA with the specific inhibitor Y-27632 (Hirose et al. 1998), restored pMLC levels to that of controls. It has also been shown that p190 RhoGAP, a GTPase activating factor, is a substrate of Fyn and that its activity increases during the differentiation of oligodendrocytes (Wolf et al. 2001). We found that inhibition of Fyn prevents MLC dephosphorylation observed during OPC development (Wang et al. 2008). Thus, OPC maintained in proliferating conditions (+PDGF) have higher levels of pMLC compared to mature oligodendrocytes (+T3). Treatment with Fyn inhibitor PP2 resulted in increased MLC phosphorylation in T3-treated cultures, to levels that were comparable to those seen in proliferating conditions. Similarly, knockdown of Fyn using siRNA also resulted in increased levels of pMLC in OPC maintained in either PDGF or T3. Taken together these results indicate

that inactivation of NMII downstream of Fyn/RhoA/ROCK signaling promotes oligodendrocyte process extension via downregulation of MLC phosphorylation. Interestingly, although high levels of pMLC resulting from Fyn inactivation or downregulation prevent process extension, the expression of differentiation markers such as Rip (data not shown) or MBP (Figure III 1B) was not affected by these treatments. These results are in agreement with previous data (Osterhout et al. 1999), showing that OL morphological and molecular differentiation can be uncoupled *in vitro*.

In an effort to establish if other known pathways controlling cytoskeletal rearrangement also regulate NMII activity in OL, we investigated the role of Cdk5 in OL morphological differentiation. We confirmed that roscovitine, an inhibitor of Cdk5 had a negative impact on OL morphological complexity and differentiation. Furthermore we were able to show that this effect was prevented by the knockdown of NMIIB, indicating that the target of roscovitine, presumably Cdk5 and NMIIB were acting in the same pathway. To validate the specificity of these results, which implicated Cdk5 as the target of roscovitine (Miyamoto *et al*, 2007), we repeated the experiment using siRNA against Cdk5. We could not replicate the effects of roscovitine on OL morphology with knockdown of Cdk5 using siRNA, while Miyamoto et al., used shRNA viral transfection and analyzed the endpoint of *in vitro* differentiation, namely MBP+ OL. This type of analysis is not feasible with the shorter lasting siRNA knockdown but we expected the results of the specific knockdown to be similar to that of the inhibitor. Furthermore we failed to detect the NMIIB splice variant (NMIIB1), which has a consensus Cdk5 phosphorylation motif. This suggests that roscovitine might be acting on some other known targets of the drug, namely Cdc2 or Cdk2 in OL to alter their morphological complexity and differentiation. Indeed looking at the

literature, Cdk2 knockout impairs OL remyelination after focal demyelination in an adult mouse (Caillava *et al.,* 2011). Collectively, our data suggests that NMII might be a common effector of cytoskeletal remodeling of multiple signaling pathways implicated in OL morphological differentiation. In addition, while the Fyn-ROCK-MII pathway has a clear impact on OL branching, its effect on differentiation is not clear, further confirming the importance of investigating other pathways regulating NMII activity in glial cells. Of note, our lab has found that MLCK, another regulator of NMII activity is found predominantly in the nucleus of Schwann cells. This was a surprise finding for a protein mainly involved in cytoskeletal regulation. Furthermore, knockdown of MLCK in Schwann cells leads to upregulation of several pro-myelinating genes, while at the same time causing a failure to myelinate (Leitman *et al.* 2011). In OL, during optic nerve development, MLCK staining coincides with MBP expression and is localized primarily in the nucleus (M. Urbanski, E. Leitman unpublished data). This leads us to speculate that MLCK/NMII might participate in gene regulation, aside from their function as cytoskeletal regulating proteins. Further work remains to distinguish which pathways feed into NMII regulation through MLCK and ROCK, as well as their contribution to differentiation of OL versus branching.

Significance and future directions:

Potentiation of remyelination

The ability to potentiate a new subset of cells to aid in myelin regeneration in the context of demyelinating diseases such as MS is an exciting possibility. It is well known that MS etiology includes the depletion of OPC as continuous lesion progression and relapse takes a toll

on not just the remyelinating cells but also the neurons that become atrophied and eventually lead to the debilitating outcomes of the disease. Another useful note here is that other studies have shown that inhibitors of ROCK, a known regulator of NMII activity, ameliorated disease progression in mice with EAE (Huang *et al.* 2011). It is important to note that those studies showed that inhibition of inflammation played a key role in the therapeutic effect of the ROCK inhibitor. We are not discounting this conclusion, rather we add that mechanistically it is advantageous for remyelination to also inhibit the activity of NMII in OL, and that this therapeutic strategy might have a synergistic effect in a disease that benefits from a repression in the damaging insult, namely inflammation and the attack of the immune system on myelin, as well as an enhancement of remyelination in regions that did get damaged. It is important to note that other lines of evidence support our hypothesis that NMII activity is inhibitory to OL development. It has been found for example that cell crawling from soft to stiff extracellular matrix increases myosin phosphorylation (Raab *et al.* 2012). Unpublished work from our laboratory by Mateusz Urbanski also implicates extracellular matrix stiffness and NMII activity in OL development. In his experiments, OL plated on soft versus stiff substrates display an increase in complex morphology, an increase in MBP expression and a reduced level of MLC phosphorylation that is dependent on ROCK. While there have not been any studies directly measuring the stiffness of a demyelinated lesion, one can assume that the formation of the glial scar would present the OL with a stiffer and less permissive environment than the one experienced in normal developmental myelination. This to an extent has been shown to be true in EAE where OPCs are observed alongside hypertrophic astrocytes at the borders of chronic demyelinated lesions but not within them (Bannerman *et al,* 2007). In agreement with the

hypothesis that changes in OL environment have an impact on NMII, we have shown in preliminary immunochemical studies of lesion tissue isolated from the corpus callosum that the levels of NMIIB raise in a lysolecithin induced lesion compared to normal uninjured tissue. This could partly explain why cKO of NMIIB favors OL development and myelination in an MS like-environment.

There are two interesting ways to follow up the results of our experiments. One is to challenge the NMIIB cKO animals with EAE, a model of MS, which is different mechanistically from the focal lysolecithin model used in our studies. As opposed to lysolecithin where the toxin dissolves myelin, EAE is akin to actual MS where immune cells are sensitized to myelin antigen and cause systemic lesions, which have debilitating effects on the animals and can be clearly analyzed and graded for severity. This model would lend itself to determining if the acceleration of lesion repair by NMIIB ablation has the possibility to ameliorate disease progression in MS. EAE would give us an idea of the impact that NMIIB cKO has on actual neuronal injury which results from progressive relapsing forms of MS and is modeled in the mouse.

There is also the question of what effect would ablation of NMIIB in earlier OL progenitors, such as A2B5 or NG2 positive, would have on remyelination in the lysolecithin model or EAE. From our experiments with lysolecithin in mice harboring GFP+ PLP+ OL we can conclude that the majority of remyelination is accomplished by PLP- OL progenitors. Initially the role of NMII in directed fibroblast migration made us hesitant to target early progenitors (Lo *et al.* 2004), as we feared the possibility that OPCs would not be able to properly target their migration to the site of injury, thus inhibiting remyelination as opposed to enhancing it. However, we see more NMIIB cKO OL inside lesions, and in the context of NG2+ OL

precursors inhabiting almost every region of the brain, it would preclude migration from being a limiting factor in localized lesion repair. Thus, it could be that by knocking out NMII at an earlier stage such as the NG2+ progenitor, we could further potentiate remyelination after injury.

Of importance to the proper interpretation of the data generated in our remyelination experiements, is a thorough analysis of the Cre-mediated tamoxifen-induced recombination efficiency in cells carrying multiple flox targeted alleles. One way to do this will be to cross the PLP-Cre line with two different reporters, for example: one expressing GFP after Rosa recombination and RFP after NMIIB alleles recombination. This approach would permit quantitation of double or single positive recombination events after tamoxifen induction.

Signaling pathways affecting NMII protein levels

While we established a model for the downregulation of NMII activity during OL differentiation via the Fyn-Rho-ROCK-pMLC pathway, there remains the question as to the cause of the observed decline in NMIIB levels as part of OL progressing from OPC to immature OL to mature myelinating OL. The downregulation of NMII at the protein level during OL differentiation has been demonstrated in our lab *in vitro* using western blotting techniques (Wang *et al.* 2008) as well as by others using gene expression analysis, where levels of mRNA for *Myh10,* the gene encoding the NMII heavy chain are seen to decline significantly as OL mature (Cahoy *et al.* 2008). Clearly NMII activity and protein levels are relevant to OL differentiation, and while we have shed some light on regulation of the former, how exactly the latter of the two becomes downregulated remains to be determined. A look at recent literature revealed the discovery of mutations associated with the familial platelet disorder, thrombocytopenia, in the transcription factor RUNX1 (Jalagadugula *et al.* 2010). The authors found decreased

phosphorylated MLC as well as a reduction in the transcript levels of its gene, MYL9 in a patient with the RUNX1 mutation. Furthermore the authors confirmed their hypothesis that MYL9 is a transcriptional target of RUNX1. Given the resource of the large transcriptome database for OL (Cahoy *et al.* 2008), it can be seen that RUNX1T1, a chromosomally translocated version of RUNX1 is enriched 24.5 times in OPCs versus myelinating OL. To strengthen this argument, recent work on chromatin remodeling in OL by the laboratory of Richard Lu, shows that the transcription factor Olig2, essential for OL linage specification, is associated with permissive chromatin near the RUNX1 gene in OPCs but not myelinating OL (Yu *et al.* 2013). Thus, it is possible that RUNX1T1 acts in OPCs but not mature OL to cause transcription of MLC contributing to NMII protein levels. This still leaves the question of MYH10 levels, the gene for the heavy chain portion of NMIIB. This however can be addressed by looking again at chromatin remodeling as MYH10 in Richard Lu's work is found to be tri-methylated at H3K9, a mark of transcriptional repression. In conclusion, RUNX1 is an interesting target for further investigation into the pathways controlling NMII levels in OPCs.

Studying the role of myosin IIB in OL development in vivo

Given the usefulness of the tamoxifen inducible, OL specific knockout model, it is possible to study the role that NMIIB plays in the development of OL *in vivo.* Mice bred to generate both *PlpCre/ESR1*:*NMIIB* $^{fl/fl}$ (cKO) and NonCre:*NMIIB* $^{fl/fl}$ (Control) offspring are injected with tamoxifen while lactating, thus supplying the pups with the drug at the time of birth. Meaning we can study what effect knocking out NMIIB will have on myelination and/or proliferation of OL. We have performed some preliminary studies using this approach and found

changes in the morphology and organization of myelinated tracks in the cerebellum of cKO animals compared to that of controls at 14 days old (Figure IV 1).

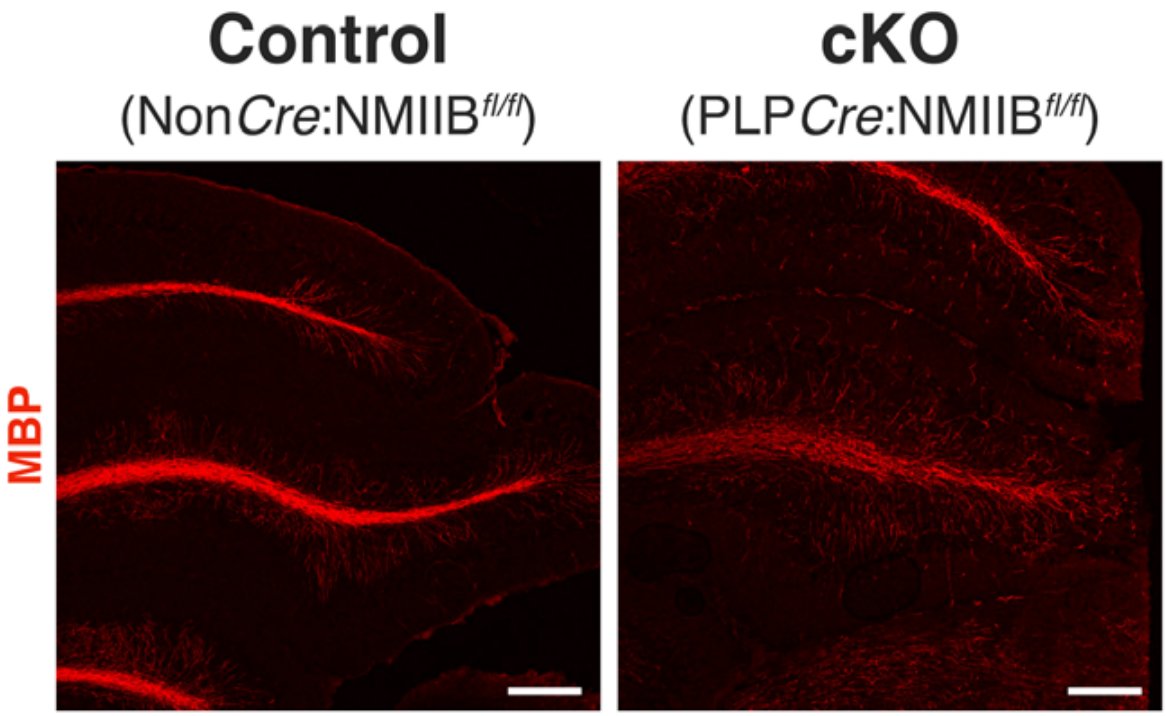
Since we have already bred mice that not only contain the inducible and OL specific transgene but also the Rosa26 mTomato/mGFP reporter gene [*PlpCre/ESR1*:Rosa26-mT/mG:*NMIIB* $^{fl/fl}$ (mT/mG; cKO) and *PlpCre/ESR1*:Rosa26-mT/mG:*NMIIB* $^{+/+}$ (mT/mG; Control)], it will be possible to repeat the aforementioned developmental experiments and know exactly which OL are deficient of NMIIB, as they will also be GFP positive. This eliminates the problems associated with studying morphology in chimerized cells, as recombination does not occur in every OL.

Concluding remarks

More than just giving shape to cells, we are learning that nearly all cellular processes depend in some way on the function of the cytoskeleton. The oligodendrocyte is defined by its stunning feats of cellular contortionism and the cytoskeleton was an obvious place to look when attempting to understand its biology. Our work focusing on non-muscle myosin II has identified it as a novel regulator of oligodendrocyte morphological differentiation that is important both in development of myelin as well as its repair.

Figure IV 1. Conditional ablation of NMIIB in early postnatal development affects the organization of myelinated tracks in the cerebellum.

Sagittal frozen sections from 14 d old cKO and control littermate brains stained with antibodies to MBP. Cre-mediated recombination was induced in mice during the first postnatal week. Myelinated tracts in the granular layer of cKO mice appear thicker and more defasciculated compared to control. Scale bars = 100μm.



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