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The role of β1 integrin in oligodendrocyte development and CNS myelination

A Dissertation Presented

by

Tom Thanh Van Nguyen

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Abstract of the Dissertation

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Myelin is essential for proper human neurophysiology, but the precise molecular mechanisms underlying the myelination process remain poorly understood. One potential effector molecule, the β1 integrin, has been suggested to play a critical role in CNS myelination. In support of this, α6β1 integrin has been shown to modulate oligodendrocyte development *in vitro* and the loss of an integrin ligand, laminin, causes myelin defects *in vivo*. Here, we use transgenic mice (*β1-CNSko*) engineered to excise the β1 integrin gene at the neural progenitor stage to study how β1 integrin modulates oligodendrocyte

development and myelination. Electron micrographs of the spinal cord and corpus callosum from the *β1-CNSko* mouse revealed hypomyelination compared to wildtype littermate controls. Biochemical and immunohistochemical analyses of cerebral cortices showed less MBP in the *β1-CNSko* compared to wildtype littermate controls. Oligodendrocytes derived from mutant mice are unable to efficiently extend myelin sheets and do not fully activate AKT, a kinase that regulates axonal ensheathment. The inhibition of PTEN, a negative regulator of AKT, or the expression of a constitutively active form of AKT, restores myelin sheet outgrowth in cultured β1-deficient oligodendrocytes. Our data suggest that β1 integrins play an instructive role in CNS myelination by promoting myelin wrapping in a process that depends on AKT. In addition, β1 integrins were found to regulate oligodendrocyte process dynamics, such that Sholl analysis of process complexity in *β1-CNSko* mutants showed a reduction in process outgrowth and branching. Also, *in vitro* differentiation studies indicated no differences in β1-KO oligodendrocyte lineage progression, survival, and but the newly-formed β1-deficient oligodendrocyte population had significantly more cell death compared to wildtype controls. Our findings reveal a role for β1 integrin in oligodendrocyte development and CNS myelination, and suggest that myelin abnormalities in laminin-deficiencies may be in part due to loss of β1 integrin signaling.

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Chapter I: General introduction

Oligodendrocytes, the myelinating cells of the CNS

Myelination is essential to the normal physiology and function of the central nervous system (CNS). As an insulating layer that surrounds the axons of the central nervous system, myelin provides trophic support and allows the efficient conduction of electrophysiological signals through saltatory conduction (Baumann and Pham-Dinh, 2001). The importance of myelin becomes particularly evident in some pathological conditions. For example in multiple sclerosis (MS), the progressive loss of myelin is believed to contribute to axonal atrophy and dysfunction (Dutta and Trapp, 2010; Stadelmann et al., 2008).

CNS myelin is formed by a population of specialized glial cells called oligodendrocytes, whereas PNS myelination is carried out by Schwann cells (Baumann and Pham-Dinh, 2001). Oligodendrocytes arise from restricted zones in the developing telencephalon and spinal cord (Kessaris et al., 2005). Under the influence of several transcription factors (e.g., the Olig1/2 and Sox family of proteins) and morphogens (e.g., Sonic hedgehog and BMPs) (Li et al., 2007;

Ligon et al., 2006; Menn et al., 2006; Wegner, 2007), neural precursors specialize into oligodendrocyte precursor cells (OPCs) and migrate toward regions in the CNS. These proliferating OPCs express stage-specific proteins, such as PDGFαR and proteoglycan NG2 (figure I-1), and remain responsive to mitogens (e.g., PDGF) during migration (Raff et al., 1988; Richardson et al., 1988). Upon reaching their target, OPCs begin to differentiate into pre-myelinating oligodendrocytes and extend multiple processes to make contact with up to 50 axon segments (Baumann and Pham-Dinh, 2001). The establishment of this glial-axon interaction is a critical point in oligodendrocyte differentiation and mediates target-dependent oligodendrocyte survival (Barres et al., 1992; Fernandez et al., 2000). Ultimately, myelinating oligodendrocytes begin membrane expansion and synthesis of myelin components such as myelin basic protein (MBP), and will ensheath surrounding axons with myelin (Akiyama et al., 2002; Simons and Trotter, 2007).

Multiple steps in oligodendroglial development have been shown to involve complex glial-matrix and glial-axon interactions. For instance, cell culture studies have shown that oligodendrocyte differentiation and membrane expansion are enhanced by the extracellular matrix protein laminin (Buttery and ffrench-Constant, 1999). In several genetic models, including invertebrates, glial cell function is influenced by neuronal contact. For example, in the *C. elegans* nervous system, although myelin is not formed, the glial ensheathment of neurons

regulates synapse formation and function (Shaham, 2006). In the drosophila CNS, the development of midline glia is tightly regulated by cell-cell contact with adjacent neurons (Crews, 2010). Also, in a study using rat-mouse cultures, mouse oligodendrocytes have enhanced proliferation and survival in the presence of rat dorsal root ganglia compared to when cultured alone (Shaw et al., 1996). However, the factors and mechanisms that regulate axon-glial interactions during mammalian oligodendrocyte development and myelination *in vivo* remain largely uncharacterized. One group of molecules, however, that has been implicated in axon-glial interactions is the integrin family of receptors.

The integrin family

Integrins are transmembrane receptors that bind to a variety of extracellular matrix molecule (ECM) ligands, including laminins, collagens, and fibronectin (Hynes, 2002). Integrins exist in a heterodimeric form and are composed of one alpha (α) and one beta (β) subunit, the differential combination of which confers ligand selectivity to each type of integrin receptor (figure I-2) (Hynes, 2002). The largest and most diverse subclass of integrins is the β1 integrin family encompassing 12 of the 24 different integrins. In the absence of a stimulus, integrins exist in a closed, inactive conformation, with ligand-binding

domains lying in proximity to the membrane. Upon ligand binding, integrins undergo a conformational change to an open, active state where the integrin receptor extends its ligand-binding domain (Calderwood, 2004). This open state also causes the receptor's transmembrane and cytoplasmic domains to separate and become available to bind intracellular molecules that can activate downstream pathways (Calderwood, 2004; Hynes, 2002). In addition to the ability to potentiate extracellular signals (i.e., outside-in signaling), integrins are also relatively unique in their ability to relay intracellular signals that affect the extracellular environment (i.e., inside-out signaling). This is driven, in part, by an intracellular protein called talin. Talin can bind to the inactive closed confirmation of integrins and contains at least two integrin binding sites that include a FERM domain (Gingras et al., 2007; Moser et al., 2009; Ziegler et al., 2008). Binding to integrin by talin via the FERM domain increases integrin receptor affinity for extracellular ligands. Ablation of talin 1 and 2 in mice leads to defects in myoblast fusion, similar to defects in muscle lacking β1 integrins (Calderwood et al., 2001; Calderwood et al., 2002; Conti et al., 2009). Through these properties, integrins are able to regulate cell adhesion, basement membrane assembly, cytoskeletal dynamics, and intracellular signaling cascades. In light of these diverse functions, understanding how β1 integrins regulate oligodendrocyte biology could provide more specific insights on the role of adhesion receptors in CNS myelination.

Integrins, the actin cytoskeleton, and cell adhesion

One of the best known functions of integrins is the ability to influence the organization of the actin cytoskeleton and thus affect cell morphology, differentiation, and migration (Abelson and Simon, 2007; Milner and Campbell, 2002). However, integrins do not bind to actin directly and instead regulate actin dynamics by recruitment of adaptor proteins that form the integrin-associated protein complex. This involves essential proteins such as focal adhesion kinase (FAK), integrin-linked kinase (ILK), and talin (Abelson and Simon, 2007). FAK has been implicated in key cellular processes, such as focal adhesion turnover and cell migration, and has been shown to function as a structural scaffold for other signaling molecules. Activation of integrins allows the recruitment of FAK (Schlaepfer and Hunter, 1998) and the phosphorylation of FAK at its autocatalytic site, Y397, which recruits Src family kinases (SFKs) that in turn further phosphorylate FAK at Y576 (Fox et al., 2004; Schlaepfer and Hunter, 1998). This enhancement of FAK phosphorylation allows FAK to have dual roles as a scaffold protein and as a kinase. For instance, FAK can recruit the adaptor molecule growth factor receptor-bound protein 2 (Grb2), which leads to enhanced

signaling via the MAPK pathway that is involved in cell differentiation ((Mitra et al., 2005; Schlaepfer and Hunter, 1998).

In addition, FAK can phosphorylate paxillin to modulate the actin cytoskeleton for cell adhesion and morphology (Mitra et al., 2005). The analysis of live cells lacking FAK, the SFKs Src, Yes and Fyn, p130Cas or paxillin, showed that focal complexes assembled normally, but were distinctly slower in their rate of disassembly (Jovic et al., 2007), consistent with observations that removing these proteins results in focal adhesions larger than normal. Similarly, ILK has been suggested to bind directly to the cytoplasmic tail of integrins to regulate cell adhesion and migration (Hannigan et al., 2005; Lock et al., 2008). The role for integrins in down-regulating actin structures was revealed in mice where ILK was knocked out and cells of the early mouse embryo lacking ILK contained abnormally elevated levels of actin at the integrin junctions (Nakrieko et al., 2008).

Other actin regulatory proteins, small Rho GTPases, rho, rac1 and cdc42, have been shown to be downstream effectors of activated integrins that act as positive regulators of actin cytoskeleton remodeling (Burridge and Wennerberg, 2004; Guan, 2004). In addition to organizing preformed actin filaments, integrins can trigger new synthesis of actin filaments, as suggested by the recruitment of

actin nucleating proteins, such as the Arp2/3 complex, to sites of integrin adhesion (Soderling, 2009).

Integrin signaling and cell phenotype

The cellular phenotype can be influenced by the extracellular environment, and cell surface receptors such as integrins are in prime position to relay extracellular cues and activate intracellular signaling cascades (Guilak et al., 2009; Wiesner et al., 2006). Integrins affect cell phenotype in several ways including ligand-binding activation and cooperation with growth factors and their receptors.

Integrins have also been directly implicated in promoting cell survival. Integrin-ECM ligation leads to activation of the PI3K/AKT pathway, inhibiting pro-apoptotic molecules such as Bcl-xL-antagonist causing cell death (BAD) (Paez and Sellers, 2003). In cultured neurospheres integrins have been shown to control cell survival, proliferation, and differentiation (Leone et al., 2005). Moreover, Blaess et al. (2004) showed that laminin-integrin interactions enhance the proliferative response of cerebellar granule cells to the morphogen, sonic hedgehog (SHH) (Blaess et al., 2004; Mills et al., 2006). Studies have shown that

the failure of certain integrins to bind their ECM ligands can induce cell death (Kim et al., 2002; Stupack and Cheresh, 2002). Although adhesion-mediated cell survival has been observed *in vitro*, the molecular mechanism by which this occurs *in vivo* is likely more complex and remains poorly characterized.

In addition to promoting cell survival, integrins cooperate with growth factors in regulating the cell cycle by transmitting signals via the Ras/Raf/Mek/Erk signaling pathway. Erk activation leads to increased in cyclin D1 transcription, and this depends on integrin-mediated Rac activation for cellular proliferation (Bill et al., 2004; Ung et al., 2008). In human capillary endothelial cells, integrins are important for promoting cell cycle exit through regulation of the cyclin D kinase inhibitor p27, allowing the activation of cyclin E–cdk2 complexes (Bill et al., 2004; Collins et al., 2005).

β1 integrins in neural development

Integrins and their ligands are widely expressed in the nervous system, and have been shown to have diverse roles in neural development (Milner and Campbell, 2002). Integrins have a number of ways to modulate brain development. They have been shown to control survival and proliferation of some

populations of neural progenitor cells (Milner and Campbell, 2002). For example, the presence of β1 integrins is required for the normal morphology and function of radial glia; this property may underlie the neuronal lamination deficits observed in mutant mice of genes encoding basal lamina constituents and their receptors, including integrins (Anton et al., 1999; Graus-Porta et al., 2001).

Effects of the loss of β1 integrin function on various populations of neural cells have been documented both *in vitro* and *in vivo* (Hagg, 2005; Prestoz, 2001). In cultured neurospheres, β1 integrins were shown to affect neural cell development, such that genetic deletion of the β1 subunit resulted in reduced migration, decreased proliferation, and increased neuronal progenitor death (Gu et al., 2009). In the developing cerebral cortex, the transient administration of a β1 integrin blocking antibody to the embryonic ventricular zone disrupted the structural integrity of this neurogenic niche, causing defects in neural stem cell adhesion and cortical layer formation. These defects were also observed in embryonic brains in which the expression of an α6β1 integrin ligand, laminin α2, had been prevented (Loulier et al., 2009). In mice where β1 integrin expression was genetically ablated in all cells of neural origin through a Cre-lox strategy (using the *Nestin* promoter as the Cre-driver), the cerebral cortices formed an abnormal basement membrane, resulting in radial glial endfeet detachment, neuronal migration defects, and cobblestone lissencephaly. Cerebellar defects also emerged during early postnatal development in this β1 integrin-knockout mouse,

where cerebellar hypoplasia became evident by P7 (Graus-Porta et al., 2001). Collectively, these findings indicate that β1 integrins mediate diverse functions in the cell populations of the developing brain.

β1 integrins, oligodendrocytes and myelination

Myelination is essential for proper neurological communication in mammals. In the CNS, oligodendrocytes (OLGs) are the myelinating glial cells, with the primary function of ensheathing axons. The myelin sheath provides the axon with a discontinuous insulation of myelin lipids (mainly composed of galactocerebrosides) required for saltatory conduction to occur (Sherman and Brophy, 2005). The destruction of myelin occurs in numerous neurodegenerative diseases, such as Multiple Sclerosis and Alzheimer's disease (Hoozemans et al., 2006; Stadelmann et al., 2008). However, small-scale myelin repair occurs throughout adulthood in healthy brains, and it remains unclear why this intrinsic repair capacity fails during diseases (Calza et al., 2009). To understand the process of myelination, however, we must understand the molecular mechanisms that contribute to the development of oligodendrocytes.

Ongoing research is attempting to more fully understand oligodendrocyte biology and the mechanisms of CNS myelination. For instance, several groups have reported that oligodendrocyte biology is controlled by the physiological environment via cell surface receptors. For example, laminins are extracellular matrix molecules that have been found to enhance oligodendrocyte differentiation (Buttery and ffrench-Constant, 1999; Colognato et al., 2002). Patients with MDC1A, a congenital muscular dystrophy caused by laminin mutations, have white matter abnormalities of unknown etiology in the CNS (Farina et al., 1998). Several studies have indicated that the laminin receptor, α6β1 integrin, may be responsible for the outside-in signaling from the extracellular matrix to the developing OLGs (Buttery and ffrench-Constant, 1999; Colognato et al., 2002).

Although OLGs express another laminin receptor, the non-integrin receptor dystroglycan, as well as other non-laminin-binding integrins (αvβ3, αvβ5, αvβ1), there are several reasons to focus on the role of α6β1 integrin as a regulator for myelination in the CNS. For instance, mice that lack the α6 integrin subunit have an increased level of OLG death in the developing brain stem (Colognato et al., 2002; Georges-Labouesse et al., 1996). *In vitro* studies have shown that laminin substrates promote oligodendrocyte myelin sheet formation, and that this effect is α6β1 integrin-dependent (Buttery and ffrench-Constant, 1999; Chun et al., 2003). Disruption of integrin function by integrin-blocking antibody resulted in decreased areas of myelin membrane formation in

oligodendrocytes grown on laminin, compared to cells grown on the non-physiological substrate poly-D-lysine (PDL), indicating a possible alteration in signaling components that regulate the cytoskeleton, such as the small GTPases rho, rac1, and cdc42.

Small GTPases are a class of GTP-binding proteins consisting of the Ras and Rho super-families of GTPases. These small GTPases are well known to regulate cell motility and morphology via the actin cytoskeleton (Liang et al., 2004). Recent evidence suggests that rac1 and cdc42 of the Rho family of GTPases have critical roles in neurite extension, axon guidance, and myelination (Nodari et al., 2007; Thurnherr et al., 2006). Knockout mice have shown that myelin sheath thickness in Schwann cells is modulated by rac1 and cdc42 and, independently, Liang et al. (2004) found that dominant-negative forms of rac1 or cdc42 alter OLG morphology in culture. β1 integrin has been shown to activate rac1 in Schwann cells during myelination by regulating radial lamella during axonal sorting (Nodari et al., 2007). In general, rac1 is believed to be involved in lamellopodia formation and cdc42 is involved in filopodia formation. Conditional knockout of cdc42 or rac1 in OLGs led to abnormal accumulation of cytoplasm in the inner tongue of the OLGs' processes. The double knockout of cdc42 and rac1 in OLGs have higher frequency of myelin membrane outfolding, indicating that cdc42 and rac1 are required for normal CNS myelination in a cooperative manner by regulating the same effectors of myelin (Thurnherr et al., 2006). Recently,

Benninger et al., (2006) and Nodari et al., (2007) showed that laminin signaling activates rac1 in Schwann cells, leading to myelination of axons in the PNS.

Other signaling mechanisms seem likely to mediate the ability of integrins to modulate myelination. For instance, the phosphatase PTPα has been reported to promote OLG differentiation, with reduction in MBP expression in the forebrain of PTPα-null mice (Wang et al., 2009). In addition, oligodendrocytes expressing a constitutively active form of a small GTPase, R-Ras, a known positive regulator of integrin inside-out signaling, exhibit an even bigger increase in myelin membrane sheet formation when cultured on a laminin substrate (Kwong et al., 2003). Conversely, oligodendrocytes infected with a lentiviral-vector coding for the dominant negative form of R-Ras formed smaller myelin sheets on laminin, compared to wildtype R-Ras-infected cells (Olsen and ffrench-Constant, 2005). Collectively, these findings indicate a role for β1 integrins in allowing laminin-mediated activation of small GTPases.

Several studies in culture indicate that integrins have profound effects by enhancing oligodendrocyte myelin sheet membrane grown on laminin (Buttery and ffrench-Constant, 1999; Olsen and ffrench-Constant, 2005). Moreover, antibody blocking of OLG β1 integrin yielded smaller processes and less myelin membrane sheet formation (Liang et al., 2004). Despite these studies, the effect of integrin loss on oligodendrocyte development and myelination in the CNS

appears to be modest. For instance, in mice lacking β1 integrins in mature OLGs, myelination appears to be completely normal during development and following injury in the adult (Benninger et al., 2006). During development there is elevated apoptosis of premyelinating oligodendrocytes, but this does not prevent successful myelination. No obvious reduction in myelination in mice lacking either the αV or β8 integrins, although these mice have not been examined in the same detail as the studies performed using mice lacking the β1 integrin subunit (McCarty et al., 2005; Proctor et al., 2005). Previous work indicates that the α6β1 integrin ligand, laminin, is required for normal myelin sheet formation (Buttery and ffrench-Constant, 1999; Colognato et al., 2002).

Moreover, mice engineered to overexpress a dominant negative β1 integrin subunit have aberrant myelination in the spinal cord, including increased OLG death and thinner myelin sheaths (Lee et al., 2006). On the other hand, mice engineered to excise a floxed β1 integrin gene using CNP-Cre have normal myelin sheaths in the spinal cord (Benninger et al., 2006). More recently, Camara et al. (2009) reported a transient hypomyelination phenotype of the optic nerves in mice expressing a dominant negative transgene form of β1 integrin and suggested that β1 integrins function in myelin initiation and determining the axon caliber size that is appropriate for myelination. Thus, conflicting evidence on the role of β1 integrin in myelination has been found, and, furthermore, the signaling mechanisms by which the β1 integrin receptor may regulate myelination remain

poorly characterized. It remained to be determined how and when β1 integrin can influence OLG development and CNS myelination. The studies in the current thesis addressed some of these unresolved questions regarding integrin function.

Figure I-1. Oligodendrocytes express different stage-specific cell markers during their development.

A schematic of oligodendrocyte developmental stages in rodents, where proliferative progenitor cells of early embryonic stage express proteoglycan, NG2 and platelet derived growth factor α receptor (PDGFαR) and migrate to target destination. Then, these cells exit the cell cycle and differentiate into newly formed oligodendrocytes expressing proteolipoprotein (PLP), APC (CC1), GalC, and CNP. Myelinating oligodendrocytes express MBP, a major component in myelin. Immature sheets are only present in cell culture and oligodendrocytes form myelin wraps around axons upon axon contact *in vivo*. (Image modified from (Baron et al., 2005) and (Nishiyama, 2007)).

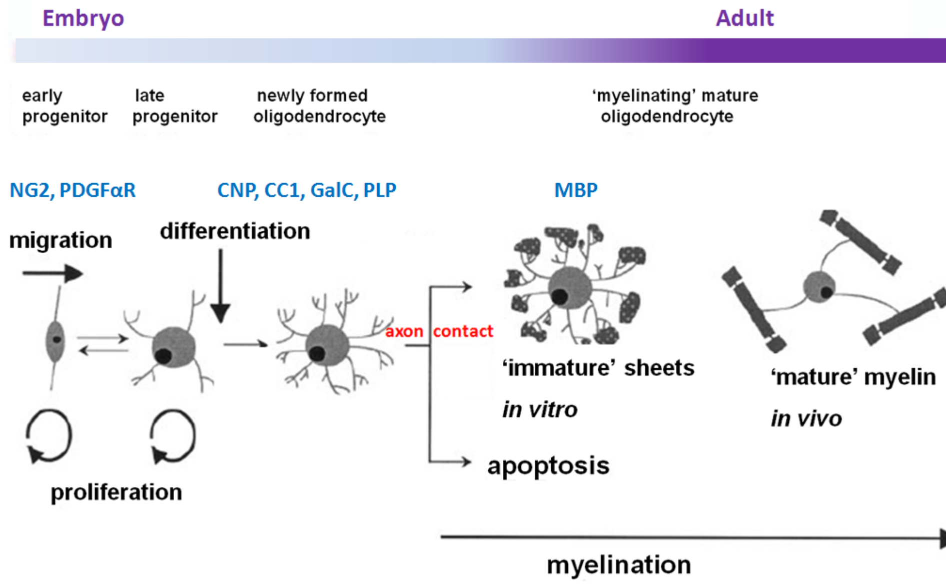
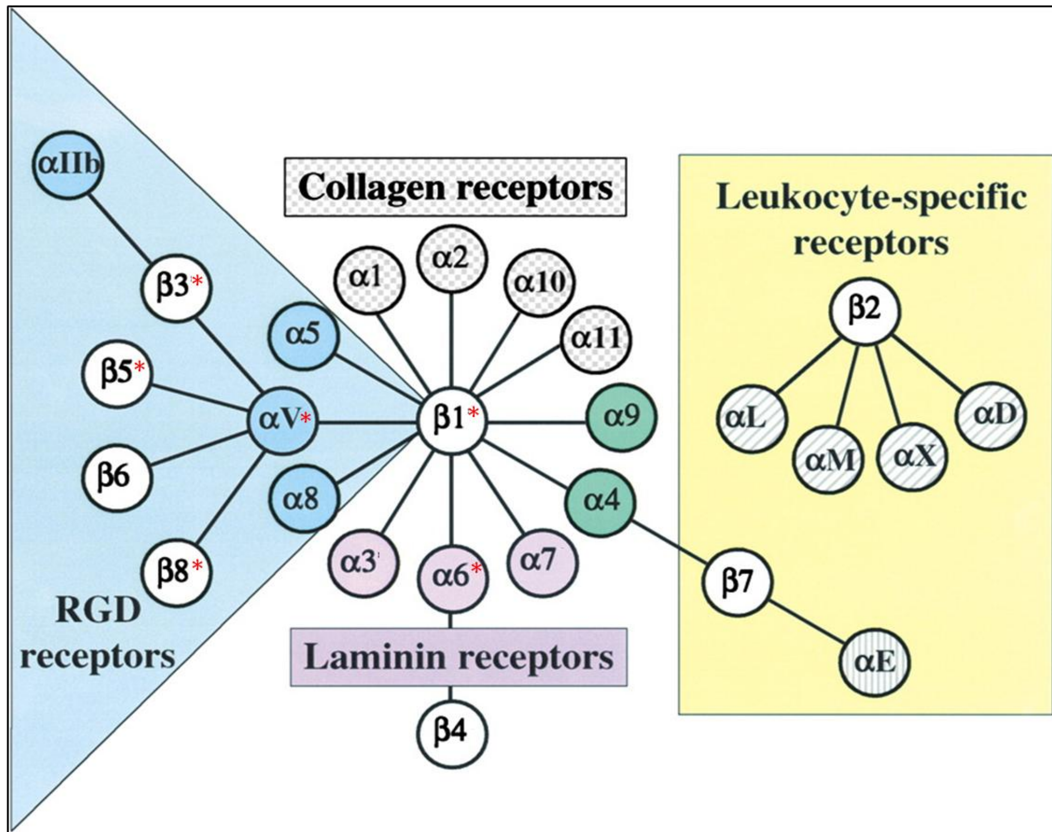

CNS Maturation
Embryo
Adult
early progenitor
late progenitor
newly formed oligodendrocyte
'myelinating' mature oligodendrocyte
NG2, PDGFαR
CNP, CC1, GalC, PLP
MBP
migration
differentiation
axon contact
'immature' sheets
in vitro
'mature' myelin
in vivo
apoptosis
proliferation
myelination

Figure I-2. The integrin family of receptors.

Integrin diversity and ligand specificity to extracellular matrix proteins laminin, collagen, and fibronectin. The β1 subfamily of integrins comprises 12 of the 24 different integrins with ligand specificity to laminin, collagen, and fibronectin. The β2 subfamily of integrins are expressed primarily in leukocytes. Asterisk indicates integrin subunits express in oligodendrocytes. (Image modified from (Hynes, 2002)).


Collagen receptors
Leukocyte-specific receptors
Laminin receptors
RGD receptors
αIIb
β3*
β5*
β6
β8*
αV*
α5
α8
α1
α2
α10
α11
β1*
α9
α4
α3
α6*
α7
β4
β2
αL
αM
αX
αD
β7
αE

Chapter II: General methods and materials

Animals

All mouse lines have been described: *Itgb1*$^{+/-}$ (Stephens et al., 1995), *Itgb1*^{flox} (Graus-Porta et al., 2001), *nestin-Cre* (Tronche et al., 1999) and protocols for genotyping have been described (Graus-Porta et al., 2001). Briefly, *Itgb1*$^{+/-}$ were crossed with *nestin-Cre* mice to generate *Itgb1*$^{+/-}$*nestin-Cre*$^{+/-}$. *Itgb1-CNSko* mice were generated by crossing *Itgb1*$^{+/-}$*nestin-Cre*$^{+/-}$ with *Itgb1*$^{flox/flox}$ mice. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Stony Brook University Institutional Animal Care and Use Committee.

Antibodies

The following antibodies were used: myelin basic protein (MBP, rat monoclonal IgG, 1:100, Serotec, Raleigh, NC); neurofilament (NF, mouse

monoclonal IgG, 1:100, Sigma); Plp (Serotec), chondroitin sulfate proteoglycan 4 (NG2, rabbit polyclonal IgG, 1:100, Chemicon); APC (CC1, mouse monoclonal IgG, 1:50, Calbiochem); galactocerebroside (GalC, mouse IgG, 1:50, Sigma); β1 integrin (rat IgG, Cell Signaling); AKT phospho-S473 and AKT (Cell Signaling); Texas Red-X phalloidin against F-actin (1:40, Invitrogen); proliferating cell nuclear antigen (PCNA, mouse monoclonal IgG, 1:100, Cell Signaling); 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP, mouse monoclonal IgG, 1:100, Sigma); PDGFαR (BD Biosciences); Olig2 (Chemicon); Cleaved Caspase-3 (Cell Signaling); α-tubulin (mouse monoclonal IgG, 1:100, Upstate); and β-actin (mouse monoclonal IgG, 1:100, Sigma). Secondary antibodies were AlexaFluor 488-, AlexaFluor 568-conjugated (Molecular probes), FITC-, Texas Red-, Cy3-, and Cy5-conjugated donkey antibodies against rat, rabbit, and mouse IgG were used as secondary antibodies (Jackson Immuno).

Primary oligodendrocyte cultures

Mouse or rat (Sprague-Dawley rat pups) oligodendrocyte precursor cell (OPC) cultures were cultured (37 ^{o}C, 7.5% CO_2) in high glucose DMEM with 10% fetal calf serum (FCS) on poly-D-lysine (PDL)-coated flasks from postnatal

day 2 animals as previously described (Colognato et al., 2007; Colognato et al., 2004; McCarthy and de Vellis, 1980), and isolated with an additional lectin panning procedure to remove microglia as previously described (Cahoy et al., 2008). Briefly, OPCs were isolated from 10-days *in vitro* (DIV) mixed glial cultures by 20-hour mechanical dissociation on MaxQ 400 cell shaker, followed by 30-minute differential adhesion on uncoated petri dishes, and unattached cells were subjected to two rounds of immunopanning on lectin-coated dishes. Lectin-coated dishes were prepared by incubating petri dishes with 2.3μg/ml BS-lectin-1 (BSL1, Vector Laboratories) in Dulbecco's PBS (D-PBS) for 4 hours at room temperature, followed by four washes with D-PBS immediately prior to use. Purified OPCs were added to PDL- or laminin-coated Permanox chamber slides in a modified SATO's medium (SATO with 0.5% FCS for differentiation experiments). Human placental laminin (laminin 211, Sigma) was used to coat the surfaces of slides and dishes at 10μg/ml in PBS for 4 hours at 37 ^{o}C. Surface coating with PDL (Sigma) coating was performed at 10μg/ml in dH_2O.

For AKT stimulation and myelin sheet formation studies, mixed glial cultures from neonatal spinal cords were grown on PDL-coated chamber slides and switched to SATO plus 0.5% FCS to differentiate at 10-DIV. Recombinant protein comprising the EGF-like domain of neuregulin-1 was used at 100ng/ml (Peprotech). PTEN inhibitor bpV(pic) (Schmid et al., 2004) was used at 31nM, the IC_{50} for PTEN inhibition (Calbiochem). Wild-type and β1-KO mixed glial

cultures were transfected using FUGENE (Roche) as described (Colognato et al., 2004) with either Myr-AKT-EGFP (CA-AKT) or control ECFP plasmids (A kind gift of Dr. Bing-Hua Jiang, West Virginia University). Transfected oligodendrocytes were differentiated within the mixed glial cultures and identified 6-days-post-transfection using combined GFP and MBP immunocytochemistry. A minimum of 25 fields per transfection were evaluated by morphometric analysis.

Electron microscopy and morphometric analysis

Electron microscopy (EM) of spinal cords and cerebellar samples were prepared as previously described (Graus-Porta et al., 2001). EM images were acquired using a Philips CM100 microscope (FEI, Hillsborough, OR). MetaMorph software (Universal Imaging Corporation) was used for morphometric measurements and analysis of nerve fibers. Separately, corpus callosum EM samples were prepared from *Itgb1–CNSko* and wildtype littermates as previously described (Relucio et al., 2009). Briefly, animals were perfused with 4% EM grade paraformaldehyde (Electron Microscopy Sciences), 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1M phosphate buffered saline. The brains were postfixed overnight in perfusate. Cortical coronal sections

of 50-60µm were cut on a Leica VT-1000 Vibratome. Vibratomed sections were processed using standard transmission electron microscopy techniques. Briefly, vibratomed sections were placed in 2% osmium tetroxide in 0.1M phosphate buffer, washed in 0.1M phosphate buffer, and dehydrated in a graded series of ethyl alcohol. Sections were vacuum infiltrated in Durcupan resin (Durcupan ACM Epoxy, Electron Microscopy Sciences) overnight, flat embedded between two pieces of ACLAR film (Ted Pella), and polymerized in a 60 ^{o}C oven for 48–72 hours. Areas of interest were blocked and ultrathin (~70–80nm) sections were cut on a Reichert-Jung Ultracut E ultra-microtome. Ultrathin sections were placed on formvar-coated copper slot grids and counterstained with uranyl acetate and lead citrate. Samples were viewed with a FEI Tecnai BioTWIN G^2 transmission electron microscope. Digital images were acquired with an AMT XR-60 CCD digital camera system. Unmyelinated axon counts were done using cell count measurement in Adobe Photoshop CS3 and g-ratio (ImageJ (NIH)) of myelinated axons was determined by dividing the axon diameter by the myelin diameter. A minimum of 100 axons per animal was measured for each region.

AxioVision Interactive Measurement Module was used for myelin sheet and process length measurements. To ensure consistent analysis across different experiments, MBP-positive cells and MBP-positive, sheet-bearing cells were determined using intensity thresh-holding relative to background intensity (AxioVision). Morphometric analysis to determine the area coverage of myelin

sheets was performed by tracing the outer perimeter of MBP-positive, sheet-bearing oligodendrocytes (AxioVision, Zeiss). Process length measurements were performed on corresponding phase micrographs by tracing from the cell body/process border to the tip of the longest process (AxioVision).

SDS–PAGE and immunoblotting

Western blots were carried out as previously described (Colognato et al., 2004; Graus-Porta et al., 2001). Briefly, cortical tissues were collected from juvenile and adult *Itgb1–CNSko* and wildtype littermate mice and lysed immediately in extraction buffer (20mM Tris pH 7.4, 1% sodium dodecyl sulfate (SDS); 1 ml buffer per 200mg tissue) preheated to 95 ^{o}C; cocktails of protease and phosphatase inhibitors (Calbiochem) were added immediately prior to use. Tissue solutions were incubated at 95 ^{o}C for 10 minutes with occasional trituration, centrifuged at 14,000 rpm, and protein supernatant was isolated. Protein concentration was determined (detergent compatible protein assay, BioRad) and the lysates were reduced on a 95 ^{o}C heat block for 5 minutes in Laemmli Solubilizing Buffer (LSB) and 4% β-mercaptoethanol (βME). Proteins were separated by SDS-PAGE using 7.5, 10, or 15% acrylamide minigels and

transferred onto 0.45μm nitrocellulose. Membranes were blocked with 4% bovine serum albumin (BSA) or 1% non-fat milk in 0.1 % Tween-20, and Tris buffered saline (TBS-T) for 1 hour at room temperature, followed by primary antibodies in blocking buffer on rocker overnight at 4 ^{o}C. Membranes were washed 4 times for 15 minutes in TBS-T, incubated for 1 hour in HRP-conjugated secondary antibodies in blocking buffer, washed in TBS-T, then developed using enhanced chemiluminescence (Amersham). Relative densitometries were determined using the NIH ImageJ Processing and Analysis Program.

Fluorescent immunohistochemistry on perfused tissues

Immunohistochemistry was carried out as previously described (Belvindrah et al., 2007; Relucio et al., 2009). Briefly, mice were anesthetized with Avertin and carried intracardic perfusion with 1 ml saline solution, followed by 50 ml 4% paraformaldehyde (PFA) in PBS. Brains were harvested, post-fixed for 1 hour in PFA, and cryopreserved overnight in 30% sucrose at 4 ^{o}C. Tissues were embedded in Tissue-Tek OCT (Sakura Finetek) and cryosectioned to a thickness of 25μm. To visualize oligodendrocyte precursor cells (OPCs) or oligodendrocytes, cortical sections were incubated with NG2 or CC1 primary

antibody, respectively, in block buffer (5% donkey serum in PBS, 0.1% Triton-X100 detergent) overnight at 4 ^{o}C. Sections were washed 4 times in block buffer and incubated with Cy3-conjugated anti-rabbit or anti-mouse secondary antibodies in block buffer without detergent for 1 hour at room temperature. To visualize myelinated axon tracts, sections were double immunostained for MBP and NF. Sections were post-fixed in ethanol: acetic acid (95:5) for 30 minutes at -20 ^{o}C. Sections were washed 4 times with 1x PBS and blocked with 0.05% Triton-X100 in PBS for 1 hour before co-incubating with anti-NF and anti-MBP primary antibodies overnight at 4 ^{o}C. FITC- and Texas Red-conjugated secondary antibodies were incubated together in block buffer without Triton X-100 for 1 hour. All sections were incubated in 10 μg/ml DAPI or Topro-3 (Molecular Probes) in PBS for 10 minutes to visualize nuclei before mounted with SlowFade Gold antifade reagent (Invitrogen).

Fluorescent immunocytochemistry

Cells were fixed in 4% PFA (15 minutes) or 100% MeOH (5 minutes; -20 ^{o}C). Block and primary antibody incubations were in PBS, 10% donkey serum (with 0.1% Triton for PFA-fixed cells). Cells undergoing GFP/MBP

immunocytochemistry were blocked in PBS, 10% donkey serum, and 0.05% Triton. For GalC staining, live cells were labeled with antibody for 30 minutes in DMEM, 1% FCS, followed by washes and PFA fixation. Primary antibodies were NG2, β1 integrin MAB1997 (Chemicon), GalC, CNP, MBP, GFP (Molecular Probes) and Olig2. Secondary antibodies were FITC- or Texas Red-conjugated (Jackson). Nuclei were stained with DAPI. Images were collected on an Olympus Fluoview 500 confocal microscope, an Olympus AX70 microscope, and a Zeiss Axioplan microscope. TUNEL assays were carried using ApopTag Red *InSitu* Apoptosis Detection Kit (Chemicon).

Process complexity

To determine whether the β1 integrin deficiency in β1-KO oligodendrocytes affected the number of primary process and process complexity, β1-KO and wildtype OPCs were first differentiated for 2- and 4-days *in vitro* or rat OPCs were treated with β1 integrin blocking (clone Ha2/5, Pharmingen) or isotype control IgM (Sigma) antibodies, at 20μg/ml daily. Oligodendrocyte morphology was visualized using Texas Red-X phalloidin to label F-actin (Invitrogen). The total number of primary processes per cell was determined,

while process outgrowth and complexity were assessed by semi-automated Sholl analysis (Gensel et al., 2010; Sholl, 1953) using the NIH ImageJ Processing and Analysis Program. Briefly, the cell of interest was manually outlined to exclude processes from adjacent cells and densitometric black and white threshold was set to identify detailed cellular processes. Each image was converted into 8-bit binary, noise despeckled, and single pixels of immunoreactive labeling above the threshold of detection were removed to reduce false positives. Missing process segments were traced in using paintbrush tool and the image was skeletonized. The epicenter of concentric circles (r_{start}=10μm, $r_{step\ size}$=10μm, r_{span}=0, r_{end}=100μm, span type=median) was placed onto the center of cell nucleus (DAPI). The total number of pixels above threshold intersecting each circle was tallied using the Sholl Analysis automated macro in the NIH ImageJ Processing and Analysis Program. The sum of the number of intersections (process complexity) for all rings were generated for each cell and compared between groups.

Survival assay

To determine whether the β1 integrin deficiency in *Itgb1–CNSko* mice affected OPC and oligodendrocyte survival, 4% PFA perfused cortical sections (25μm) were fixed in 4% PFA for 30 minutes at room temperature, followed by ethanol: acetic acid (95:5) for 20 minutes at -20 ^{o}C. Sections were quickly washed 4 times with 0.5% BSA in 10X PBS (wash buffer) and blocked with 10% donkey serum in 1XPBS for 1hour at room temperature. Immunohistochemical detection of OPCs (anti-NG2) and mature oligodendrocytes (anti-CC1 primary antibody) in block buffer were carried out overnight at 4 ^{o}C. Sections were washed 4 times in wash buffer then incubated with Cy5-conjugated donkey against rabbit and FITC-conjugated donkey against mouse secondary antibody in block buffer for 1hour at room temperature. To identify apoptotic oligodendrocytes, TUNEL assay was carried out using indirect immunofluorescence to visualize nicked DNA according to the manufacturer instructions (Apoptag Red, Intergen, Norcross, GA) on the CC1- and NG2-immunostained sections. Nuclei were visualized using 10 μg/ml DAPI in PBS and slides were mounted with SlowFade Gold antifade reagent (Invitrogen). Cell survival was determined by the percentage of TUNEL-negative, CC1-positive or TUNEL-negative, NG2-positive cells in a given condition.

Fluorescence microscopy and image acquisition

Sections were visualized using a Zeiss Axioplan inverted fluorescence and confocal (model LSM510; Carl Zeiss MicroImaging, Inc.) fluorescent microscopes fitted with 10X eyepiece magnification using 5X (0.16 N.A.), 10X (0.3 N.A.), 20X (0.5 N.A.), 40X (0.75 N.A.), and 63X (1.4 N.A.) objectives. Images were acquired using a Zeiss Axiocam MRM digital camera with Zeiss AxioVision and LSM 510 Meta imaging software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, Microsoft Excel, and SigmaPlot. Statistical significance of data sets was determined using the Student's two-tailed, paired *t*-test, and Mann-Whitney *U* test. Graphs depict the mean ± standard error of the mean (mean ± SEM). Statistical significance was set at $p<0.05$ (*, significant), $p<0.01$ (**, very significant) and $p<0.001$ (***, highly significant).

Chapter III: β1 integrins are required for normal CNS myelination and promote AKT-dependent myelin outgrowth

Abstract

Myelin is essential for proper neurodevelopment. In the CNS, myelination is carried out by oligodendrocytes and in the PNS this process is done by Schwann cells. β1 integrins control myelin ensheathment of peripheral nerves, but their underlying function during the myelination process of axonal tracts in the CNS remains unclear. One potential effector molecule, β1 integrin, has been suggested to play a critical role in CNS myelination. Here, we show that axonal tracts of the spinal cord, cerebellum, and optic nerve in genetically modified mice lacking β1 integrins in the CNS have thinner myelin sheaths with normal oligodendrocyte lineage development compared to their wild-type littermates. Moreover, *in vitro* data show that β1 integrins regulate the outgrowth of myelin sheaths. Oligodendrocytes derived from mutant mice are unable to efficiently extend myelin sheets and do not fully activate AKT, a kinase that regulates axonal

ensheathment. Myelin sheet outgrowth in cultured β1-deficient oligodendrocytes was restored by inhibiting PTEN, a negative regulator of AKT, or the expression of a constitutively active form of AKT. These findings suggest that β1 integrins play an instructive role in CNS myelination by promoting myelin wrapping in a process that depends on AKT.

Chapter III is done in collaboration with Drs. Claudia S. Barros and Ulrich Muller. Experiments performed by and with our collaborators are noted as such at the beginning of the sections describing findings from these experiments.

Introduction

Oligodendrocytes (OLGs) have an essential physiological role in myelinating axons of the central nervous system (CNS). The myelination process is needed in order for neuron networks to communicate via saltatory conduction, a process that enables rapid propagation of action potentials traveling down axons (Sherman and Brophy, 2005), but the precise molecular mechanisms underlying the myelination process remain poorly understood. One potential effector molecule, the β1 integrin, has been suggested to play a critical role in CNS myelination.

Integrins are heterodimeric cell surface receptor proteins, consisting of α and β subunits, that bind to a variety of extracellular matrix ligands, including laminins (Hynes, 2002). Laminins are extracellular matrix molecules that have been found to enhance oligodendrocyte differentiation. Patients with MDC1A, a congenital muscular dystrophy caused by laminin mutations, have white matter abnormalities in the CNS (Farina et al., 1998). Integrins containing the β1 subunit are expressed in Schwann cells, the myelinating glial cells of the peripheral nervous system, where they mediate interactions with laminin that are critical for myelination (Berti et al., 2006). Oligodendrocytes express a limited repertoire of

integrins including the α6β1 integrin, a laminin receptor. Several studies have indicated that the laminin receptor, α6β1 integrin, may be responsible for the outside-in signaling from the extracellular matrix to the developing OLGs. *In vitro* studies show that the αvβ1 integrin promotes the migration of oligodendrocyte progenitors, while α6β1 regulates oligodendrocyte survival and myelin membrane formation in response to laminin-2 (Buttery and ffrench-Constant, 1999; Frost et al., 1999; Relvas et al., 2001). Mice that lack the α6 integrin subunit have an increased level of oligodendrocyte death in the developing brain stem (Chun et al., 2003; Colognato et al., 2002).

However, since α6-deficient mice die at birth (Georges-Labouesse et al., 1996), it has remained unclear whether the defects in oligodendrocyte survival lead to persistent changes in myelination. In fact, genetic studies addressing the function of β1 integrins in oligodendrocytes have led to contradictory results. Mice engineered to overexpress a dominant negative (β1ΔC) β1 integrin subunit have aberrant CNS myelination (Lee et al., 2006). On the other hand, mice engineered to excise a *floxed β1 integrin* gene using *CNPase-Cre* have normal myelin sheaths (Benninger et al., 2006). In addition, a more recent study, Camara et al. (2009), showed that inhibition of integrins by dominant negative transgene (IL-2Rα domain and β1 integrin cytoplasmic domain) expression of β1 integrin in myelinating oligodendrocytes transiently perturbs axoglial interactions that are required for smaller caliber axons to be myelinated in the CNS. The

interpretation of these experiments is complex since DN-β1 integrin can also ectopically activate integrin signaling by receptor clustering with endogenous β1 integrins, and *CNPase-Cre* induces recombination only in a subset of oligodendrocytes by postnatal day 0 (P0) (Benninger et al., 2006). To circumvent the complications associated with the previous studies, we have analyzed *Itgb1-CNSko* mice in which β1 integrins were inactivated in the ventricular neuroepithelium, including oligodendrocyte precursors.

Here, we show that mice lacking β1 integrins have thinner myelin sheaths in axonal tracts of the spinal cord, optic nerves and cerebellum. Similarly, myelination defects were also observed in the spinal cord from mice in which β1 was inactivated solely in NG2-positive oligodendrocyte precursors. Interestingly, oligodendrocyte differentiation or survival was not affected by the loss of β1 integrins. Instead, oligodendrocyte culture studies showed that myelin sheet outgrowth was regulated by β1 integrins. In addition, we show that β1 integrins regulate AKT activity in oligodendrocytes and that constitutive-active AKT rescues the defect in myelin sheet outgrowth in cultured β1 integrin-deficient oligodendrocytes.

Results

CNS myelination defects in *Itgb1-CNSko* and *Itgb1-OL-ko* mice.

[Experiments in this part of the results were done by our collaborators, Drs. Claudia S. Barros and Ulrich Muller.]

To examine the functions of β1 integrins in CNS myelination, we inactivated β1 expression in *β1-CNSko* mice engineered to excise *floxed β1 integrin* genes using *nestin-Cre* (*nestin-Cre* is expressed before oligodendroglial lineage commitment). Here, *Cre*-mediated recombination occurs early in neural precursors at embryonic day (E) 10.5 resulting in the inactivation of the *Itgb1* gene in developing neurons, astroglia and cells of all stages of oligodendrocyte development (Graus-Porta et al., 2001). Mutant mice are viable and fertile with no obvious behavioral abnormalities, and will be referred to as *Itgb1-CNSko* mice (figure II-1A). In order to study the role of β1 integrins in CNS myelination, we first confirmed recombination of the *Itgb1-flox* allele by PCR (figure II-1B); and by Western blot analysis of spinal cord lysates showed the lack of β1 protein detection from *Itgb1-CNSko* mice compared to wild-type littermates (figure II-

1C). Previous studies showed that the *loxP* sites or heterozygosity for *Itgb1-null* do not affect β1 function (Graus-Porta et al., 2001; Stephens et al., 1995). Thus, in all assays, *Itgb1-CNSko* (*Cre*$^{+}$*Itgb1*$^{floxed/-}$) mice were compared with wild-type littermates (*Cre*$^{-}$*Itgb1*$^{floxed/floxed}$, *Cre*$^{-}$*Itgb1*$^{floxed/+}$, or *Cre*$^{-}$*Itgb1*$^{floxed/-}$).

To examine myelin sheaths in *Itgb1-CNSko* mice, we carried out electron microscopy studies of the spinal cords of postnatal (P) day 30 mice. *Itgb1-CNSko* mutants have thinner myelin sheath compared to that of wild-type littermates (figure II-2A). Morphometric analysis of the nerve fibers showed a significant increase in the g-ratio (ratio between axon to fiber diameters; figure II-2B). Interestingly, the smallest caliber axons ($<0.7\mu m$ diameter) were not affected. The periodicity of myelin sheets in the mutants was unaffected, suggesting that the overall reduction in myelin thickness resulted from a decrease in the number of wraps (figure II-2A insets). We also checked for protein levels of two major CNS myelin components, myelin basic protein (MBP) and proteolipid protein (PLP) by Western blot analysis of P30 spinal cords. There were modest but significant reductions of MBP and PLP proteins in spinal cord of *Itgb1-CNSko* compared to wild-type littermates by densitometry analysis (figure II-2C; reduced by 36.5±12.1%, n=3, $p<0.05$, and 13.5±3.0%, n=3, $p<0.05$, respectively). Moreover, we investigated whether other axonal tracts in the CNS were similarly affected. The myelin sheaths enwrapping the larger caliber axons were reduced in thickness

in both the optic nerves and the cerebellum of *Itgb1-CNSko* compared to wild-type littermates (figures II-2D, II-2E, respectively).

Since β1 is expressed in neurons and other glial cells in addition to oligodendroglia, it is possible that myelin wrapping of axons is regulated extrinsically by β1 integrin from neurons or other cell types, and not by oligodendroglial β1 integrin. Ablation of *β1 integrin* by *nestin-Cre* ablates β1 integrin in neurons, astroglia and oligodendrocytes, but not in microglia. To address cell autonomy, we inactivated β1 integrin expression using *NG2-Cre* mice. In this mouse line, CRE specifically induces the recombination of floxed alleles in NG2-positive oligodendrocyte precursors (Zhu et al., 2008). Electron microscopy of axonal tracts in the spinal cord of *NG2-β1ko* mutants (named *Itgb1-OL-ko* mice) revealed a reduction in myelin thickness by g-ratio (figure II-3A). Similarly to *Itgb1-CNSko* mice, myelin thickness was affected in axons with diameters >0.7µm, although this phenotype was less severe (figure II-3B). However, it cannot be excluded that β1 integrins in axons, which were inactivated by *nestin-Cre* but not by *NG2-Cre*, also contributed to myelination. Together, our findings demonstrate that β1 integrins are required for the myelination of axonal tracts in the spinal cord, optic nerve and cerebellum, and that they act, at least in part, in oligodendrocytes to carry out their function.

Oligodendrocyte lineage development in *Itgb1-CNSko* mutants.

[The experiments in this part of the results were done in collaboration with Drs. Claudia S. Barros and Ulrich Muller. The *in vitro* experiments were performed and analyzed by our group.]

The hypomyelination defects of axonal tracts in the CNS of *Itgb1-CNSko* mutants could arise from defects in oligodendrocyte development. Thus, we analyzed oligodendrocyte development using stage-specific molecular markers. We first examined the oligodendrocyte progenitors at P0, prior to axonal ensheathment, of the spinal cords by immunostaining with PDGFαR (figure II-4A). Numbers of PDGFαR positive cells per area were comparable between *Itgb1-CNSko* mutants and wild-type littermates (figure II-4B; β*1mt*, 17.88±1.09/μm^2, WT, 17.95±1.21/μm^2, n=3, p>0.05). Similarly, at P19, when myelination is in progress, and at P60, after myelin sheaths have formed, there were no significant differences in the numbers of CC1-positive oligodendrocytes (figures II-4C, II-4D; β*1mt*, 32.71±0.89/μm^2, WT, 33.5±1.36/μm^2 and β*1mt*, 34.17±1.05/μm^2, WT, 34.89±0.78/μm^2, respectively, n=3, p>0.05). Thus, the reduction in myelin ensheathment in *Itb1-CNSko* mice is probably not caused by the loss of oligodendrocytes.

To further address the cell-autonomous function of β1 integrins in oligodendrocytes, we extracted oligodendrocyte progenitor cells from wild-type and *Itgb1-CNSko* mice forebrains and examined their differentiation *in vitro*. The efficient inactivation of β1 integrins in oligodendrocytes from *Itgb1-CNSko* mice was confirmed by immunostaining and Western blots (figures II-5A, II-5B, respectively). We found no significant difference in percentage of NG2-positive oligodendrocyte precursor cells differentiated for 1-DIV between β1-deficient and wild-type cells (figures II-5C, II-5D; β*1mt*, 46.83±3.02%, WT, 50.90±5.32%, n=4, paired *t*-test, p=0.23). We further examined later stages of oligodendrocyte development and found no difference in percentage of GALC-positive oligodendrocytes at 2-DIV or 4-DIV (figures II-5E, II-5F; β*1mt*, 67.05±2.1%, WT, 66.89±3.72%, n=4, paired *t*-test, p=0.94 and β*1mt*, 81.6±2.82%, WT, 78.87±4.29%, respectively, n=4, paired *t*-test, p=0.48), or MBP-positive mature oligodendrocytes at 4-DIV or 6-DIV (figures II-5G, II-5H; β*1mt*, 33.26±4.37%, WT, 37.39±4.54%, n=4, paired *t*-test, p=0.67, and β*1mt*, 23.28±2.95%, WT, 32.97±4.22%, n=4, paired *t*-test, p=0.24, respectively). These data are in agreement with our *in vivo* analysis and further confirm that β1 integrins are not essential for the formation of mature oligodendrocytes.

Defects in myelin sheet outgrowth in β1-deficient oligodendrocytes.

In vitro, in the absence of axons, oligodendrocytes differentiate, extend their membrane and form myelin membrane sheets by the extension of branched processes surrounded by a specialized plasma membrane enriched with myelin proteins such as MBP (Richardson et al., 2006). This process is thought to mimic many of the morphological changes that take place during oligodendrocyte ensheathment of axons *in vivo* (Simons and Trotter, 2007). While performing lineage analysis, we observed that MBP-positive cortical oligodendrocytes of *Itgb1-CNSko* mutants extended smaller myelin membrane sheets (figure II-6A). Thus, we performed morphometric analysis on MBP-positive oligodendrocytes bearing myelin sheets. There were fewer MBP-positive oligodendrocytes derived from *Itgb1-CNSko* cortex compared to wild-type littermates at 2-DIV (figure II-6B; β*1mt*, 29.98±1.81%, WT, 36.62±0.23%, n=4, paired *t*-test, p=0.020). Mutant oligodendrocytes have less elaborate and smaller myelin membrane sheets compared to wild-type cells at 2-DIV and 4-DIV (β*1mt*, 2409.12±225.79μm^2, WT, 4164.70±311.60μm^2, n=4, paired *t*-test, p=0.010 and β*1mt*, 3658.04±457.05μm^2, WT, 5557.27±806.51μm^2, n=4, paired *t*-test, p=0.029, respectively). The decrease in sheet outgrowth was also reflected by a decrease in the mean length of mutant oligodendrocyte processes at 4-DIV (figure II-6D; β*1mt*, 85.01 ± 5.59µm, WT, 115.16±3.16µm, n=4, paired *t*-test, p=0.014). In

addition, we evaluated mixed glial cultures from the spinal cord to confirm that defects in myelin sheet formation are not confined to cortical oligodendrocytes. Spinal cord oligodendrocytes derived from *Itgb1-CNSko* spinal cord formed significantly smaller myelin sheets compared to wild-type controls (figure II-6E; β*1mt*, 9863.98±719.86μm^2, WT, 12,944.54±346.64μm^2, n=4, paired *t*-test, p=0.043).

Activated AKT mediates β1 integrin function in myelin sheet outgrowth.

Several signaling molecules have been implicated in controlling myelination, including the serine/threonine kinase, AKT (Flores et al., 2008). Integrin signaling in oligodendrocytes amplifies receptor tyrosine kinase signaling and neuregulin-1 signaling, which is particularly sensitive to integrin engagement (Colognato et al., 2002; Colognato et al., 2004). To determine whether β1-deficient oligodendrocytes exhibit defects in AKT activation, we treated oligodendrocytes plated on laminin with soluble neuregulin-1 to stimulate AKT phosphorylation (figure II-7A). Upon neuregulin-1 treatment, wild-type oligodendrocytes showed a substantial increase in AKT phosphorylation at serine 473, but β1-deficient oligodendrocytes did not show significant AKT activation (figure II-7B; 105.78±6.15% versus untreated β*1mt*, n=8, *t*-test, p=0.17). In addition, a similar

AKT stimulation assay was performed using β1-integrin-blocking antibodies on wild-type rat oligodendrocytes grown on laminin (figure II-7C). Neuregulin-treated oligodendrocytes showed a significant increase in AKT phosphorylation in the presence of the control antibody, but cells treated with β1-integrin-blocking antibodies showed no significant increase (figure II-7D; 117.54±29.87%, n=4, paired *t*-test, p=0.19). To ascertain β1 integrin specificity, we also used antibodies that block the non-integrin laminin receptor dystroglycan, which is expressed in oligodendrocytes (Colognato et al., 2007). In the presence of the dystroglycan antibody, oligodendrocytes were able to activate AKT upon neuregulin-1 stimulation (figure II-7D; 150.65±10.25%, n=4, paired *t*-test, p=0.035). Thus, loss of β1 integrin protein and function affects the ability of oligodendrocytes to activate AKT phosphorylation.

We next tested if the defects in myelin sheet outgrowth in β1-deficient oligodendrocytes could be attenuated upon increased activation of AKT pharmacologically. Treatment with a PTEN inhibitor, bpV (Schmid et al., 2004), to enhance AKT phosphorylation caused mutant oligodendrocytes to grow myelin membrane sheets of an equivalent size to wild-type (figures II-7E, II-7F; β*1mt*+bpV, 5026.72±90.34μm^2 versus β*1mt* untreated, 3612.06±97.31μm^2, n=4, paired *t*-test, p=0.011; β*1mt*+bpV, 5026.72±90.34μm^2 versus WT, 4800.85±312.75μm^2, n=4, paired *t*-test, p=0.74). To further confirm directly whether AKT signaling can rescue the defect in myelin sheets in β1-deficient

oligodendrocytes, we transfected a construct of constitutively active AKT-GFP fusion protein (CA-AKT) or with a control GFP (figure II-7G) in mixed glia culture. The expression of CA-AKT dramatically increased the myelin sheet area in β1 mutant oligodendrocytes and restored it to near wild-type levels as visualized by GFP and MBP staining (figure II-7H; β*1mt*+control GFP, 2174.65±280.15μm^2 versus β*1mt*+CA-AKT, 3449.05±274.46μm^2; WT + CA-AKT, 3783.54±69.66μm^2; 2 animals per group, 12 to 25 cells measured per group, paired *t*-test, p=0.0020). Collectively, these results suggest that β1 integrins are required for normal myelin outgrowth by regulation of AKT activity.

Discussion

Here we show that the axonal tracts in the spinal cord, cerebellum, and optic nerve required β1 integrins for normal myelination. Also, we establish a link between β1 integrins and AKT in oligodendrocyte function. Myelin thickness was reduced in the spinal cord, cerebellum and optic nerve of *Itgb1-CNSko* mice without a reduction in oligodendrocyte numbers. These findings suggest that the myelination defects in mutant mice were caused by perturbations in the formation of myelin membrane sheaths. Consistent with this finding, myelin outgrowth was substantially impaired in cultured β1-deficient oligodendrocytes. Myelination was also affected in the spinal cord of *Itgb1-OL-ko* mice, providing additional evidence that β1 integrins act, at least in part, cell-autonomously in oligodendrocytes to regulate myelination. Interestingly, activation of AKT signaling was affected in cultured β1-deficient oligodendrocytes. Furthermore, myelin membrane sheet formation in the β1-mutant cultured cells was restored by inhibiting PTEN or by overexpressing constitutively active AKT. Taken together, these findings provide strong evidence that AKT is crucial for β1 integrin function during the myelination of axonal tracts in the CNS.

Previous studies have provided conflicting results regarding the function of β1 integrins in CNS myelination. Expression of a dominant-negative β1 integrin in oligodendrocytes has been reported to affect CNS myelination (Câmara et al., 2009; Lee et al., 2006). In another study meant to circumvent the drawback of dominant-negative approach that could have ectopic affects and loss of specificity, Benninger et al., (2006) showed that β1 integrin was not required for CNS myelination by using the transgenic conditional KO approach of β1 integrin where Cre-recombinase was driven by the CNPase promoter. We now report significant defects in CNS myelination in mice where β1 integrins are inactivated with *nestin-Cre*, supporting the view that β1 integrins are important for CNS myelination. In addition, we observed defects in myelination in *Itgb1-OL-ko* mice, in which integrins have been inactivated in oligodendrocytes using *NG2-Cre*. We consider it likely that the differences in the results reported here and the earlier study can be explained by differences in the efficiency and timing of CRE expression. CNPase is expressed relatively late during oligodendrocyte differentiation, and *CNPase-Cre* induces recombination in ~65% of oligodendrocytes at around P0. By contrast, NG2 is already expressed in oligodendrocyte precursors at the stage when PDGFαR is expressed, which is a marker for the earliest stages of oligodendrocyte differentiation. *NG2-Cre* has also been reported to lead to recombination in approximately 90% of NG2-positive cells. Since oligodendrocytes are generated in excess, sufficient

progenitors probably escaped gene inactivation and were able to compensate for the loss of β1 integrin protein in some progenitors. Interestingly, myelination defects in *Itgb1-CNSko* mice were more severe than in *Itgb1-OL-ko* mice. As β1 integrins in *Itgb1-CNSko* mice were inactivated in both neurons and oligodendrocytes, β1 integrins in axons might have additional roles in myelination.

We provide here insights into the mechanisms by which β1 integrins regulate myelination in the CNS. Unlike in previous studies, which indicated that β1 integrins regulate oligodendrocyte survival, we only detected a small yet statistically insignificant trend towards increased death in the developing cerebellum of *Itgb1-CNSko* mice. Although we cannot fully explain the difference with previous studies, our findings suggest that the subtle changes in oligodendrocyte death in *Itgb1-CNSko* mice were compensated for during development. The changes in myelin thickness that we observed are therefore probably not caused by a decrease in the number of oligodendrocytes but by defects in myelin membrane outgrowth. This interpretation is consistent with our *in vitro* data, which demonstrate that the formation of myelin membrane sheets was affected in β1-deficient cultured oligodendrocytes.

Interestingly, recent findings show that constitutively active AKT enhances myelination without affecting the number of oligodendrocytes (Flores et

al., 2008). Moreover, mice lacking the P85α regulatory subunit of PI3K, an activator of AKT, show hypomyelination in the CNS (Tohda et al., 2006) and conversely, whereas knockout mice for PTEN, a negative regulator of PI3K/AKT signaling, have thickened CNS myelin sheaths (Fraser et al., 2008). The opposite effects of β1 inactivation and AKT activation on myelination prompted us to test whether the two proteins are functionally linked. Consistent with this model, the loss of β1 integrins in oligodendrocytes affected AKT activation. A second laminin receptor, dystroglycan, which also promotes myelin membrane outgrowth *in vitro* (Colognato et al., 2007), did not affect AKT activation, indicating that there is a specific link between AKT and β1 integrins. Furthermore, defects in myelin membrane outgrowth in β1-deficient oligodendrocytes were rescued upon expression of constitutively active AKT or by modulating endogenous AKT activity levels with a PTEN inhibitor. Based on these findings, we suggest that β1-deficient oligodendrocytes are unable to properly myelinate axons at least in part due to defective AKT signaling. Interestingly, FAK and ILK, mediators of integrin functions that control AKT, have also been implicated in the regulation of myelin sheet outgrowth by oligodendrocytes (Chun et al., 2003; Hoshina et al., 2007). Collectively, these data support a model in which extracellular ligands that activate β1 integrins induce AKT activity to control myelin outgrowth and axonal wrapping in the CNS. In the future, it will be important to define the downstream effectors of AKT and the extent to which they integrate biosynthetic and

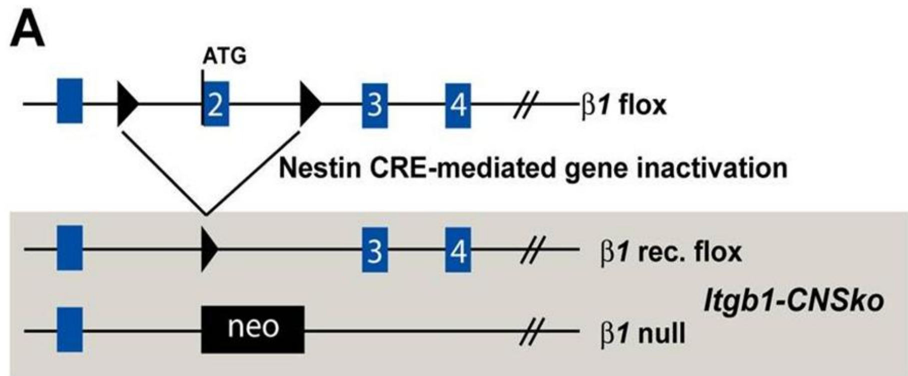
cytoskeletal pathways to control the outgrowth of myelin membranes that enwrap axonal processes in the CNS.

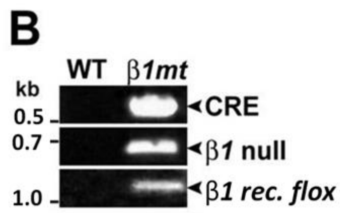
Although, I did not rule out the possibility that the reduced myelin membrane sheet area could reflect a reduction in cell size, DAPI immunoreactivity suggests that this is not the case. In addition, it is possible that myelin membrane sheet area is not reflective of myelin membrane volume. Thus, a smaller and more rounded oligodendrocyte may have a smaller myelin membrane sheet area per se, but myelin membrane volume could be comparable to a larger and flatter oligodendrocyte. A more in depth analysis by 3D reconstruction is required to shed more insight and better address this question.

Figure II-1. ***Cre*****-mediated gene inactivation leads to loss of β1 protein.**

[The experiments in this part of the results were done by our collaborators, Drs. Claudia S. Barros and Ulrich Muller.]

This figure is modified from Barros and Nguyen et al. *Development* (2009). **(A)** Diagram of *Nestin-Cre* mediated gene inactivation in *Itgb1-CNSko* mutants. *Itgb1* (*β1*) *flox* allele before and after recombination (rec.) and *Itgb1-null* allele are shown. *LoxP* sites are indicated as black triangles and exons are numbered. **(B)** PCR analysis of DNA obtained from P19 spinal cord of wild-type and *Itgb1-CNSko* (β*1mt*) mice. In DNA from *Itgb1-CNSko* mice, bands corresponding to the *Nestin-Cre* transgene, the *Itgb1-null* allele, and the recombined *Itgb1-flox* allele were detected. **(C)** Immunoblotting with β1 antibody using spinal cord extracts from mice at P7, P19 and P30 showed loss of β1 protein in the mutants. Tubulin served as loading controls.


A
ATG
2
3
4
β1 flox
Nestin CRE-mediated gene inactivation
3
4
β1 rec. flox
Itgb1-CNSko
neo
β1 null


B
WT
β1mt
kb
0.5
0.7
1.0
CRE
β1 null
β1 rec. flox

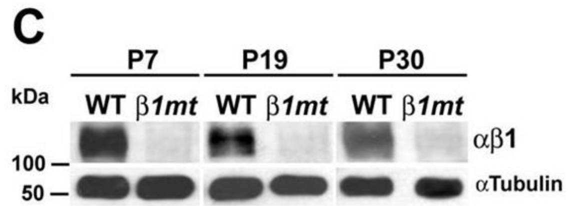
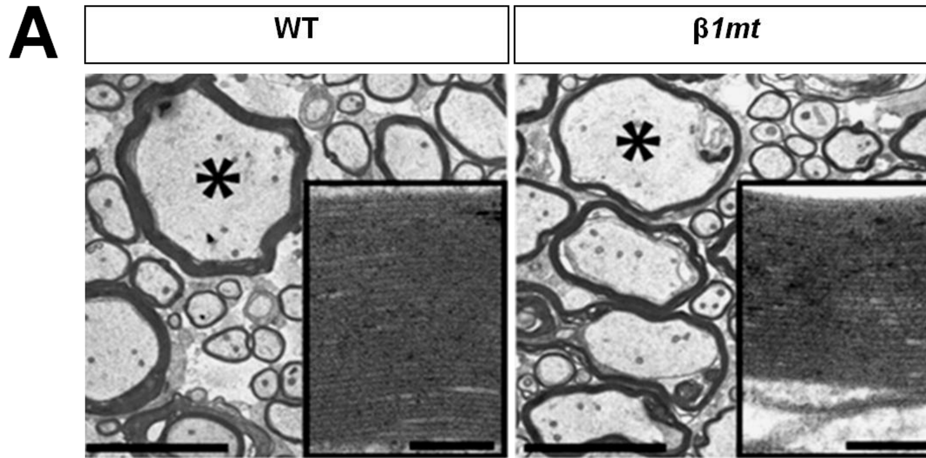
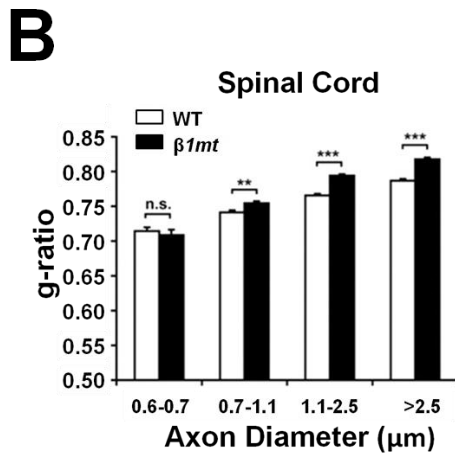

C
P7
P19
P30
kDa
WT
β1mt
WT
β1mt
WT
β1mt
αβ1
100
50
αTubulin

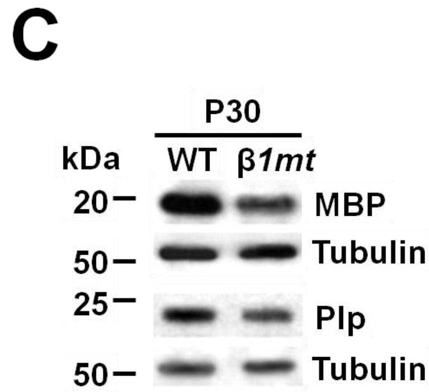
Figure II-2. Myelination defects in the spinal cords, optic nerves and cerebellum of *Itgb1-CNSko* mutants.

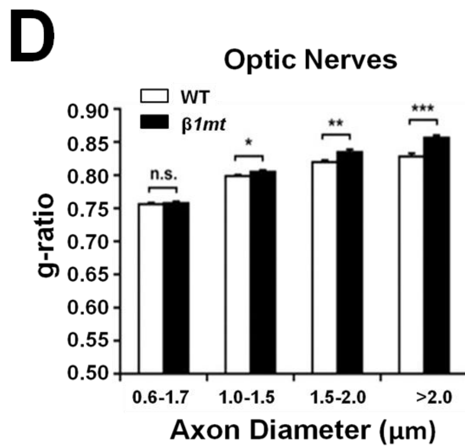
[The experiments in this part of the results were done by our collaborators, Drs. Claudia S. Barros and Ulrich Muller.]

This figure is modified from Barros and Nguyen et al. *Development* (2009). All values are shown as mean±SEM; n=3; n.s.=not significant; *p<0.05; **p<0.01; ***p<0.001; and scale bars=5μm and 100nm for insets. **(A)** Electron microscopy analysis of myelinated fibers in spinal cord sections from P30 *Itgb1-CNSko* show thinner myelin compared to wild-type mice. Asterisks (*) indicate axons of similar size. Insets in lower panels show higher magnifications of myelin wraps from indicated axons reveal no defect in myelin periodicity. **(B)** G-ratios (diameter axon/diameter fiber) quantification of fibers grouped by axon diameter from spinal cords. Myelinated fibers of *Itgb1-CNSko* spinal cord have significantly higher g-ratios indicating thinner myelin compared to wild-type. **(C)** Immunoblotting for MBP and PLP in spinal cord extracts from P30 mice. *Itgb1-CNSko* mice express less myelin components, MBP and PLP compared to wild-types. Tubulin served as controls for loading. **(D)** The g-ratios of optic and **(E)** cerebellum nerves fibers grouped by axon diameter are also shown. Similarly, myelinated fibers of *Itgb1-CNSko* optic nerves and cerebellum also have significantly higher g-ratios indicating thinner myelin compared to wild-type.


A
WT
β1mt


B
Spinal Cord
WT
β1mt
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
g-ratio
n.s.
**
***
***
0.6-0.7
0.7-1.1
1.1-2.5
>2.5
Axon Diameter (μm)


C
P30
kDa
WT
β1mt
20
MBP
50
Tubulin
25
Plp
50
Tubulin


D
Optic Nerves
WT
β1mt
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
g-ratio
n.s.
*
**
***
0.6-1.7
1.0-1.5
1.5-2.0
>2.0
Axon Diameter (μm)

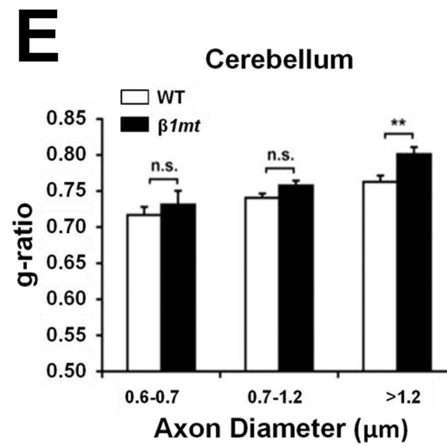
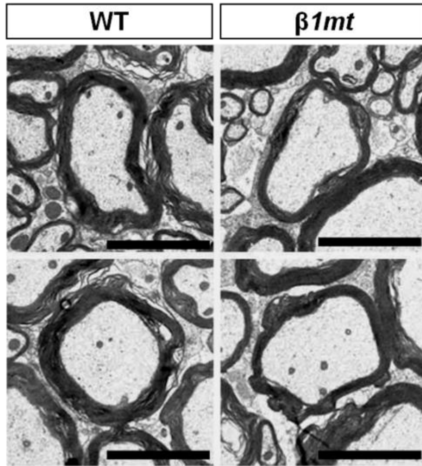

E
Cerebellum
WT
β1mt
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
g-ratio
n.s.
n.s.
**
0.6-0.7
0.7-1.2
>1.2
Axon Diameter (μm)

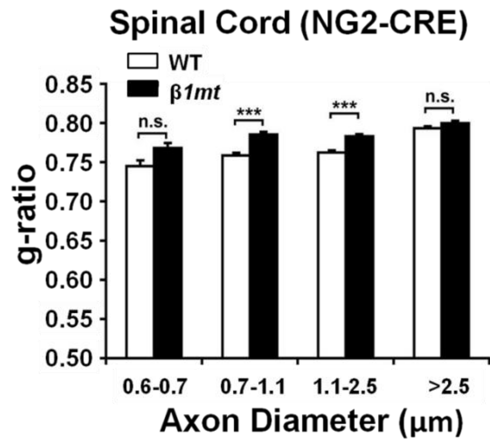
Figure II-3. Myelination defects in the spinal cords of *Itgb1-OL-ko* mutants derived using *NG2-Cre*.

[The experiments in this part of the results were done by our collaborators, Drs. Claudia S. Barros and Ulrich Muller. In order to be consistent with the spinal cord g-ratio analysis, I perfused and harvested the samples for our collaborators for EM imaging and analysis.]

This figure is modified from Barros and Nguyen et al., *Development* (2009). All values are shown as mean±SEM; from 2 mutant and wild-type littermate pairs; n.s.=not significant; *p<0.05; ***p<0.001; and scale bars=2.5μm. **(A)** Electron micrographs of myelinated fibers from P30 spinal cord sections of *Itgb1-OL-ko* and wild-type littermates generated using the *NG2-Cre* driver. **(B)** G-ratios of fibers grouped by axon diameter. Although moderate, but significantly higher g-ratios in *Itgb1-OL-ko* spinal cord fibers suggesting the myelin defects in the *Itgb1-CNSko* mice are, at least partially, due to the loss of β1 integrins in oligodendrocytes.



A
WT
β1mt



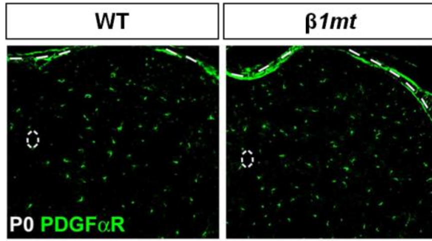
B
Spinal Cord (NG2-CRE)
WT
β1mt
n.s.
***
***
n.s.
g-ratio
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.6-0.7
0.7-1.1
1.1-2.5
>2.5
Axon Diameter (μm)

Figure II-4. Normal oligodendrocyte lineage progression in *Itgb1-CNSko* mice.

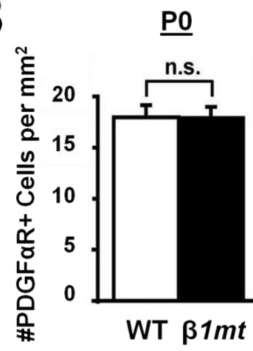
[The experiments in this part of the results were done by our collaborators, Drs. Claudia S. Barros and Ulrich Muller.]

This figure is modified from Barros and Nguyen et al. *Development* (2009). All values are shown as mean±SEM; n=4; n.s.=not significant; and scale bars=100µm. **(A)** Spinal cord cross sections from *Itgb1-CNSko* and wild-type mice immunostained with antibodies to the oligodendrocyte progenitor marker PDGFαR (green) at P0 and **(C)** with antibodies to the mature oligodendrocyte marker APC (CC1, green) at P19. Dotted lines mark spinal cord borders and central canal. **(B)** Cell density quantifications showed no significant difference in the numbers of PDGFαR-positive progenitors or **(D)** in the numbers of mature CC1-positive cells between P19 and P60 *Itgb1-CNSko* mutants and wild-type.

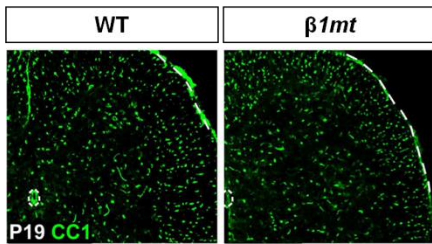
A



B



C



D

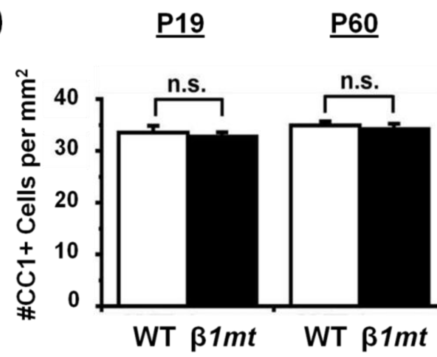
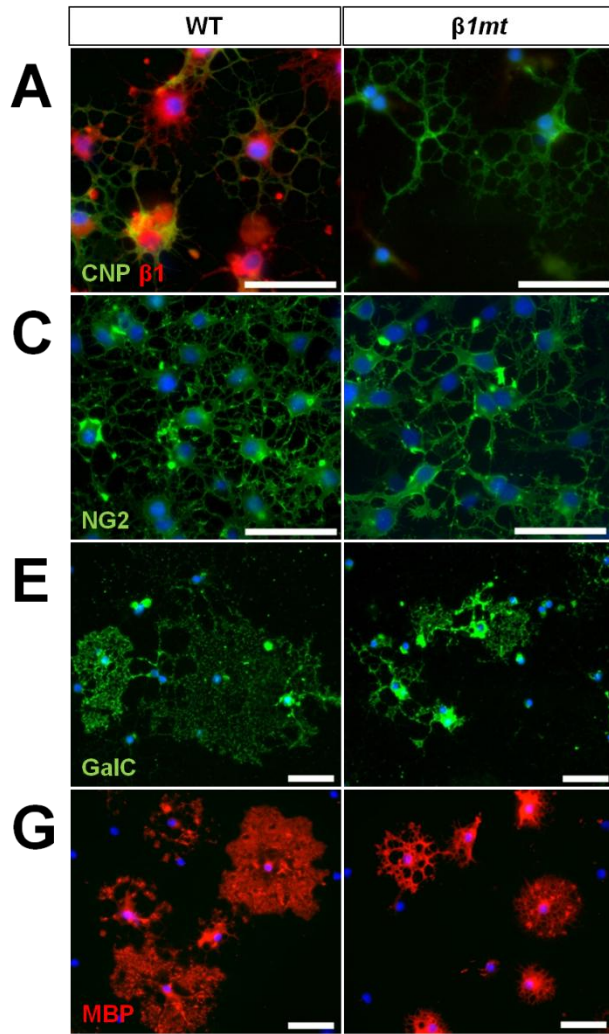
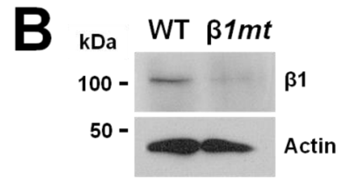
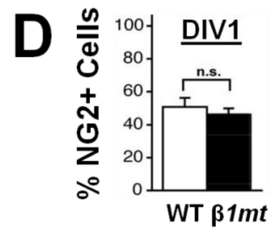


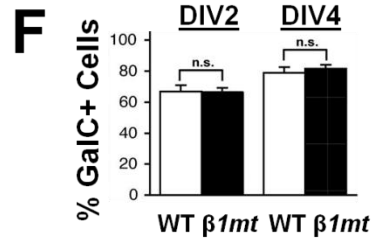
Figure II-5. Normal lineage progression in cultured *Itgb1-CNSko*-derived oligodendrocytes.

This figure is modified from Barros and Nguyen et al. *Development* (2009). All values are shown as mean±SEM; n=4; n.s.=not significant; *p<0.05; and scale bars=50μm. **(A)** Immunocytochemistry was used to visualize the oligodendroglia marker CNPase (green) and β1 integrin (red) at 2-DIV to confirm the lack of β1 integrin protein detection in oligodendrocytes cultured from *Itgb1-CNSko*, but not wild-type mice. All images show counterstaining with DAPI to visualize nuclei (blue). **(B)** Immunoblotting with β1 antibody using oligodendrocytes cell extracts derived from P2 pups. Western blot analysis of lysates obtained from oligodendrocytes purified from wildtype and *Itgb1-CNSko* mice revealed a severe reduction in the levels of β1 integrin protein in mutant cells (β*1mt*). Actin blots were performed as a loading control. **(C)** Immunocytochemistry was used to visualize the oligodendrocyte progenitor marker NG2 (green) at 1-DIV, **(E)** mature oligodendrocyte marker GalC (green) at 4-DIV, and **(G)** mature oligodendrocyte marker MBP (red) at 4-DIV and counter stained with DAPI to visualize nuclei. **(D)** Quantifications of percentage of NG2-positive oligodendrocytes, **(F)** GalC-positive oligodendrocytes, and **(H)** MBP-positive oligodendrocytes show no significant differences between β*1mt* and wild-type oligodendrocytes at 1-DIV, 4-DIV and 6-DIV, respectively.


WT
β1mt
A
CNP β1
C
NG2
E
GalC
G
MBP


B
kDa
WT β1mt
100 -
50 -
β1
Actin


D
DIV1
% NG2+ Cells
100
80
60
40
20
0
n.s.
WT β1mt


F
DIV2
DIV4
% GalC+ Cells
100
80
60
40
20
0
n.s.
n.s.
WT β1mt WT β1mt

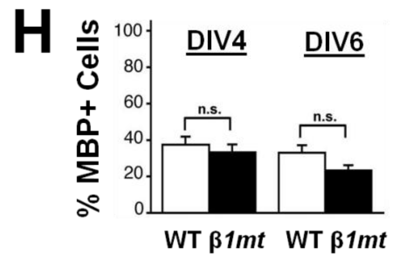
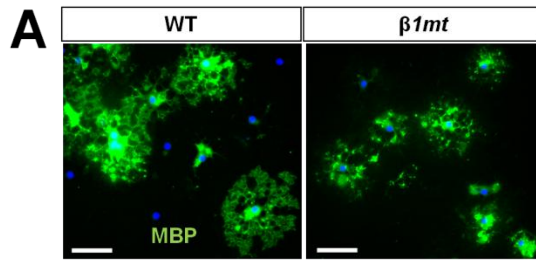
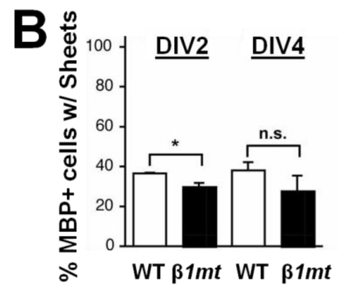
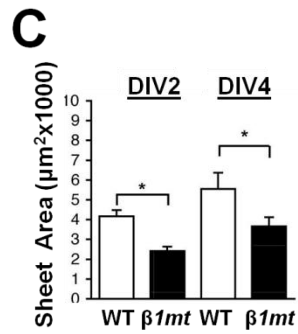

H
DIV4
DIV6
% MBP+ Cells
100
80
60
40
20
0
n.s.
n.s.
WT β1mt WT β1mt

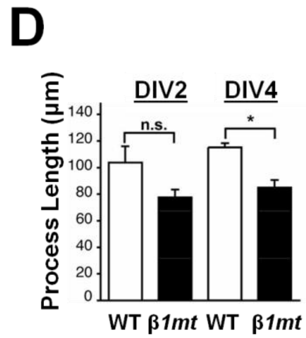
Figure II-6. ***Itgb1-CNSko*** **oligodendrocytes have smaller myelin membrane sheets.**

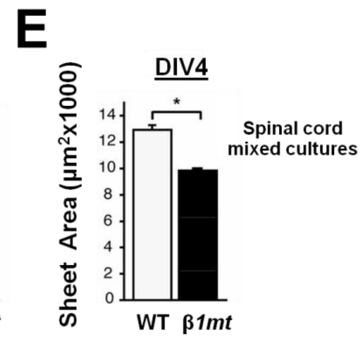
This figure is modified from Barros and Nguyen et al. *Development* (2009). All values are shown as mean±SEM; n=4; n.s.=not significant; *p<0.05; and scale bars=50μm. **(A)** Images of oligodendrocytes derived from wild-type and *Itgb1-CNSko* mice that were differentiated for 4-DIV, then visualized using MBP immunocytochemistry (green) and counterstained with DAPI (blue). **(B)** Graph of percentage of MBP-positive oligodendrocytes containing visible sheet-like morphology at 2-DIV and 4-DIV. **(C)** Graph of the mean area of MBP-positive myelin membranes at 2-DIV and 4-DIV. **(D)** Graphs show the length of the longest process within each myelin membrane sheet at 2-DIV and 4-DIV. **(E)** Graph of the area of MBP-positive myelin sheets from 2-DIV and 4-DIV mixed glial cultures obtained from spinal cords of P0 wild-type and *Itgb1-CNSko*. **(F)** Example pictures depicting morphometric analysis performed on MBP-positive wild-type (F-F'') and β1 mutant (GG'') oligodendrocytes.


A
WT
β1mt
MBP


B
% MBP+ cells w/ Sheets
DIV2
DIV4
*
n.s.
WT β1mt WT β1mt


C
Sheet Area (μm²x1000)
DIV2
DIV4
WT β1mt WT β1mt


D
Process Length (μm)
DIV2
DIV4
n.s.
WT β1mt WT β1mt


E
DIV4
Sheet Area (μm²x1000)
Spinal cord
mixed cultures
WT β1mt

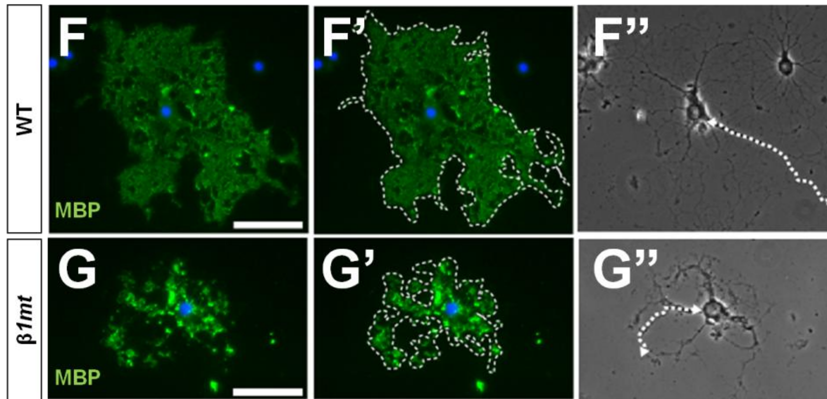
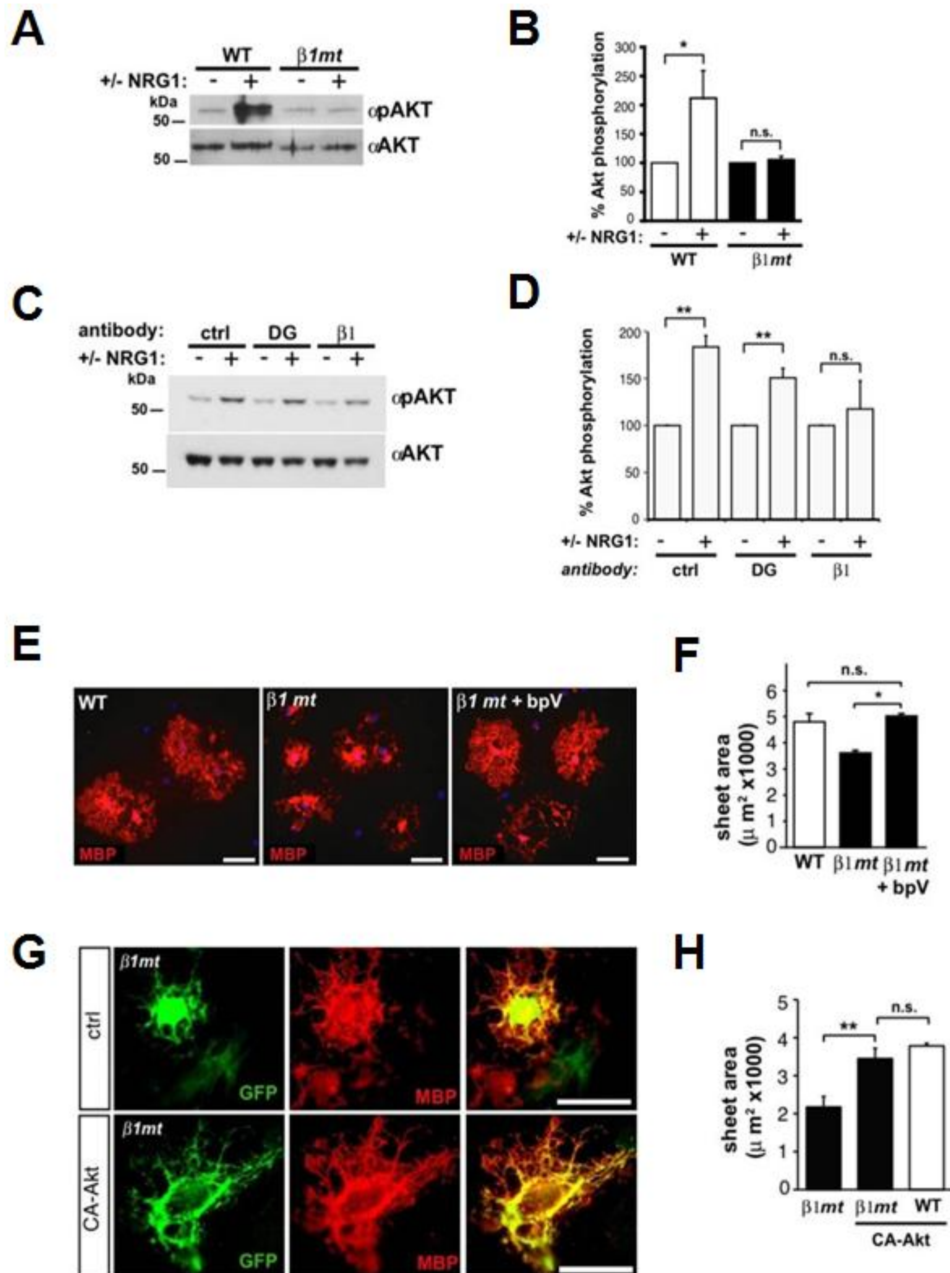

WT
β1mt
F
F'
F''
G
G'
G''
MBP
MBP

Figure II-7. β1 integrin function in myelin sheet outgrowth is mediated by AKT.

This figure is modified from Barros and Nguyen et al. *Development* (2009). All values are shown as mean±SEM; n=3 (2 animals per group, 12 to 25 cells measure per group, paired *t*-test for CA-AKT experiments in G and H); n.s.=not significant; *p<0.05; **p<0.01; and scale bars=50μm. **(A)** Lysates from neuregulin-1 (NRG1) stimulated 1-DIV oligodendrocytes derived from wild-type and *Itgb1-CNSko* brains and evaluated by Western blot to detect pAKT (phospho-Ser473) and total AKT. **(B)** Densitometric analysis of relative AKT phosphorylation (ratio of pAKT/total AKT) in which β*1mt* lack AKT phosphorylation response upon treating with NRG1 as seen with wild-type oligodendrocytes. **(C)** Lysates from 1-DIV rat oligodendrocytes treated with control (ctrl), dystroglycan (DG), or β1 integrin blocking antibodies followed by stimulation with neuregulin-1 (NRG1) and evaluated by Western blot to detect pAKT (phospho-Ser473) and total AKT. **(D)** Densitometric analysis of relative AKT phosphorylation (ratio of pAKT/total AKT). Blocking β1 integrin function with blocking β1 integrin antibody also has similar non-AKT phosphorylation response as seen in β*1mt*. This is not the case when blocking DG where NRG1 treatment yields similar AKT phosphorylation response as seen in wild-type controls. **(E)** Representative images of MBP^+ oligodendrocytes (red) from wild-type and β1-deficient oligodendrocytes differentiated for 4-DIV in the presence or absence of PTEN inhibitor, bpV, Nuclei are visualized using DAPI (blue). **(F)** Graph of the area of MBP-positive myelin sheets showed that mutant oligodendrocytes treated with bpV have sheet area similar to untreated wild-type oligodendrocytes. **(G)** Representative images of MBP^+ (red) and GFP^+ (green) oligodendrocytes from wild-type and β1-deficient oligodendrocytes transfected with either CA-AKT-GFP or control CFP constructs. **(H)** Graph of the area of MBP-positive myelin sheets showed that mutant oligodendrocytes transfected with CA-AKT have significantly larger sheets than mutant oligodendrocytes transfected with control CFP.


A
WT
β1mt
+/- NRG1:
kDa
50
αpAKT
αAKT
B
% Akt phosphorylation
n.s.
WT
β1mt
C
antibody:
ctrl
DG
β1
αpAKT
αAKT
D
antibody:
E
MBP
β1 mt + bpV
F
sheet area (μ m² x1000)
+ bpV
G
ctrl
CA-Akt
GFP
H
CA-Akt

Chapter IV: β1 integrins regulate the ability of oligodendrocytes to myelinate axons in the corpus callosum

Abstract

Myelination in the central nervous system (CNS) is carried out by specialized glia termed oligodendrocytes. In the previous chapter we show that in the CNS, β1 integrins are required to achieve normal levels of myelin wrapping and, in oligodendroglia, are able to promote AKT-dependent myelin sheet outgrowth. Here, in addition to thinner myelin, genetically modified mice lacking β1 integrins in the CNS have a substantial decrease in the percentage of myelinated axons in the corpus callosum, a major white matter tract of the cerebral cortex. This is in contrast to the optic nerve, cerebellum, and spinal cord, where normal percentages of myelinated axons are achieved. Although β1 integrin mutant mice exhibited modest delays in oligodendrocyte generation during initial phases of myelination, the number of mature oligodendrocytes in the cerebral cortex of mutant mice eventually reached levels equivalent to those of

wild-type mice. These findings indicate that the reduced numbers of myelinated axons observed in mutant cerebral cortices were not caused by a lack of oligodendroglia, but instead, oligodendrocytes derived from mutant mice were found to extend fewer primary processes, exhibit shorter process lengths, and have decreased process complexity, i.e. branching, suggesting that each individual mutant oligodendrocyte may be incapable of interacting with appropriate numbers of axons. Together, these data suggest that in some regions of the CNS, β1 integrins influence myelination by promoting oligodendrocyte process development to ensure an adequate ratio of myelinated axon to oligodendrocyte.

Introduction

Myelination in the CNS first requires the proper development of oligodendrocyte progenitor cells to premyelinating oligodendrocytes. Premyelinating oligodendrocytes then have to initiate glial-axon contact with nearby axons (Bozzali and Wrabetz, 2004; Nave and Trapp, 2008). Oligodendrocytes can extend multiple processes to make contact with up to 50 axon segments (Pfeiffer et al., 1993). Once glial-axon contact is established, oligodendrocytes undergo myelin wrapping that requires membrane expansion (Bozzali and Wrabetz, 2004; Nave and Trapp, 2008; Pfeiffer et al., 1993). This process has been proposed to rely on bidirectional communication between glial cells and axons that could be mediated by cell surface molecules such as integrins.

Regulation of oligodendrocyte development has been studied *in vitro* where adhesion molecules, such as integrins, have been reported to play pivotal roles in controlling OPCs proliferation, survival and differentiation (Benninger et al., 2006; Frost et al., 1999). Oligodendrocyte culture studies have also shown that signal transduction cascades involving laminin-2, β1 integrin, and ILK are required for myelin sheet formation (Chun et al., 2003; Colognato et al., 2002; Frost et al., 1999; Liang et al., 2004). Furthermore, integrins have been suggested

to be important in axon-glial contact (Câmara et al., 2009; Colognato et al., 2002). Also, recent studies have demonstrated that β1 integrins are required for Schwann cell myelination, specifically for radial sorting (Grove et al., 2007; Nodari et al., 2007; Pereira et al., 2009). Together, these studies suggest that integrins may play roles in oligodendrocyte-axon interactions to ensure proper CNS myelination.

CNS myelination proceeds in a caudal-to-rostral fashion from the spinal cord to the forebrain (Foran and Peterson, 1992). Interestingly, previously described genetic mouse models display regional CNS myelination defects. Knockout mice that lack the Src Family Kinase *fyn* (Sperber et al., 2001) and the *dy/dy* (laminin-deficient) dystrophic mice have hypomyelination in the forebrain but not the spinal cord (Chun et al., 2003; Relucio et al., 2009). Conversely, *PDGF-A* null mice are severely hypomyelinated in the optic nerve and spinal cord (Fruttiger et al., 1999). In addition, transgenic mice expressing a dominant negative (*Itgb1*Δc) form of β1 integrin in oligodendrocytes exhibit hypomyelination in the optic nerve and spinal cord but not in the forebrains (Lee et al., 2006). This prompted further investigation in another region of the brain, the corpus callosum.

In this chapter, I studied the role of β1 integrins in oligodendrocyte development and myelination specifically in the corpus callosum, a major white matter tract of the cerebral cortex using genetically modified mice lacking β1

integrins in the CNS. Axons of the corpus callosum of mutant mice were highly unmyelinated in contrast to what was observed in the optic nerve, cerebellum, and spinal cord (see previous chapter), where percentages of myelinated axons were comparable to wild-type animals. Oligodendrocytes derived from mutant mice were found to extend fewer primary processes and have decreased process complexity, i.e. branching, suggesting that each individual mutant oligodendrocyte may be incapable of interacting with appropriate numbers of axons.

Results

Myelination defects in the corpus callosa of *Itgb1-CNSko* mice.

To examine myelin sheaths in the forebrains of *Itgb1-CNSko* mice, I analyzed the corpus callosum, a major white matter tract of the cerebral cortex, by electron microscopy (figure III-1A). In adult mutants, the thickness of myelin surrounding axons was reduced compared to wild-type littermates. Morphometric analysis (figure III-1D) of the nerve fibers revealed a statistically significant increase in the ratio of axon diameter to fiber diameter (g-ratio) (WT, 0.75±0.0037; β*1mt*, 0.85±0.0022, n=3, Mann-Whitney test, $p<0.001$). Individual corpus callosum g-ratios were plotted as a function axon diameter (figure III-1F) and linear regression showed mutants have increased in g-ratios (thinner myelin) especially in the smaller diameter axons. This was similar to other regions of the CNS (Chapter III), where increased g-ratios were also observed. Interestingly, however, in the corpus callosum there was also a significant increase in the percentage of unmyelinated axons (figure III-1C) in the mutants compared to wild-type littermates (49.87±11.58%; 79.35±4.16%, respectively, n=3, paired *t*-test, $p=0.043$), but not in the largest caliber axons (>1.0µm diameter). This was in

contrast to what was observed in other CNS regions i.e. spinal cord, cerebellum, and optic nerve, where no change in the percentage of myelinated axons was observed. In addition, there were no differences in the distribution of axon sizes (figure III-1E); however, there was an increase in overall axon density in the mutants. This maybe a consequence of increased numbers of unmyelinated small caliber axons per area in the mutants as myelinated axons occupy more space compared to unmyelinated axons.

The overall organization of the corpus callosum was examined by using myelin basic protein (MBP) immunoreactivity to visualize the white matter tracts of juvenile postnatal (P) day 22 and adult P85 mice. Mutant mice had thinner MBP-positive tracts in the corpus callosum, and fibers appeared sparser compared to wild-type littermates (figure III-2A). Consistent with a pattern of fewer myelinated axons, levels of MBP protein were significantly less in the mutant cerebral cortices compared to wild-type littermates at P20 and P80 (40.72±0.25, n=3, paired *t*-test, p=0.037; 46.71±0.17, n=3, paired *t*-test, p=0.032, respectively). *Itgb1-CNSko* mice lack β1 integrins in both glial cells and neurons, and thus it is possible that reduced MBP level could be due in part to fewer cortical neurons. To address this possibility, immunohistochemistry against neurofilament (NF) was used to visualize axons in P22 and P85 corpus callosum (figure III-2C). There appears to be less NF immunoreactivity in the mutants compared to the WT. In addition, NF immunoreactivity is absent from striated fibers. This appears

to contradict the axon density findings from our EM analysis. Since our NF antibody recognizes the mature phosphorylated form of neurofilament, it is possible that the less mature axons are not detectable at the immunostaining level. Also, that the loss of axonal integrins could have a negative impact on cortical neuron maturation and the myelination process.

Corpus callosa of *Itgb1-CNSko* mice have normal oligodendroglial lineage progression.

One possibility is that the reduced level of myelination in the corpus callosum of *Itgb1-CNSko* mutants could arise from defects in oligodendrocyte development. I therefore analyzed oligodendrocyte development using lineage stage-specific markers. The number of NG2-positive oligodendrocyte progenitors (figures III-3A, III-3B) at P20 was significantly reduced in corpus callosum of mutant mice compared to wild-type (443.91±23.63/μm^2, 572.04±40.42/μm^2, respectively, n=3, paired *t*-test, **p=0.012). Interestingly, NG2-positive oligodendrocyte progenitors at P5 and P10 of age, which is prior to the peak of myelination in the corpus callosum, showed a trend towards fewer cells, but this was not statistically significant (P10; WT, 944.53±118.83/μm^2, β*1mt*, 876.56±146.74/μm^2, n=3, paired *t*-test, p=0.77). However, *Itgb1-CNSko* brain

lysates from juvenile P20 mice were consistently observed to have lower levels of NG2 protein (figures III-3E, III-3F; 73.42±11.02%, n=3, paired *t*-test, p=0.16), but were comparable to wild-type animals at adult age, P80.

As oligodendrocyte progenitor cells exit the cell cycle and differentiate into mature oligodendrocytes, APC (CC1) is highly expressed (Baron et al., 2005). CC1 immunohistochemistry was used to further examine if the reduction in NG2-positive cells persisted in later stages of oligodendrocyte development. Similarly, there was a significant reduction in mature CC1-positive oligodendrocytes (figures III-3C, III-3D) at P20 in mutant mice compared to wild-type (1326.97±129.00/μm^2, 2072.64±89.34/μm^2, respectively, n=3; paired *t*-test, **p=0.0063). Brain lysates from juvenile *Itgb1-CNSko* P20 animals were also observed to have significantly lower levels of the oligodendrocyte protein, CNP (figures III-3E, III-3G; 50.91±11.02%, n=3, paired *t*-test, **p=0.0026), a trend that persisted at P80. However, CC1 counts in P80 mutant mice appear to be fewer when compared to those of wild-type littermates, but were not statistically significant (figure III-3D). These data suggested that the reduced numbers of myelinated axons observed in mutant corpus callosum could be a result of having fewer oligodendrocytes during the appropriate window for normal myelination.

Oligodendrocyte proliferation and cell death in the corpus callosa of *Itgb1-CNSko* mice.

Several reports have indicated that α6β1 integrin is important for oligodendroglial proliferation and survival (Benninger et al., 2006; Colognato et al., 2002), properties that could contribute to the myelination defects observed in the *Itgb1-CNSko* mutant corpus callosum. First, I tested if the lack of β1 integrin affected oligodendrocyte progenitor cell proliferation using immunohistochemistry to visualize proliferating cell nuclear antigen (PCNA) in conjunction with the oligodendrocyte precursor cell marker, NG2 (figures III-4A, III-4C). Counts of double positive $NG2^{+}PCNA^{+}$ showed a minor reduction in proliferative oligodendrocyte progenitors of P5 mutants. Proliferation of OPCs was reduced at P10, with a 16.71% reduction in double positive $NG2^{+}PCNA^{+}$ in mutants compared to wild-type littermates ($54.49 \pm 2.24/\mu m^2$, $65.42 \pm 1.30/\mu m^2$, respectively, n=3; paired *t*-test, p=0.032). At P20, however, mutants had a substantial fewer proliferative oligodendrocyte progenitors, but were not statistically significant when compared to wild-type littermates ($36.08 \pm 1.90/\mu m^2$, $48.04 \pm 6.33/\mu m^2$, respectively, n=3; paired *t*-test, p=0.092). Thus, while very modest differences existed in *Itgb1-CNSko* mutant oligodendrocyte progenitor cell proliferation, it was unlikely to be an underlying factor in myelination abnormalities. However, it does not rule out the possibility of insufficient or

dysregulated OPC proliferation prior to the critical time, P14, of CNS myelin development at which is the peak of myelination in the corpus callosum.

Next, I evaluated the role of β1 integrin in oligodendrocyte death using terminal TdT-mediated dUTp nick end labeling (TUNEL), both *in vitro* and *in vivo*. β1-KO oligodendrocytes derived from P2 *Itgb1-CNSko* mice were allowed to differentiate for 4-days *in vitro*, followed by TUNEL in conjunction with a immunocytochemistry for GalC, a marker for oligodendrocytes (figure III-4E). The percentage of $TUNEL^{+}GalC^{+}$ cells were significantly increased in oligodendrocytes lacking β1 integrin compared to that in wild-type mice (figure III-4F; 41.47±3.225%, 29.98±1.67%, respectively, n=4, paired *t*-test, **p=0.0054). The percentage of $TUNEL^{+}MBP^{+}$ oligodendrocytes was also increased, showing that the increase in apoptotic cell death persisted in mature myelinating oligodendrocytes of β1-KO differentiated for 4-days *in vitro* (figure III-4G; 33.11±2.84%, 20.61±1.81%, respectively, n=4, paired *t*-test, **p=0.0015). In contrast, however, *in vivo* analysis showed no statistically significant differences in the numbers of apoptotic cells, either in progenitor or mature stages of oligodendrocyte development (figures III-4B, III-4D). The above data suggested that cell death is not a major component contributing to the myelination defects in the corpus callosum of *Itgb1-CNSko* mice.

Oligodendrocyte process dynamics and outgrowth.

Glial-axon contacts are thought to be initiated by oligodendrocyte process extension towards axons (Pfeiffer et al., 1993). Integrins are known to regulate the actin cytoskeleton that, together with the plasma membrane (Anitei and Pfeiffer, 2006; Krämer et al., 2001), is extensively remodeled during oligodendrocyte process extension. I hypothesized that the β1 integrin could influence the process outgrowth and complexity required for oligodendrocytes to interact with axons and initiate myelination. To address this, β1 integrin ligand binding was inhibited by treating rat oligodendrocytes with β1 integrin blocking antibody (Ha-2/5). Primary rat OPCs were isolated from P2 pups and differentiated on laminin substrate for 1-DIV and 2-DIV. Ha-2/5 blocking antibody was added 10 minutes post seeding of rat OPCs onto laminin-coated chamber slides. Differentiation media with fresh Ha-2/5 blocking antibody was changed daily. An isotype matched antibody was used in control conditions. Semi-automatic Sholl analysis was then performed on images of phalloidin-labeled oligodendrocytes to best visualize processes. I found that by blocking β1 integrins, oligodendrocytes process complexity (i.e. branching) was significantly decreased compared to control treated oligodendrocytes (figures III-5C, III-5D). Next, I asked if the lack of β1 integrin affected the number of primary processes in maturing oligodendrocytes. Here, isolated β1-KO OPCs derived from P2 *Itgb1-CNSko* pups

were differentiated for 2-DIV and 4-DIV on laminin-coated chamber slides. β1-KO oligodendrocytes had significantly fewer primary processes and decreased process complexity when compared to wild-type cells (figures III-6B, III-6F). Our data indicate that individual β1 mutant oligodendrocytes may display abnormal process dynamics. This defect may, in turn, cause these mutant oligodendrocytes to be incapable of interacting with the appropriate number of axon segments during development *in vivo*.

Discussion

Here I present evidence that mice lacking β1 integrin gene expression, in addition to thinner myelin globally in the CNS, have fewer myelinated axons in the corpus callosum. Myelin basic protein (MBP), a major protein in myelin was reduced in *Itgb1-CNSko* mice. β1-deficient oligodendrocytes were also found to extend fewer primary processes and to have less process branching. In addition, although *in vitro* studies showed significantly increased apoptosis in β1 integrin-deficient cortical oligodendrocytes, the corpus callosa of *Itgb1-CNSko* mice appear to have higher levels of oligodendroglial death, although these changes were not statistically significant. Finally, oligodendroglial lineage progression is normal. Taken together, these findings suggest that, while dispensable to generate oligodendroglia, β1 integrins promote oligodendrocyte process extension and branching to ensure the appropriate ratio of myelinated axons to oligodendrocytes.

Oligodendrocytes extend numerous processes that contact and myelinate up to 50 axonal segments (Baumann and Pham-Dinh, 2001). Thus, process outgrowth is important to ensure the appropriate ratio of oligodendrocytes to axons. In this study, β1 integrin-deficient oligodendrocytes extended fewer

primary processes. In addition to having fewer primary processes, oligodendrocytes that are deficient in β1 integrins exhibited decreased complexity of process branching, as determined by Sholl analysis. It remains unclear; however, which integrin-regulated downstream effectors are dysregulated by the absence of oligodendroglial integrins. However, studies have linked several candidate effector molecules to oligodendroglial integrins, include focal adhesion kinase (FAK) (Forrest et al., 2009), small Rho family GTPases (Liang et al., 2004), and other cytoskeleton modulators such as WAVE1 (Kim et al., 2006). In the case of FAK, the phosphorylation of FAK, a scaffold and kinase protein shown previously to act downstream of integrins, has been shown to regulate oligodendroglial process outgrowth (Forrest et al., 2009; Schlaepfer and Hunter, 1998). In addition, the knockdown of FAK expression in the CG4 oligodendrocyte cell line suppressed process outgrowth on laminin, an integrin ligand (Hoshina et al., 2007). Also, the optic nerve of oligodendrocyte-specific FAK knockout mice has a significant reduction in the number of myelinated fibers, while the oligodendrocytes themselves had fewer primary processes (Forrest et al., 2009). Moreover, reducing FAK activity using a dominant negative FAK strategy (overexpression of the partial FAK protein, FRNK) markedly abolished rho GTPase cdc42 activity in MDCK cells (Yeh et al., 2009). In oligodendrocytes themselves, the ablation of Cdc42 leads to a dysregulation of

process outgrowth, correlating with the formation of abnormal myelin outfolding (Thurnherr et al., 2006).

A common feature of integrin-mediated intracellular signaling is the activation of cytoskeletal regulators. For instance, the actin nucleation factors N-WASP and Arp2/3 work together to initiate microfilament branching that in turn widen membrane protrusions (Soderling, 2009). The Wiskott-Aldrich syndrome protein family verprolin homologous (WAVE) proteins have been shown to mediate process dynamics in oligodendrocytes. Oligodendrocytes expressing a dominant-negative form of WAVE1 exhibit impaired process outgrowth and lamellopodia formation *in vitro* (Kim et al., 2006). Similarly, WAVE1-deficient oligodendrocytes formed fewer processes (Kim et al., 2006). Given that our findings indicate an important role for integrin in oligodendrocyte process dynamics, it may be that integrins act, at least in part, by modulating cytoskeletal regulators such as WAVE1.

During development, the myelination of the CNS proceeds in a caudal-to-rostral fashion (Foran and Peterson, 1992). Besides this temporal gradient in myelination, however, there is considerable debate as to whether the regulatory mechanisms that control myelination are equivalent in different regions of the brain. In support of regional variability, region-specific myelination defects have been observed in various gene loss-of-function mouse models. For instance, in the

optic nerves and spinal cords of mice expressing a dominant negative form of β1 integrin, white matter deficits include thinner myelin and increased percentage of unmyelinated axons. However, the myelin in the corpus callosum did not show these abnormalities (Lee et al., 2006). Laminin-deficient (*dy/dy*) mice also exhibit regional variations, with an increase in unmyelinated fibers in the corpus callosum, whereas other CNS regions having normal percentages of myelinated fibers, albeit with thinner myelin (Chun et al., 2003; Relucio et al., 2009). Moreover, Fyn-knockout mice display profound regional differences in their myelination defects (Sperber et al., 2001). Similarly, I observed regional variation in myelination defects in the *Itgb1-CNSko* mouse. Thus, while the spinal cord and other regions contain normal percentages of myelinated axons, but have thinner myelin, the corpus callosum is unique in that it has an increase in unmyelinated fibers.

A possible explanation behind the variation in observed myelin deficits is that axons in the different brain regions exhibit various mean calibers, with the spinal cord having a large average axon diameter and the corpus callosum being composed mostly of small diameter axons (Sherman and Brophy, 2005). In the PNS, myelin wrapping has been shown to be regulated by axonal neuregulin (Brinkmann et al., 2008), a property that correlates with axonal diameter, such that larger caliber are associated with higher levels of surface neuregulin and with thicker myelin (Galin V. Michailov et al., 2004). Also, the observation of a

transient hypomyelination phenotype found in the optic nerves of mice expressing a dominant negative chimeric form of β1 integrin suggests a threshold axon caliber size requirement for myelination (Câmara et al., 2009). This may be mediated in part by the presence of the integrin ligand laminin found in association with some premyelinating white matter tracts and, upon α6β1 integrin ligation, has been reported to amplify growth factor signaling by NRG (Colognato et al., 2002). This suggests that there may be heterogeneous oligodendrocyte populations from different CNS regions and/or oligodendrocyte myelination is regulated by extrinsic factors such as, components of the extracellular matrix, growth factors, and axonal cues. It is beyond the scope of this study, but will be interesting to investigate if this is the case.

My *in vitro* study with β1 integrin-deficient oligodendrocytes showed that oligodendrocyte cell death is increased *in vitro*. Interestingly, there is an apparent increase in oligodendrocyte cell death in the corpus callosum of the *Itgb1-CNSko* mouse. However, this is not statistically significant due to high variability in TUNEL detection. Alternatively, I could examine and score for cleaved caspase 3 for apoptosis. Although there was a decrease in OPC and newly formed oligodendrocyte cell numbers at P20, the numbers of these cells normalize to wildtype levels by P80. This suggests that other factors such as growth factors and extracellular matrix molecules and the present of axons *in vivo* may compensate for the lack of β1 integrins to promote oligodendrocyte survival. In agreement

with our findings, studies with dominant negative forms of integrin and oligodendrocyte-specific knockouts of β1 integrins reported no significant changes in either oligodendrocyte proliferation or cell death (Benninger et al., 2006; Câmara et al., 2009; Lee et al., 2006). Moreover, signaling through growth factors such as NRG has been shown to be amplified by α6β1 integrin to promote axonal dependent oligodendrocyte survival (Colognato et al., 2002). Also, I observed little significant change in OPC proliferation in the *Itgb1-CNSko* mice, suggesting that β1-integrins may be dispensable for OPC proliferation. This is in contrast to what is observed in cerebellar granule cells, where β1-deficiency leads to decreased proliferation (Blaess et al., 2004).

In summary, my work highlights the importance of β1 integrin in regulating oligodendrocyte process dynamics and CNS myelination. It will be interesting to learn in future studies whether a requirement for β1 integrin exists in the adult brain during myelin repair. A prediction is that, given the role of integrins in potentiating growth factor signaling, and the relatively limited availability of growth factors in the adult brain, integrins may be more important during myelin repair than during development.

Figure III-1. Myelination defects in the corpus callosa of *Itgb1-CNSko* mutants.

All values are shown as mean±SEM; n=3; *p<0.05; **p<0.01; n.s.=not significant; and scale bars=500nm. **(A)** Representative electron micrographs of myelinated fibers in the corpus callosum cross sections from P85 wild-type and *Itgb1-CNSko* littermate. **(B)** Graph depicts percent of unmyelinated axons and **(C)** Graph depicts percent of unmyelinated axons versus axon diameter from wild-type and *Itgb1-CNSko* adult (P80-90) animals. *Itgb1-CNSko* corpus callosum has significantly more unmyelinated axons, mainly of smaller axon caliber, compared to wild-types. **(D)** G-ratios grouped by axon diameter revealed significantly thinner myelin in *Itgb1-CNSko* cortical axons of all sizes. **(E)** Graph depicts the number of axons against axon diameter from 3 pairs of wild-type (solid line) and *Itgb1-CNSko* (broken line). **(F)** Cortical g-ratios were plotted as a function of axon diameter for wild type (open circles) and *Itgb1-CNSko* (open triangles) and increased mean g-ratios were observed overall in *Itgb1-CNSko* corpus callosum (0.761 in wild-type versus 0.848 in *Itgb1-CNSko*).

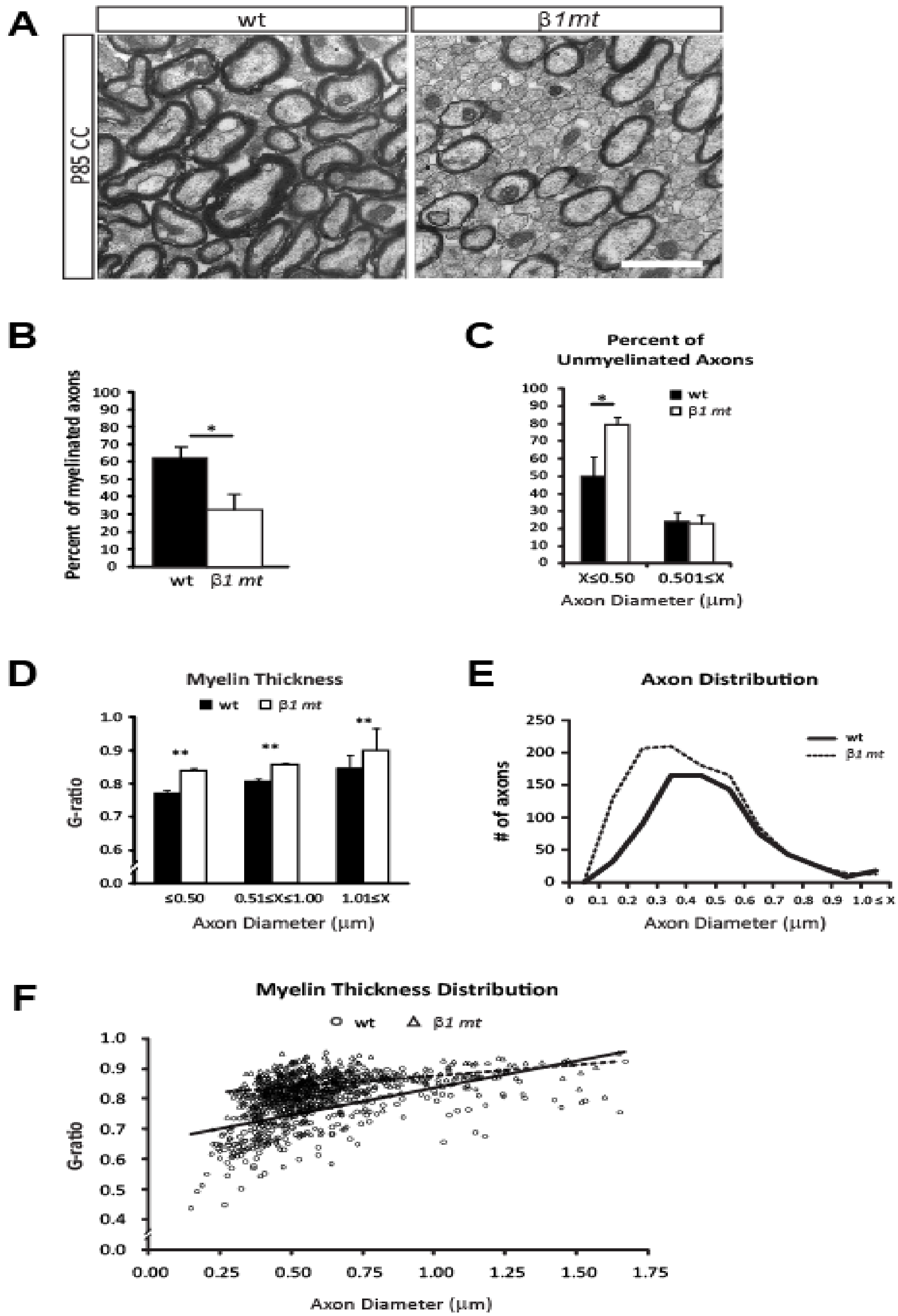
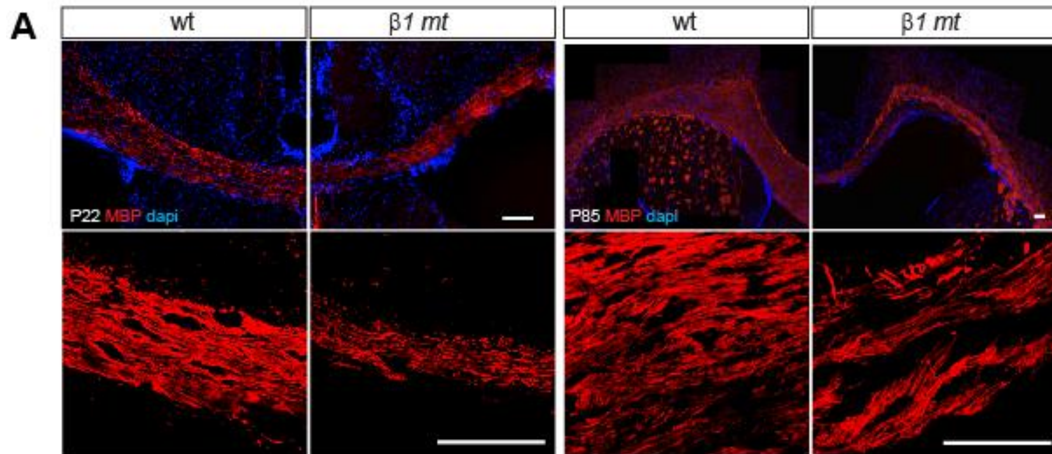
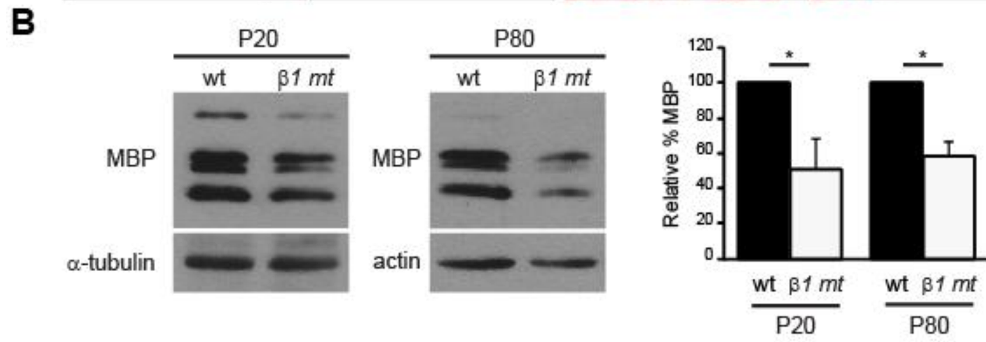

A
wt
β1mt
P85 CC
B
Percent of myelinated axons
wt
β1 mt
C
Percent of Unmyelinated Axons
wt
β1 mt
X≤0.50
0.501≤X
Axon Diameter (μm)
D
Myelin Thickness
wt
β1 mt
G-ratio
≤0.50
0.51≤X≤1.00
1.01≤X
Axon Diameter (μm)
E
Axon Distribution
wt
β1 mt
# of axons
Axon Diameter (μm)
F
Myelin Thickness Distribution
wt
β1 mt
G-ratio
Axon Diameter (μm)

Figure III-2. Less MBP and thinner corpus callosa in *Itgb1-CNSko* mutants.

All values are shown as mean±SEM; n=3; *p<0.05; and scale bars=100µm. **(A)** Top panels, representative images of MBP immunocytochemistry (red) in the corpus callosum of P22 and P85 wild-type and *Itgb1-CNSko* mutant littermates. Nuclei are visualized using DAPI (blue). Bottom panels, higher magnification representative images of MBP immunocytochemistry. **(B)** Densitometric analysis of MBP from P20 and P80 cerebral cortical lysates. Actin and α-tubulin were used for loading controls. Representative blots from P20 and P80 wild-type (wt) and *Itgb1-CNSko* mutant (β*1mt*) littermates. MBP protein levels were normalized to actin or α-tubulin protein levels. **(C)** Top panels, representative images of MBP immunocytochemistry (red) and NF (green) in the corpus callosum of P22 and P85 wild-type and *Itgb1-CNSko* mutant littermates suggesting axons are unaffected by the lost of β1 integrins. Nuclei are visualized using DAPI (blue). Together, the corpus callosa of *Itgb1-CNSko* mutants are thinner and MBP protein expression is significantly less compared to wild-type littermates.


A
wt
β1 mt
wt
β1 mt
P22 MBP dapi
P85 MBP dapi


B
P20
wt
β1 mt
MBP
α-tubulin
P80
wt
β1 mt
MBP
actin
Relative % MBP
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100
80
60
40
20
0
*
*
wt
β1 mt
wt
β1 mt
P20
P80

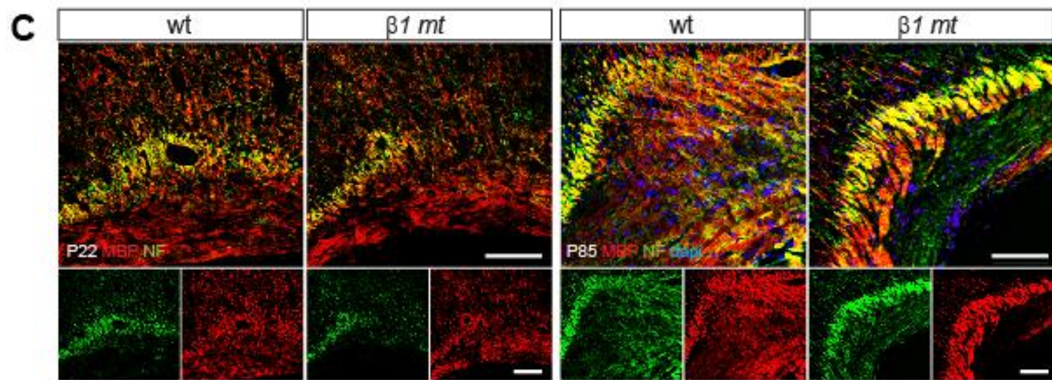
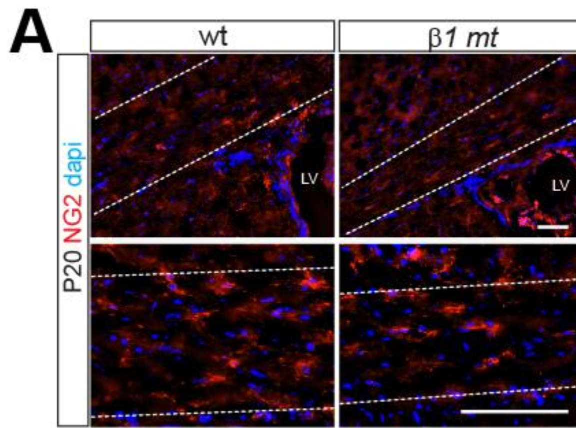
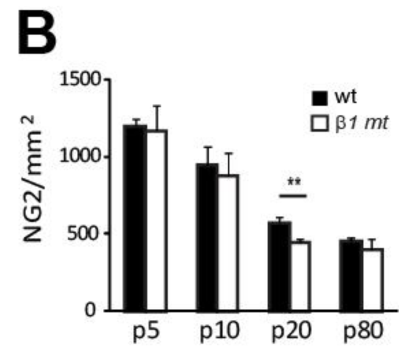
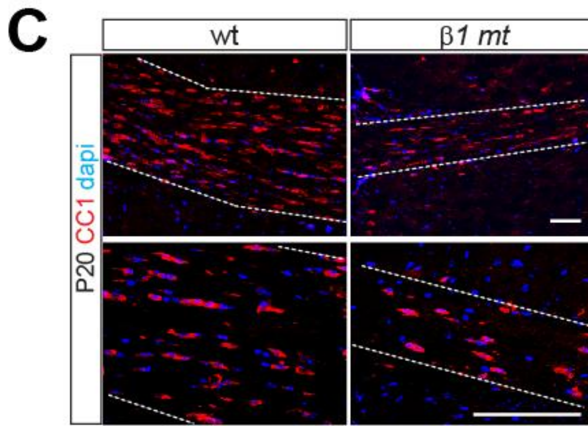

C
wt
β1 mt
wt
β1 mt
P22 MBP NF
P85 MBP NF dapi

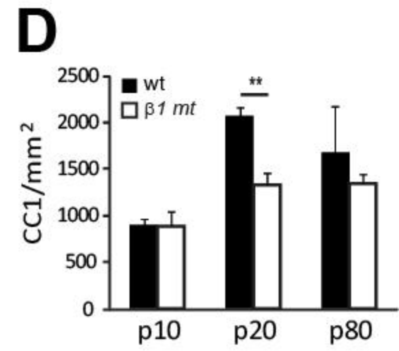
Figure III-3. Normal oligodendrocyte lineage progression *in vivo*.

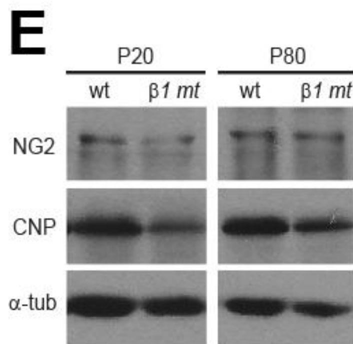
All values are shown as mean±SEM; n=3; **p<0.01; n.s.=not significant; and scale bars=100µm. **(A)** Representative images of NG2^{+} oligodendrocyte progenitors (red) and **(C)** of CC1^{+} oligodendrocytes (red) in the corpus callosum of P20 wild-type and *Itgb1-CNSko* mutant littermate. Nuclei are visualized using DAPI (blue). **(B)** Oligodendrocyte progenitors and **(D)** mature CC1^{+} oligodendrocytes per square millimeter in the corpus callosum were measured in wild-type (wt; black bars) and *Itgb1-CNSko* mutant (β*1mt*; white bars) littermates at P5, P10, P20, and P80. Graphs depict mean±SEM counts obtained from 5 different areas of the corpus callosum. Oligodendrocyte lineage progression appears to be normal despite a significant decrease of both progenitors and mature cells at P20, but normalized at P80. This suggests that the myelin defects in the cortex are unlikely due to insufficient number of oligodendroglia. **(E)** Lysates from P20 and P80 cerebral cortices were evaluated by Western blot to detect NG2, CNP, and α-tubulin. Representative blots from P20 and P80 wild-type (wt) and *Itgb1-CNSko* mutant (β*1mt*) littermates. **(F)** Densitometric analysis of NG2 and **(G)** CNP. NG2 and CNP protein levels were normalized to α-tubulin protein levels.

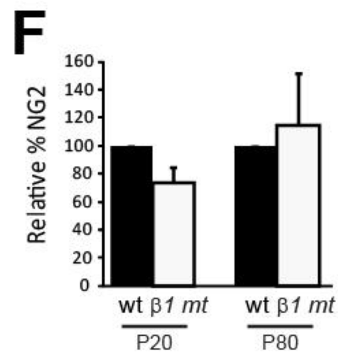

A
wt
β1 mt
P20 NG2 dapi
LV
LV


B
NG2/mm²
wt
β1 mt
**
p5
p10
p20
p80


C
wt
β1 mt
P20 CC1 dapi


D
CC1/mm²
wt
β1 mt
**
p10
p20
p80


E
P20
P80
wt
β1 mt
wt
β1 mt
NG2
CNP
α-tub


F
Relative % NG2
wt β1 mt
wt β1 mt
P20
P80

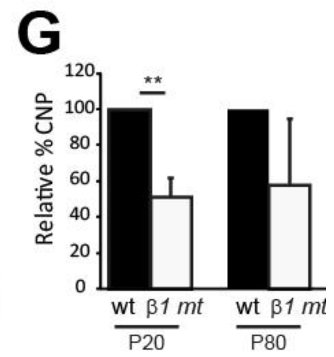
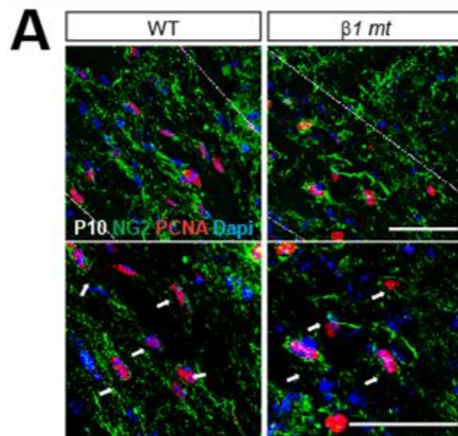
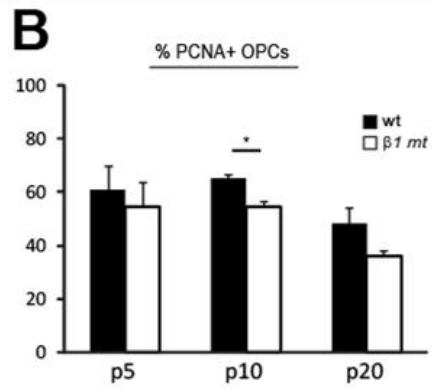
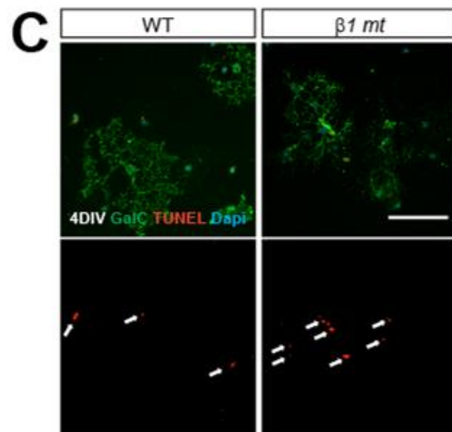

G
Relative % CNP
**
wt β1 mt
wt β1 mt
P20
P80

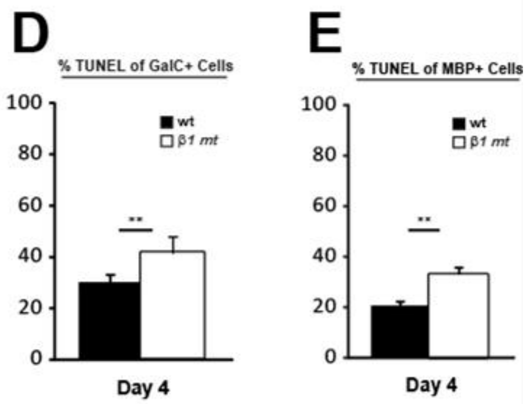
Figure III-4. β1 integrin and oligodendrocyte progenitor proliferation and survival.

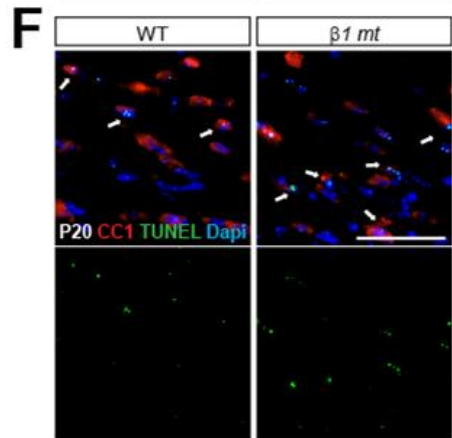
All values are shown as mean±SEM; n=4; *p<0.05; **p<0.01; and scale bars=50μm. **(A)** Top panels are representative images of $NG2^+$ oligodendrocyte progenitors (green) and $PCNA^+$ (red) in the corpus callosum of P10 wild-type and *Itgb1-CNSko* mutant littermate. Nuclei are visualized using DAPI (blue). Bottom panels show higher magnification images. Double positive oligodendrocyte progenitors in S-phase ($NG2^+PCNA^+$) are indicated by white arrows. **(B)** Percent $NG2^+PCNA^+$ oligodendrocyte progenitors in the corpus callosum were measured in wild-type (wt; black bars) and *Itgb1-CNSko* mutant (β*1mt*; white bars) littermates at P5, P10, and P20. Graphs depict mean±SEM counts obtained from 5 different areas of the corpus callosum. It appears that *Itgb1-CNSko* mutants tend to have less percentage of $PCNA^+$OPCs, but only at P20 that there was a statistical significant difference in oligodendrocyte progenitor proliferation in the corpus callosum of *Itgb1-CNSko* mutant compared to its wild-type littermate. **(C)** Representative images of myelinating oligodendrocytes derived from wild-type and *Itgb1-CNSko* mutant littermate. Oligodendrocytes were differentiated for 4-DIV and stained using α-GalC (green) and $TUNEL^+$ (red). Nuclei were visualized using DAPI (blue). Double positive oligodendrocytes ($GalC^+TUNEL^+$) are indicated by white arrows. **(D)** Percent $GalC^+TUNEL^+$ and **(E)** MBP^+TUNEL^+ oligodendrocytes were measured in wild-type (wt; black bars) and *Itgb1-CNSko* mutant (β*1mt*; white bars) littermates at 4-DIV. **(F)** Representative images of mature $CC1^+$ oligodendrocytes (red) and $TUNEL^+$ (green) in the corpus callosum of P20 wild-type and *Itgb1-CNSko* mutant littermate. Nuclei were visualized using DAPI (blue). Double positive oligodendrocytes ($CC1^+TUNEL^+$) are indicated by white arrows. **(G)** Percent $NG2^+TUNEL^+$ oligodendrocyte progenitors and percent $CC1^+TUNEL^+$ in the corpus callosum were measured in wild-type (wt; black bars) and *Itgb1-CNSko* mutant (β*1mt*; white bars) littermates at P10 and P20. Graphs depict mean±SEM counts obtained from 5 different areas of the corpus callosum. Despite the increased in oligodendrocyte death observed *in vitro*, there were no significant differences in oligodendrocyte progenitors or mature oligodendrocyte survival *in vivo*. This discrepancy could be due, in part, to the presence of axons *in vivo*.


A
WT
β1 mt
P10 NG2 PCNA Dapi


B
% PCNA+ OPCs
wt
β1 mt
100
80
60
40
20
0
*
p5
p10
p20


C
WT
β1 mt
4DIV GalC TUNEL Dapi


D
% TUNEL of GalC+ Cells
wt
β1 mt
100
80
60
40
20
0
**
Day 4
E
% TUNEL of MBP+ Cells
wt
β1 mt
100
80
60
40
20
0
**
Day 4


F
WT
β1 mt
P20 CC1 TUNEL Dapi

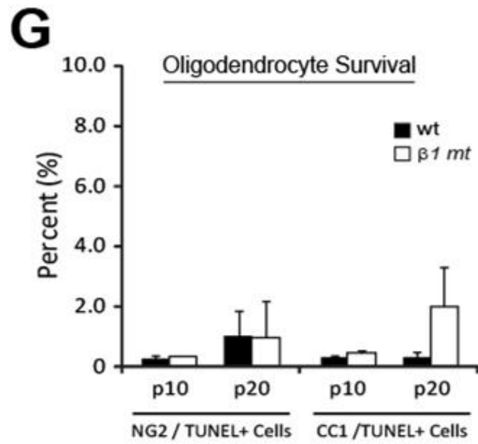
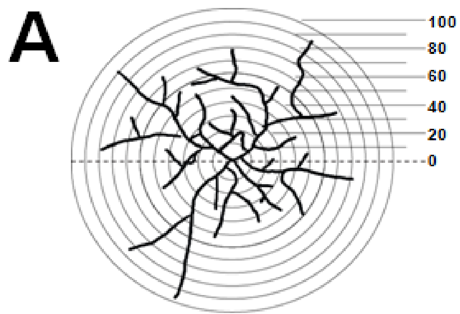
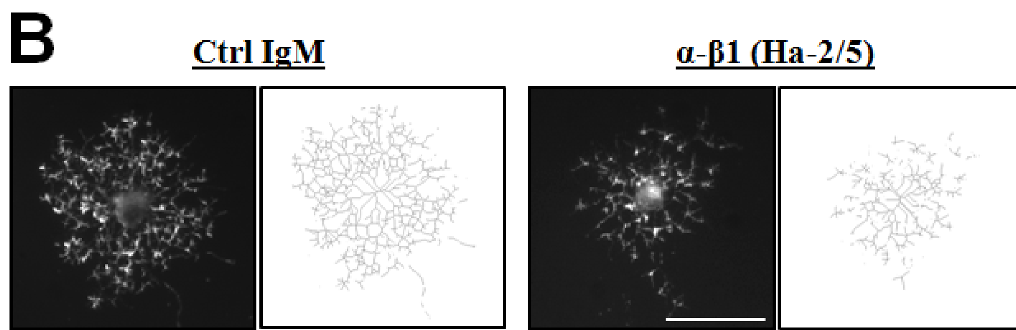
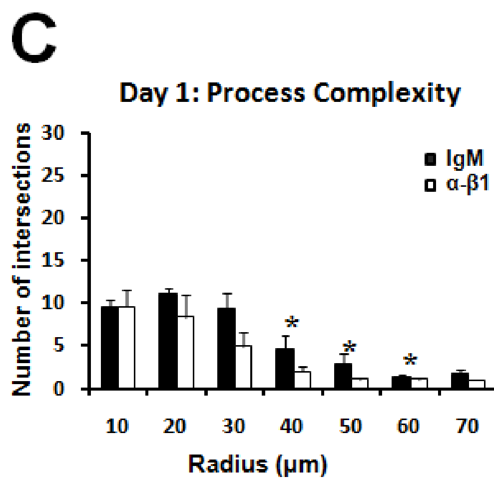

G
Oligodendrocyte Survival
wt
β1 mt
Percent (%)
10.0
8.0
6.0
4.0
2.0
0
p10
p20
p10
p20
NG2 / TUNEL+ Cells
CC1 /TUNEL+ Cells

Figure III-5. Inhibition of β1 integrin function reduces process complexity in rat oligodendrocytes.

All values are shown as mean±SEM; n=3; *p<0.05; and scale bars=50µm. **(A)** Diagram of semi-automatic Sholl analysis. Concentric circles are set at 10µm intervals from 0µm to 100µm, with the epicenter at the cell nucleus. **(B)** Representative images of phalloidin (F-actin) from isotype IgM control and Ha-2/5 (β1 integrin blocking antibody) treated 2-DIV rat oligodendrocyte cultures. Image J program was used to skeletonized phase images to get binary picture for Sholl analysis. **(C)** Quantification of Sholl analyses comparing 1-DIV and **(D)** 2-DIV oligodendrocytes treated with Ha-2/5 (gray bars), isotype IgM control (white bars) or untreated (black bars) for 1-day and 2-days, respectively. Although, there is an increase in process complexity from 1-DIV to 2-DIV oligodendrocytes, however, in the present of β1 integrin blocking antibody there is a significant decrease in process complexity compared to IgM isotype control treated.


A
100
80
60
40
20
0


B
Ctrl IgM
α-β1 (Ha-2/5)


C
Day 1: Process Complexity
Number of intersections
Radius (μm)
IgM
α-β1

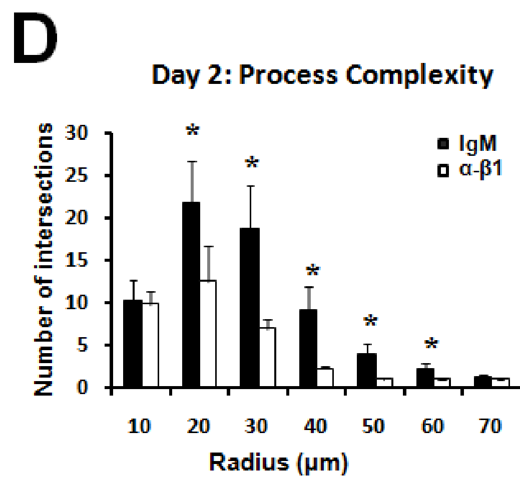
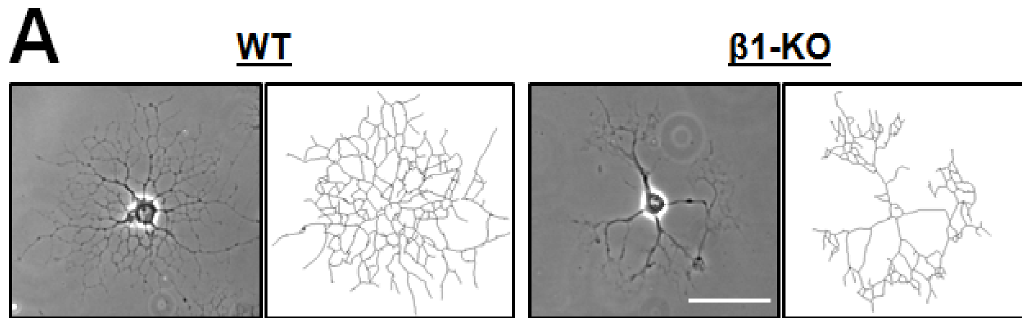
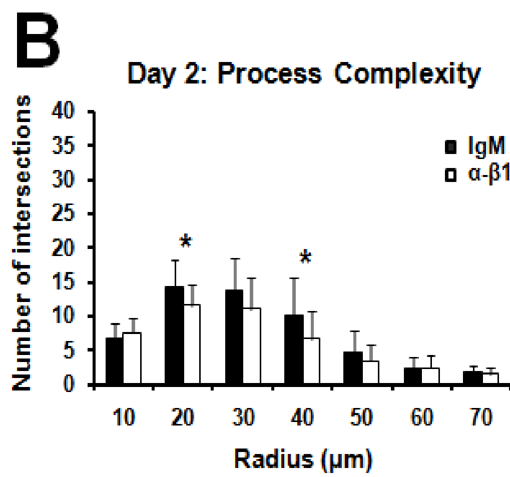
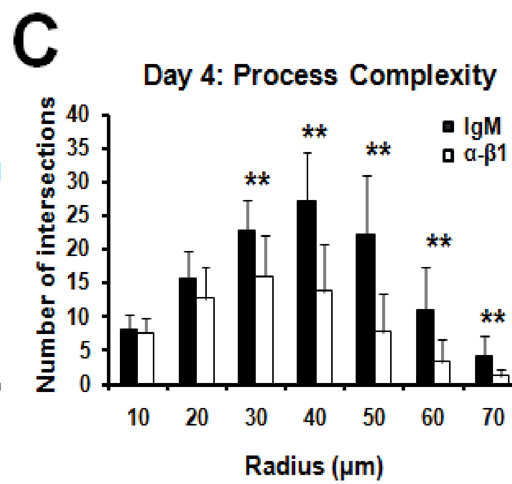

D
Day 2: Process Complexity
Number of intersections
Radius (μm)
IgM
α-β1

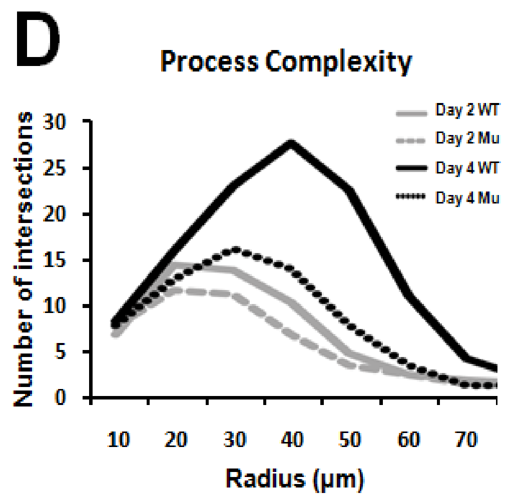
Figure III-6. β1-KO oligodendrocytes have fewer primary processes and decreased process complexity.

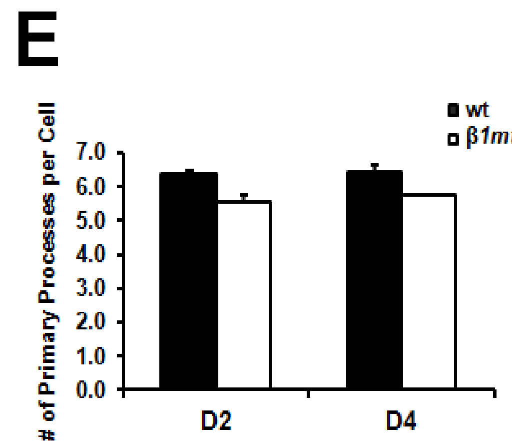
All values are shown as mean±SEM; n=3; *p<0.05; **p<0.01; and scale bars=50μm. **(A)** Phase images of 4-DIV mouse wt and β*1mt* oligodendrocytes. Images were skeletonized for Sholl analyses. **(B)** Quantification from Sholl analyses of 2-DIV and **(C)** 4-DIV oligodendrocytes comparing wt (white bars) and β*1mt* (black bars) oligodendrocytes. **(D)** Line graphs comparing 2-DIV and 4-DIV wt (solid lines) and β*1mt* (dash lines) oligodendrocytes. 4-DIV β*1mt* oligodendrocytes have decreased process complexity comparable to 2-DIV. **(E)** Primary processes were counted and β*1mt*, on average, have fewer numbers of primary processes per cell.


A
WT
β1-KO


B
Day 2: Process Complexity
Number of intersections
IgM
α-β1
Radius (μm)


C
Day 4: Process Complexity
Number of intersections
IgM
α-β1
Radius (μm)


D
Process Complexity
Number of intersections
Day 2 WT
Day 2 Mu
Day 4 WT
Day 4 Mu
Radius (μm)


E
# of Primary Processes per Cell
wt
β1mt
D2
D4

Chapter V: Summary and future directions

Oligodendrocytes (OLGs) have an essential physiological role in myelinating axons of the CNS. The myelination process is needed in order for neuron networks to communicate via saltatory conduction, a process that enables rapid propagation of action potentials traveling down axons (Sherman and Brophy, 2005). In my project, I focused on understanding how β1 integrins can influence oligodendrocyte morphology and CNS myelination. These issues were examined using transgenic mice (β1-CNSko) engineered to excise the β1 integrin gene at the neural progenitor stage, as well as using primary mouse and rat OLG cell cultures. β1 integrins were found to have an instructive role in CNS myelination. The CNS of β1-CNSko mice was found to have thinner myelin, as well as having significantly fewer myelinated axons in the corpus callosum. Although *in vitro* studies have shown that β1-null OLGs have increased programmed cell death, the loss of β1 integrins did not affect oligodendrocyte lineage progression or survival *in vivo*. OPC proliferation has an apparent reduction, but was only significant at P10 and not P5 nor P20. In chapter III, we demonstrated that OLGs derived from mutant mice were unable to efficiently extend myelin sheets and did not fully activate AKT when stimulated with soluble

NRG. Together these results suggested that β1 integrins play an instructive role in CNS myelination by promoting myelin wrapping in a process that depends on AKT. In addition, during my analysis I noticed that the corpus callosa of the β1-CNSko mice had an additional myelin phenotype: fewer myelinated axons. In chapter IV, I therefore examined the mechanism underlying this corpus callosum defect further. I found that β1-KO oligodendrocytes have reduced process complexity. This reduction in process complexity may prevent each individual oligodendrocyte from myelinating the appropriate number of axons. In summary, understanding how β1 integrins influence oligodendrocyte development can help us understand the mechanisms through which myelination occurs.

Crosstalk between β1 integrins and RTKs

Myelination in all its complexity could not be regulated by any one protein, but is likely modulated by various receptors, kinases, and effector molecules working together to temporally and spatially regulate this process. Another extracellular OLG ligand, neuregulin (NRG), has also been implicated in myelination. Mice lacking one copy of neuregulin 1 ($NRG1^{+/-}$) showed a reduction in myelination (Chen et al., 2006; Wolpowitz et al., 2000) indicating that the myelination process is very sensitive to NRG signal strength. In chapter

III we activated AKT in OLGs by treatment with soluble NRG and rescued myelin sheet defect in β1 integrin knockout OLGs by expression of CA-AKT, suggesting that there may be cross-talk between β1 integrin and NRG signaling. Previous reports showed that the NRG receptors, ErbB2 and ErbB4, but not ErbB3 (Schmucker et al., 2003), are important for spinal cord OLG development and are suggested to control CNS myelination (Vartanian et al., 1999). Also, OLGs expressing a dominant-negative form of ErbB4 are found to have the same numbers of primary process, but number of branch point, maximum branch order, and total process length are significantly reduced compare to wild type (Roy et al., 2007). In fact, my preliminary data suggested that in total cortical lysates, phosphorylation and total levels of ErbB4 receptor proteins were altered by the lack of β1 integrins. However, conditional knockout mutants of ErbB3 or/and ErbB4 specifically in OLGs exhibit normal CNS myelination (Brinkmann et al., 2008). Thus, in contrast to Schwann cells, OLGs may not require NRG signaling during CNS myelination, but may still require other non-NRG axonal signals.

Previous reports showed that the trophic effects of NRG towards OLGs are enhanced in the presence of laminin, an α6β1 integrin ligand. Also, laminin can enhance the signaling and functional response to NRG in cultured OLGs, and this can be blocked by anti-β1 integrin antibody (Colognato et al., 2002). My cortical g-ratio data in chapter IV support this model in which axonal neuregulins are recognized by oligodendrocyte ErbB receptors and that integrins are required

to amplify neuregulin signaling. Thus, small-diameter axons are more dependent on integrins amplification, whereas large-diameter axons have higher levels of axonal NRG. β1 integrin and NRG receptors both share common downstream signal transduction effector molecules and pathways, such as Src Family Kinases, MAPK and the PI3K/AKT pathways. It would be interesting to further investigate if there is any modulation in NRG receptor level, phosphorylation, or proteolytic products in the β1-null OLGs.

Rho GTPases

In chapter III and IV, we reported that β1 integrins influence CNS myelination by regulating myelin sheath formation and process dynamics, suggesting a possible role for small Rho GTPases. Small Rho GTPases, rac1 and cdc42, are downstream effectors of activated integrins, and act as positive regulators of actin cytoskeleton remodeling in other cell types (Burridge and Wennerberg, 2004; Guan, 2004). β1 integrins are required for rac1 activation in radial sorting by Schwann cell in the PNS (Nodari et al., 2007). In addition, a conditional double knockout of cdc42 and rac1 in OLGs led to the formation of an abnormal accumulation of cytoplasm in the inner tongue of the OLGs' processes, indicating that cdc42 and rac1 are required for normal CNS myelination

(Thurnherr et al., 2006). Moreover, combined rac1 and cdc42 deficiencies in OLGs cause aberrant myelination. Recently, Benninger el al., (2007) and Nodari et al., (2007) showed that laminin signaling activates rac1 in Schwann cells leading to myelination of axons in the PNS. However, the connection between laminins and small GTPase activity remains to be examined in CNS myelination. If we identify that β1 integrin regulates particular signaling effectors such as rac1 or cdc42, we will then predict that a constitutively active form of rac1 or cdc42 may rescue, or partially rescue, β1 integrin-deficient phenotypes.

Also, the mouse model used in this thesis in which the *β1 integrin* gene deletion was driven by *Cre* recombinase under the control of the *nestin* promoter, thus, β1 integrins was ablated in oligodendrocytes, astrocytes and neurons. In chapter III, the thinner myelin defect observed with the *Itgb1-OL-ko* mice (*NG2-Cre*) was less severe compared to the *Itgb1-CNSko* mice (*Nestin-Cre*) suggest β1 integrins in axons could contribute to CNS myelination. It will be interesting to examine nodal formation, axon integrity, myelin segment formation, and neurophysiology in these two distinct mouse models.

Laminin receptors: integrins and dystroglycan

Integrins are widely expressed and the loss-of-function of even one subunit can have deleterious effects (Czuchra, 2006; Fassler et al., 1996). Because each integrin heterodimer binds only to a limited repertoire of ECM ligands, attachment to any given matrix molecule requires the expression of at least one type of integrin heterodimer appropriate for that ECM protein (Hynes, 2002). For example, loss of the β1 subunit in the epidermal epithelial cells of the skin causes loss of expression of three out of the four major laminin-binding integrins (i.e., integrin α3β1, α6β1, α6β4 and α7β1) and in turn causes keratinocyte detachment from the laminin-rich basal lamina, leading to increased cell death (Lotti et al., 2010; Tiberio et al., 2002). On the contrary, the overlapping ligand binding of different integrin heterodimers makes it difficult to study a specific integrin function due to compensatory mechanism in the absence of a particular integrin subunit. Integrins can also cooperate with non-integrin receptors to mediate basement membrane assembly. This is the case in which β1 integrin receptors and dystroglycan can cooperate to promote the polymerization of laminin molecules into a network that subsequently incorporate collagen matrices and other components together to form the basement membrane (Tsiper and Yurchenco, 2002; Yurchenco and Patton, 2009). Such double knockout will be important to

delineate between β1 integrin-specific and dystroglycan-specific roles for laminin in oligodendrocyte myelination.

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