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Investigating the role of Dystroglycan in Oligodendrocyte Development

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Abstract of the Dissertation

Investigating the role of dystroglycan in oligodendrocyte development

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The extracellular matrix (ECM) protein, laminin-2 regulates the ability of oligodendrocytes to orchestrate myelin synthesis in the central nervous system (CNS). However, the mechanism by which this occurs remains unclear. While integrin receptors are known to transmit laminin signals in oligodendrocytes, their role in laminin-mediated myelinogenesis appears to be limited as oligodendrocytes with deleted or disrupted β1 integrin exhibit only marginal myelin defects compared to those observed in laminin-deficient animals. Here, we report that oligodendrocytes also express the non-integrin laminin receptor, dystroglycan, and reveal it as a novel regulator of myelin formation. Using primary oligodendrocytes, we find that dystroglycan expression correlated with and was necessary for optimum differentiation. That is, higher levels of dystroglycan were found in cells expressing myelin specific proteins while the ablation of dystroglycan resulted in a significant reduction in myelin specific

proteins. Subsequent to these findings, we revealed a novel functional connection between dystroglycan and insulin-like growth factor-1 (IGF-1) signaling. As described previously, treatment with exogenous IGF-1 caused an increase in myelin-specific proteins that was preceded by activation of PI3K (AKT) and MAPK (ERK) signaling pathways. Moreover, laminin-2 was shown to potentiate the effect of IGF-1 on oligodendrocyte differentiation, whereas dystroglycan depletion reversed it. In addition, depletion of dystroglycan led to a reduction in the ability of IGF-1 to activate MAPK, but not PI3K signaling pathways. Consistent with these observations, pharmacological inhibition of MAPK signaling prevented IGF-1-induced increases in myelin-specific proteins in the presence of laminin, indicating that MAPK signaling was necessary to drive IGF-1-mediated enhancement of oligodendrocyte differentiation. Finally, we found that dystroglycan, the adapter protein GRB2, and insulin receptor substrate-1 (IRS1), were associated in a protein complex. Taken together, we've identified the non-integrin receptor dystroglycan as a novel mediator of laminin-2-induced myelinogenesis in oligodendrocytes. We've also provided evidence that the effects of dystroglycan are attributed, at least in part to its ability to promote IGF-1-induced activation of MAPK/ERK signaling, possibly through a structural association with IRS1 and GRB2.

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CHAPTER 1 – General Introduction

Myelin

Myelin is the insulating layer of axons in the central nervous system (CNS) and the peripheral nervous systems (PNS). The myelin sheath, composed of approximately 80% lipids and 20% proteins, enwraps axons in multiple layers with the number of layers (thickness of the sheath) correlating with the diameter of the underlying axon (Friede and Samorajski 1967; Dyck, Lambert et al. 1971; Schroder, Bohl et al. 1978). Lengthwise, axons are insulated by myelin in a segmented pattern. Areas covered by myelin are called internodes while the narrow unmyelinated gaps separating the internodes are the nodes of Ranvier (Fig 1.1).

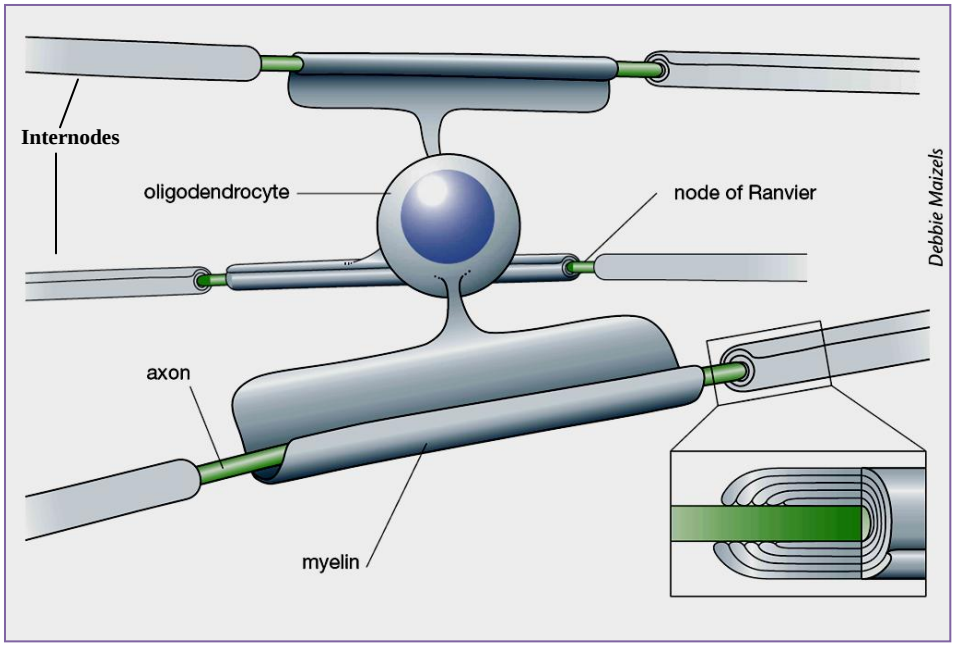


Fig 1.1 Myelin in the CNS featuring oligodendrocytes, nodes of Ranvier and internodes (Modified (Popko 2003))

In addition to providing physical protection to the underlying axon, myelin blocks cytoplasmic ion leakage along the internodes enabling pump driven ion exchange to be localized to the nodes. This allows action potentials to jump from node to node in a rapid and efficient process known as saltatory conduction. Without myelin, the speed and effectiveness of the nervous system would be substantially compromised as ion exchange and action potentials would be wavelike (propagating throughout the length of axons) rather than saltatory (jumping from node to node) (Hartline and Colman 2007). Moreover, the speed of action potentials in myelinated axons is proportional to axon diameter (Moore, Joyner et al. 1978) while that in unmyelinated axons is proportional to the square root of axon diameter (Hodgkin 1954). Hence, to achieve the same speed of impulse transmission, unmyelinated axons would have to be substantially thicker than their myelinated counterparts. Taken together, the space and energy advantages afforded by myelin make it a critical feature of vertebrate evolution (Zalc and Colman 2000). Perhaps the significance of myelin may be best demonstrated in demyelinating pathologies such as multiple sclerosis (MS). Herein, the breakdown of myelin from inappropriately activated immune cells and their inflammatory products can disrupt the propagation of action potentials and ultimately cause debilitating functional deficits.

Interventions designed to enhance myelin repair could be effective treatments for MS and other demyelinating pathologies. Crucial to the development of such treatments is to increase understanding of the functional biology of oligodendrocytes, the myelin-producing cells in the CNS.

General overview of oligodendrocyte development

In the developing nervous system, neural stem cells in the ventricular/subventricular zones and ventral spinal cord give rise to oligodendrocyte progenitor cells (OPCs) (Warf, Fok-Seang et al. 1991; Noll and Miller 1993; Ono, Yasui et al. 1997), modestly branched migratory proliferative cells marked by the expression of platelet derived growth factor receptor (PDGFR) (Pringle and Richardson 1993; Pringle, Yu et al. 1996) and the chondroitin sulfate proteoglycan, nerve-glia 2 (NG2) (Levine and Stallcup 1987). Differentiation of these cells into multi-branched non-proliferating and non-migratory early oligodendrocytes is marked by the presence of O4, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and the glycosphingolipid, galactocerebroside (GalC). The extension of processes onto unmyelinated axons induces target mediated survival of these early olidgodendrocytes (Barres, Schmid et al. 1993). This, along with developmental cues put forth by an ensemble of other regulatory factors, guides their progression into fully differentiated oligodendrocytes capable of synthesizing and assembling myelin. While continuing to express some of the early oligodendrocyte markers such as O4 and GalC, these cells also express myelin specific proteins such as myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), and myelin/oligodendrocyte glycoprotein (MOG) (Fig 1.2).

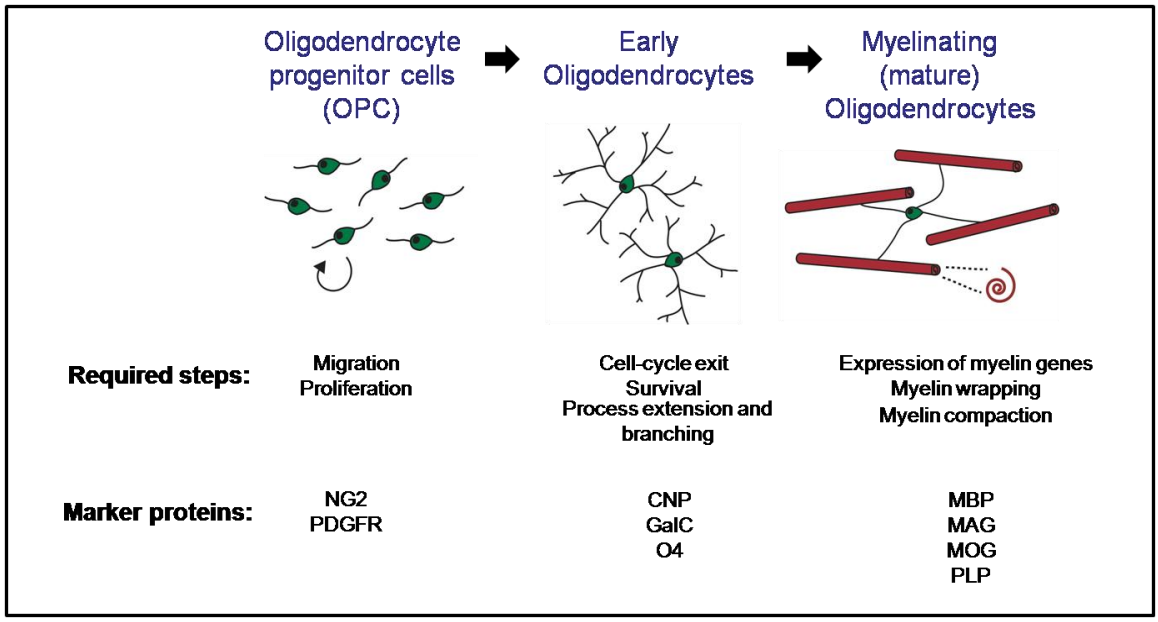


Fig 1.2 Major steps of oligodendrocyte lineage progression with relevant stage marker proteins (modified (Colognato, ffrench-Constant et al. 2005))

Among the diverse array of molecules regulating this complex process of oligodendrogenesis are extrinsic factors including growth factors, cytokines, adhesion molecules and extracellular matrix (ECM) molecules, as well as intrinsic factors including growth factor receptors, cell adhesion receptors, signaling molecules, transcription factors, chromatin structure and chromatin modifying molecules. A comprehensive description of every factor known to influence oligodendrocyte development exceeds the scope of this dissertation. However, those most relevant to present work will be expanded upon in appropriate sections.

Platelet derived growth factor (PDGF), fibroblast growth factor (FGF), neuregulin (NRG) and insulin-like growth factor-1 (IGF-1) represent prominent growth-factor mediators of oligodendrocyte lineage progression. Fibronectin, vitronectin and laminin-2 represent prominent ECM protein mediators of oligodendrocyte lineage progression (Baron, Colognato et al. 2005). Table 1.1

summarizes the influence of these key growth factors and ECM proteins on developmental processes in oligodendrocytes (data was compiled from various sources: (Noble, Murray et al. 1988; Bogler, Wren et al. 1990; Barres, Schmid et al. 1993; Mayer, Bogler et al. 1993; McMorris, Mozell et al. 1993; Canoll, Musacchio et al. 1996; Osterhout, Ebner et al. 1997; Buttery and ffrench-Constant 1999; Stariha and Kim 2001; Colognato, Baron et al. 2002; Chun, Rasband et al. 2003; Dupont and Holzenberger 2003; Colognato and ffrench-Constant 2004; Hsieh, Aimone et al. 2004; Baron, Colognato et al. 2005; Colognato, ffrench-Constant et al. 2005; Siskova, Yong et al. 2009))

	PDGF	FGF	NRG	IGF	Fn/Vn	Ln-2
Migration	+	+	NE	NE	+	NE
Proliferation	+	+	+	+	+	NE
Differentiation	+/-	+/-	+/-	+	+	+
Survival	+	NE	+	+	NE	+
Myelination	+/-	NE	NE	+	NE	+

Table 1.1 Regulation of specific oligodendrocyte developmental processes by extrinsic factors (Fn=fibronectin, Vn=vitronectin and Ln-2=laminin-2, positive effect (+) negative effect (-), no effect determined (NE))

Downstream of growth factor and ECM protein receptors, intracellular signaling cascades including mitogen associated protein kinase (MAPK, both extracellular-related kinase (ERK1/2) and p38), phosphotidyl-inositol-3 kinase (PI3K), src family kinase (SFKs, Fyn and Lyn), and protein kinase c (PKC) have all been determined to play important roles in the regulation of oligodendrocyte lineage progression. Table 1.2 summarizes processes mediated by these different signaling pathways and effector molecules in oligodendrocytes (data

gathered from numerous sources (Althaus, Hempel et al. 1997; Stariha, Kikuchi et al. 1997; Baron, Metz et al. 2000; Flores, Mallon et al. 2000; Stariha and Kim 2001; Dupont and Holzenberger 2003; Colognato and ffrench-Constant 2004; Cui and Almazan 2007; Flores, Narayanan et al. 2008; Haines, Fragoso et al. 2008; Rajasekharan 2008; Frost, Zhou et al. 2009)

	ERK1/2 MAPK	P38/ MAPK	PI3K	SFK	PKC
Migration	+	NE	NE	+	NE
Proliferation	+	+	+	+	+
Survival	+	NE	+	+	NE
Differentiation	+	+	+	+	+/-
Myelination	+	+	+	+	+/-

Table 1.2 Regulation of specific developmental processes by intracellular signaling pathways (positive effect (+) negative effect (-), mixed effects (+/-), no effect determined (NE)).

Adding to the complexity of oligodendrocyte regulation is the capacity of individual extrinsic and intrinsic factors to influence multiple and sometimes contradictory processes throughout oligodendrocyte development (see tables 1.1 and 1.2). For instance, while PDGF blocks differentiation and promotes migration and proliferation in oligodendrocyte progenitors (Noble, Murray et al. 1988; Armstrong, Friedrich et al. 1990; Friedrich, Gohring et al. 1999), it loses its mitogenic capacity and serves as a survival factor critical for differentiation in early oligodendrocytes (Barres, Schmid et al. 1993)(table 1.1). Similar findings have been made for NRG which also loses its mitogenic capacity and becomes a survival factor in early oligodendrocytes (Colognato and ffrench-Constant 2004)(table 1.1). IGF-1 enhances proliferation in progenitors, survival in early oligodendrocytes, and differentiation and myelin synthesis in late

oligodendrocytes (Barres, Schmid et al. 1993; McMorris, Mozell et al. 1993; Hsieh, Aimone et al. 2004)(table 1). Parallel observations have been made for intracellular signaling effects. MAPK/ERK and SFK signaling have each been linked to the regulation of every major developmental process in the oligodendrocyte lineage (see table 2). These seemingly paradoxical responses are often attributed to synergistic signaling between different extrinsic and/or intrinsic factors acting in concert. Such phenomena are not only fairly common but have proven pivotal in regulating oligodendrocyte behavior throughout development. For example, interaction between laminin-2 and α6β1 has been shown to alter the effects of NRG signaling by triggering a switch from PI3K driven survival and proliferation, to MAPK/ERK driven survival and differentiation (Colognato, Baron et al. 2002). Similarly, interaction with αVβ1 integrin and the SFK Lyn has been observed to enhance PDGF-induced proliferation of OPCs, while interaction with α6β1 integrin and the SFK Fyn has been observed to enhance PDGF-induced survival and differentiation in early oligodendrocytes (Colognato and ffrench-Constant 2004). Such spatial regulation is critical to progenitor cells undergoing transition into early oligodendrocytes.

As a novel example of interdependence between ECM receptors and growth factor signaling, we provide evidence that dystroglycan mediates laminin-2-induced differentiation through an association with the IGF-1 signaling cascade (chapter 4).

Extracellular matrix

The extracellular matrix (ECM) refers to secreted non-cellular materials occupying the periphery of many types of cells and cell layers. It is comprised of 3 major components: (1) fibrous proteins such as collagen IV and elastin which provide structural rigidity, (2) polysaccharides including glucosaminoglycan (gag) chains, heparin sulfates (usually existing as proteoglycans) and hyaluronic acid (not a proteoglycan) (Peach, Hollenbaugh et al. 1993) which serve to fill space and absorb shock, and (3) linking proteins such as fibronectin, vitronectin and laminins which physically join elements of the ECM with its resident cells. In general, the role of the ECM is to provide structural support and regulatory signaling to underlying tissues and/or cells. The ECM has been shown to regulate cell migration, proliferation, shape, gene expression and apoptosis (Timpl and Brown 1996). In so doing, the ECM is crucial to the development and maintenance of most types of animal tissue. One notable exception however, is in the CNS which, contrary to the PNS, has few cell types with traditional ECM or basement membranes. Nonetheless, ECM proteins such as laminin-2, fibronectin and vitrononectin have been identified in the CNS, specifically on developing white matter tracts (Chun and Shatz 1988; Neugebauer, Emmett et al. 1991; Pearlman and Sheppard 1996; Colognato, Baron et al. 2002) where they are proposed to regulate oligodendrocyte development.

Laminins

Laminins are secreted, self assembling, hetero-trimeric ECM glycoproteins with 15 known isoforms created from varied combinations of five α, three β and three γ subunits. Laminins are named according to the specific subunits they contain. For instance, the laminin comprised of α1, β1 and γ1 subunits is referred to as "laminin-111" or "laminin-1" using the more traditional nomenclature. Laminins containing the α2, β1 and γ1 subunits are referred to as "laminin-211" or "laminin-2". The characteristic cruciform structure of fully assembled laminins is formed by the 3 interlocking subunits. Together, they comprise a long arm consisting of an alpha helical triple coiled-coil at the carboxyl-terminal, and three short arms branching out at amino terminals (Fig 1.3).

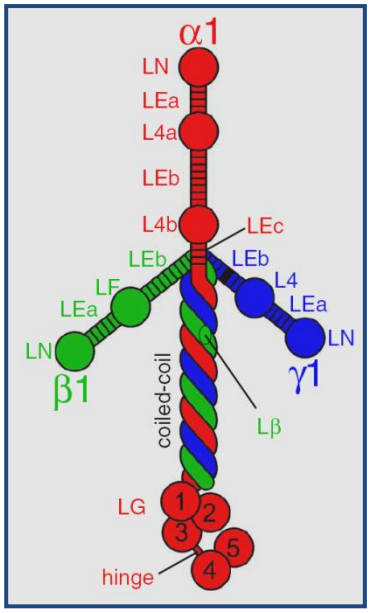


Fig 1.3 Laminin-1 as a representative laminin structure (adapted (Durbeej 2009))

The general function of the short arms is to bind laminins to other components of the ECM including collagen and other laminins. Such binding can lead to complex networks of laminin polymers (Durbeej 2009). In contrast, globular domains (LG1-LG5) found on the carboxyl terminal of α-subunits on the long arm have a binding affinity for integrin and non-integrin receptors and thus link laminins to cell surfaces. To date, eight different integrin (α1β1, α2β2, α3β1, α6β1, α6β4, α7β1, α9β1 and αvβ3) and three different non-integrin (Lutheran blood group glycoprotein, syndecans and dystroglycan) laminin receptors have been identified (Tzu and Marinkovich 2008; Durbeej 2009).

The functions of laminins in basement membranes are diverse and often dictated by the receptors of resident cells. In general terms, they provide structural linkage and regulatory signaling between basement membranes and underlying cell layers. In the ECM of muscle, laminin-2 will bind to either integrin or dystroglycan receptors. Through such binding, integrins and/or dystroglycan complexes structurally link the cytoskeleton of individual muscle cells with the ECM (discussed ahead). In the PNS, laminin-2-deficient mice show radial sorting and myelination defects as Schwann cells require laminin-2 interactions to interact normally with axons (Bradley and Jenkison 1973; Stirling 1975; Occhi, Zambroni et al. 2005). In the CNS, α2 laminin subunits have been identified on axon tracts, in capillary basement membranes of the adult sub-ventricular zone, and within small ECM structures known as “fractones” that emanate from the ventricle surface (Colognato, Baron et al. 2002; Mercier, Kitasako et al. 2003; Tavazoie, Van der Veken et al. 2008). An increasing body of evidence points to

these non-basement membrane laminins as major players in the regulation of CNS myelinogenesis. For example, in cultured oligodendrocytes, laminin-2 has been shown to enhance myelin membrane formation (Buttery and ffrench-Constant 1999; Relucio, Tzvetanova et al. in press). Mice deficient in laminin-α2 (dy/dy) show hypomyelination (Chun, Rasband et al. 2003) while humans deficient in laminin-α2 (afflicted with type 1A congenital muscular dystrophy (MDC1A)) show abnormal white matter patterns in MRIs which may be indicative of myelin deficits (Jones, Morgan et al. 2001). Of the four integrin receptors known to bind laminin-2, only α6β1 has been identified in oligodendrocytes (Milner and Ffrench-Constant 1994). Signaling transmitted through this receptor has been demonstrated to drive target-mediated survival of early oligodendrocytes (Colognato, Baron et al. 2002; Colognato and ffrench-Constant 2004)(see Table 1.1). Interestingly, compared to laminin-2 deficient mice, those in which β1-integrin was conditionally knocked out of oligodendrocytes exhibit only marginal disruptions in CNS myelin (Benninger, Colognato et al. 2006; Emery, Agalliu et al. 2009). This disparity in phenotypes suggests that laminin-2-mediated myelinogenesis is not transmitted exclusively through α6β1 integrin. leading to exploration for other. Given its role in Schwann cell regulation, we explored the possibility that dystroglycan is expressed in oligodendrocytes and plays a role in mediating laminin-induced myelinogenesis.

Dystroglycan

Dystroglycan is the membrane spanning glycoprotein product of the DAG1 gene. A single pro-peptide is translated and subsequently cleaved into 2 distinct subunits. The extracellular dumbbell shaped alpha subunit is heavily glycosylated and bound non-covalently to the membrane spanning beta sub-unit (Winder 2001)(Fig 1.4 and 1.5).

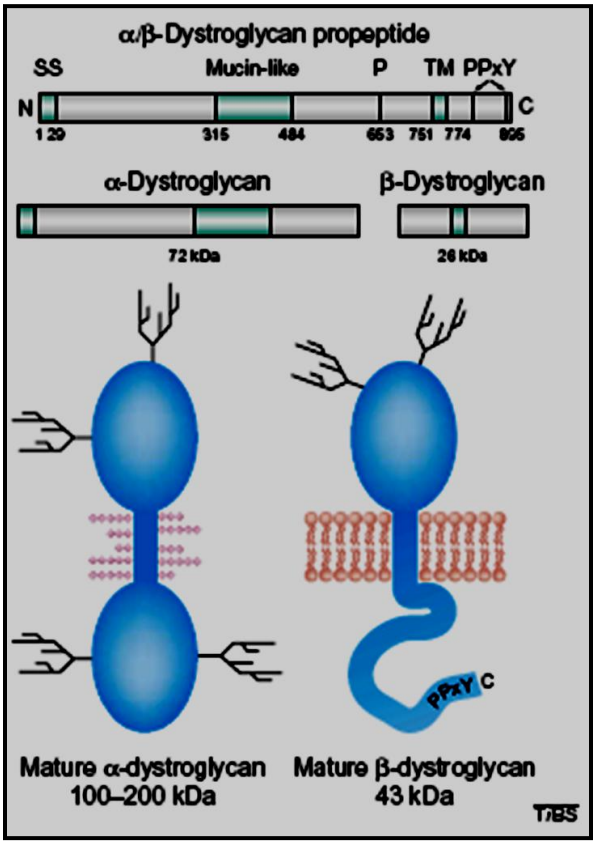


Fig 1.4 Structure of α and β Dystroglycan (Winder 2001)

Outside of the cell, mucin-rich sugar chains on the alpha subunit bind to various extracellular matrix proteins including agrin, perlecan and laminins (Barresi and

Campbell 2006). In muscle and other tissues, dystroglycan is structurally linked to the ECM through binding to specific globular domains (LG4 and LG5) on the carboxyl terminal of laminin-2 (Winder 2001) (Fig 1.5).

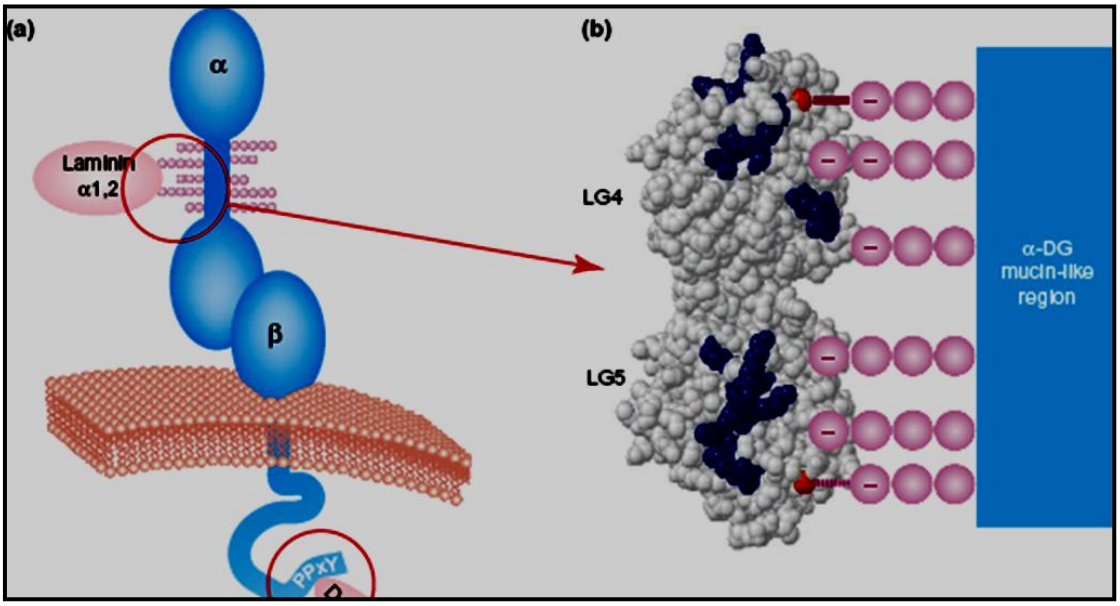
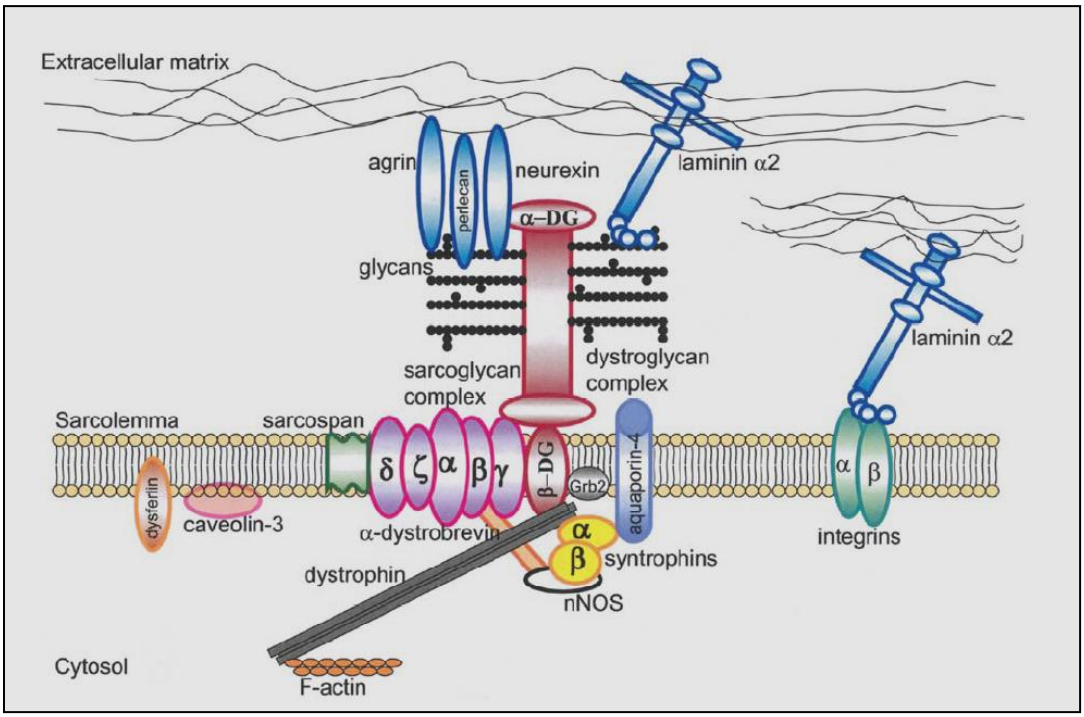


Fig 1.5 Carbohydrate chains of α-dystroglycan bind globular domains of laminin (Winder 2001)

In the cytoplasm, the beta subunit of dystroglycan links the cytoskeletal protein, actin via the chain-protein dystrophin (and/or utrophin, a more site-selective homologue of dystrophin) (Ervasti and Campbell 1993) (Fig 1.6).

Dystroglycan, dystrophin/utrophin, and laminin, together with a number of other structural, signaling and adaptor proteins including caveolin, sarcoglycans, dystrobrevin and the adaptor protein Grb2, form the dystrophin glycocoprotein complex (DGC). In muscle, this complex is a critical structural link between the cytoskeleton of individual muscle cells and the surrounding basal lamina (Fig 1.6).

Fig 1.6 Dystrophin glycoprotein complex linking the extracellular matrix with the cytoskeleton (Cohn 2005)



Various mutations disrupt the structural integrity of the DGC causing muscular dystrophy (MD), a disease marked by the progressive necrosis of muscle tissue (Matsumura and Campbell 1994). To date, there are 9 recognized forms of MD, each representing a distinct mutation relevant to some element of the DGC. Many of the congenital muscular dystrophies (CMDs) are marked by a disrupted connection between laminin-2 and alpha dystroglycan. These can result from mutations in the LAMA2 gene (expresses the alpha subunit of laminin-2) or in one or more of the genes encoding glycosyl transferases that attach specialized sugar chains that are unique to alpha dystroglycan. In many CMDs, the dystrophic phenotype includes white matter defects in the CNS (Clement, Mercuri et al. 2008) suggesting that the connection between laminin-2 and dystroglycan is critical for the proper development of myelin. Interestingly, there

are no CMDs or other diseases associated with the loss of dystroglycan. In rodents, such mutations result in early embryonic death due to structural aberrations in Reichert's membrane, which forms a critical barrier between the embryonic yolk sac cavity and the maternal blood (Williamson, Henry et al. 1997).

As a fairly ubiquitous protein, dystroglycan has been studied in numerous cell types. In Schwann cells, the myelinating cells of the peripheral nervous system (PNS), dystroglycan is required for proper clustering of sodium channels, formation of microvilli, and folding of myelin (Occhi, Zambroni et al. 2005). Several dystroglycan interacting proteins have been identified in these cells including sarcoglycans, sarcospan α-dystrobrevin 1, Dystrophin related protein 2, DP116 (alternatively spiced form of dystrophin) and α1 syntrophin (Saito, Masaki et al. 1999). In astrocytes, dystroglycan has been shown to complex with and mediate clustering of, the sodium channel Kir 4.1 and the water channel aquaporin 4 (AQP4) (Guadagno and Moukhles 2004). In fibroblasts, dystroglycan binds extra-cellular regulated kinase (ERK) and its upstream activator, mitogen activated protein kinase kinase 2 (MEK2)(Spence, Dhillon et al. 2004).

The following dissertation focuses on an examination of the function of dystroglycan in oligodendrocytes with specific emphasis on characterizing its role in laminin-2-induced myelinogenesis.

Insulin-like growth factor-1 signaling

Insulin-like growth factor-1 is a potent regulator of growth and development in nearly all tissue types. Functioning mainly as an endocrine hormone, systemic IGF-1 is produced primarily in the liver under the regulation of growth hormone (Juul 2003). However, in the CNS, it is synthesized independently of growth hormone regulation as a neurotrophic factor whose effects are the result of autocrine and paracrine signaling (Jones and Clemmons 1995). Neurons, astrocytes, and oligodedrocytes all synthesize IGF-1 and express its receptor, IGF1R. As a key regulator of myelinogenesis, IGF-1 has been shown to promote the differentiation, survival and proliferation of oligodendrocytes (Barres, Schmid et al. 1993; McMorris, Mozell et al. 1993; Hsieh, Aimone et al. 2004). In vivo work has demonstrated an increase of myelin thickness, as well as in the percentage of myelinated axons, in mice over-expressing IGF-1 (D'Ercole, Ye et al. 2002). Reduced levels of myelin are found in IGF-1-null mice (Ye, Li et al. 2002) and impaired myelin repair following cuprizone-induced demyelination has been observed following the disruption of IGF-1 receptor (IGF1R) signaling (Mason, Xuan et al. 2003). IGF-1 signaling is initiated by the binding of IGF-1 to its receptor tyrosine kinase, IGF1R. Activation of the receptor is marked by conformational changes and the phosphorylation of tyrosines along the cytoplasmic beta subunit (Gronborg, Wulff et al. 1993). Some of these phosphorylated sites serve as docking sites for insulin receptor substrates (IRS1-IRS4), a group of molecules that mediate IGF-1 signaling downstream of IGF1R.

Activated IRSs can activate MAPK and PI3K signaling through the recruitment of certain adaptor and signaling proteins. Activated IRS1 is known to recruit and interact with the adaptor protein, growth factor receptor-bound protein-2 (Grb2) (Holgado-Madruga, Emlet et al. 1996), which, as discussed, has been reported to be physically associated with β-dystroglycan. Over-expression of Grb2 in a myoblast cell line enhanced insulin-induced MAPK signaling through Ras and resulted in increased association between Grb2, Sos and IRS1, suggesting that Grb2 is a critical intermediary between insulin signaling and the MAPK pathway (Skolnik, Lee et al. 1993). Previous reports demonstrated that oligodendrocytes express IRS1, IRS2, IRS4 (Freude, Leeser et al. 2008) and Grb2 (Weinger, Gohari et al. 2008). While integrin receptors have been shown to interact with oligodendrocyte growth factor signaling pathways, the ability of dystroglycan to influence oligodendrocyte growth factor signaling has remained unknown. This issue is addressed in chapter four where we examine a structural and functional connection between dystroglycan and IGF-1 signaling molecules in oligodendrocytes.

CHAPTER 2 – Materials and Methods

Cell Culture: Neonatal rat cortices were dissociated with papain and cultured on poly-D-lysine (PDL) coated flasks in progenitor maintenance media (high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37^0C in 7.5% CO_2. Media was refreshed every 48-72 hours. A mixed glial culture, consisting of oligodendrocyte precursor cells (OPCs) and microglia bound to an astrocyte monolayer, was attained after approximately 10-12 days. OPCs were separated from mixed glial cultures by a procedure modified from the mechanical agitation and differential adhesion method described by McCarthy and De Vellis (McCarthy and de Vellis 1980; Colognato, Ramachandrappa et al. 2004). OPCs were transferred to chamber slides or Nunclon tissue culture dishes that were pre-coated with PDL or laminin-2 and incubated at 37^0C in 7.5% CO_2. To promote differentiation of oligodendrocytes**,** progenitor maintenance media was replaced with differentiation media (Sato's media (Bottenstein and Sato 1979) containing 0.5% FBS) and samples were incubated for an additional 72 hours. To assess intracellular signaling, progenitor maintenance media was replaced with serum-free DMEM for 2 hours (serum starvation conditions). Growth factor was then added to appropriate samples for indicated times prior to lysis.

For purification of astrocytes, mixed glial cultures previously used for oligodendrocyte purification, were first trypsinized for 1 minute to remove residual

oligodendrocytes, and then trypsinized for an additional 7-10 minutes to remove astrocytes. Astrocytes were transferred to Nunclon tissue culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS at 37^0C in 7.5% CO_2. Media was refreshed every 48-72 hours. Cells were passaged (1:4) every 5-7 days.

Protein Analysis: Cells were scraped from culture dishes into boil buffer (20mM Tris (pH 7.4), 1% Sodium dodecyl sulfate containing protease and phosphatase inhibitor cocktails (Calbiochem)) preheated to 95^0C. Lysates were transferred into microtubes and incubated at 95^0C for 10 minutes. Insoluble material was separated by centrifugation at 18,000 g for 10 minutes. Protein concentration of lysates was evaluated using a detergent-compatible Bradford assay (Biorad). Lysates were combined with NuPAGE® lithium dodecyl sulphate sample buffer (Invitrogen) containing 3% β-mercaptoethanol (BME) and boiled for 5 minutes. SDS-PAGE using 8%, 10%, 12% or 15% mini-gels was used to separate proteins, which were then blotted onto 0.45μm nitrocellulose membranes. To prevent non-specific binding of antibodies, membranes were incubated for 60 minutes in blocking reagent (tris-buffered saline with 0.1% Tween20 (TBS-T) containing 4% bovine serum albumin (BSA) or 1% milk). Primary antibodies were diluted in blocking reagent and incubated with membranes overnight at 4^0C. Membranes were next washed with TBS-T and incubated for 60 minutes at room temperature in HRP-conjugated secondary antibodies (Amersham) diluted (1:3000) in blocking buffer. Finally, membranes were washed and developed

using enhanced chemiluminescence (Amersham). All experiments were carried out at least 3 times. Displayed images depict representative blots.

Immunocytochemistry: Dystroglycan immunocytochemistry was performed on live cells. Media was replaced with fresh oligodendrocyte progenitor media containing 15ug/mL of IIH6 anti-α dystroglycan antibody; samples were incubated at room temperature for 45 minutes at room temperature. Samples were then washed 3 times with medium and then incubated 25 minutes with Texas Red-conjugated donkey anti-mouse IgM. This followed by an additional wash with medium (3x). Cells were then fixed with 100% methanol at -20°C for 5 minutes, incubated with DAPI, and coverslipped with SlowFade Gold (Molecular Probes). For co-immunocytochemistry of dystroglycan with additional proteins, cells were fixed and processed for standard immunocytochemistry following live labeling with dystroglycan antibodies thus, after fixation with 100% methanol at -20°C, cells were blocked for 30 minutes in phosphate-buffered saline (PBS) containing 5% donkey serum (blocking buffer), and then incubated for 60 minutes with additional primary antibodies diluted in blocking buffer. Slides were then treated for 60 minutes with FITC, Texas Red, or CY5-conjugated donkey IgG against mouse IgM (for IIH6), rabbit and/or rat IgG in blocking buffer. Washes were carried out using PBS (4 times, 5 minutes each). Slides were subjected to a final wash and mounted in Fluoromount G (Southern Biotech) or SlowFade Gold (Molecular Probes).

Microscopy and image acquisition: Cells were visualized using a Zeiss Axioplan inverted fluorescence microscope fitted with a 10X eyepiece using 20X (0.5 N.A.) or 40X (0.75 N.A.) objectives. Images were captured using a Zeiss Axiocam MRM digital camera and Zeiss Axiovision imaging software.

Quantitation and Statistical Analysis: The relative intensity of Western blot protein bands was determined using densitometry (ImageJ™). Values were normalized to appropriate loading controls and expressed as the fold change relative to control samples (controls were assigned a value of either 1 or 100%). N values ranged from three to five. Error bars represent either standard deviation or standard error of the mean (indicated in figure legends). Mean relative pixel intensity was measured from fluorescent micrographs obtained using equal exposure times and settings (Axiovision). Area coverage by immunoreactivity was determined by measuring the area of immunofluorescence above a set threshold from 3 or more images per condition. This value was divided by the total number of cells in the fields to give the immunoreactive-positive area per cell in a given image. Statistical analysis was carried out using the 2-tailed paired student's t-test.

siRNA: A mixture of 4 siRNA duplexes targeted specifically to rat DG mRNA was used to substantially diminish dystroglycan protein expression (Dharmacon). Control cells were treated with a rat-specific non-targeting pool of siRNAs (Dharmacon). Transfection into OPCs was achieved using the Nucleofector

electroporation system with the rat oligodendrocyte transfection reagent as described in the manufacturers protocol (Amaxa). Cells were transferred immediately into culture dishes or chamber slides. Cells were switched into either differentiation medium (Sato+ 0.5% FCS) or serum-free DMEM (for signaling experiments) 16 hours later.

Immunoprecipitation: Primary oligodendrocyte cultures were lysed at 4^0C using 1% Triton X-100, 150nM NaCl, 50mM Tris (pH 7.5), 5mM EDTA, and protease/phosphatase inhibitors (Calbiochem). Cell lysates containing 150µg of total protein were pre-cleared for 30 minutes using Protein A/G agarose beads (Santa Cruz) equilibrated in TBS. Antibodies (50 µg/mL) were added to pre-cleared samples (input) or lysis buffer control samples and inverted on a rotating wheel overnight at 4^0C. Samples were then combined with 30 µL of Protein A/G bead 1:1 suspension and again gently inverted overnight on a rotating wheel at 4^0C. Beads were washed four times with TBS and proteins were separated from beads by boiling in 100 µL of 1X NuPAGE® lithium dodecyl sulphate sample buffer (Invitrogen) containing 3% BME.

Pharmacological inhibitors: To assess the effects of disrupting MAPK signaling,the ERK1/2 inhibitor, PD98059 (Sigma) or equivalent volume of vehicle (75% ETOH, 25% DMSO) was added to cells 30 minutes prior to growth factor treatment, and again 24 hours later. Cells were lysed approximately 60 hours after the first inhibitor treatment. To assess the effects of endogenous IGF-1

receptor signaling in primary oligodendrocyte cultures, The IGF-1 signaling inhibitor, Tryphostin1-OMe-AG (Sigma) or the equivalent volume of vehicle control (75% ETOH, 25% DMSO) was added to cells., For signaling experiments, inhibitor or vehicle was added to oligodendrocytes following a two-hour incubation in serum-free media. Cells were then lysed after 30 minutes. For differentiation experiments, inhibitor or vehicle was added to OPCs at the time of plating. Samples were incubated in differentiation media for 3 days prior to lysis.

Reagents:

Antibodies: The following antibodies were used for Western blotting: MBP (rat IgG; Serotec), 2',3'-cyclic nucleotide 3'-phosphadiesterase (CNP) (rat IgG; Sigma), β-dystroglycan (mouse IgG; Novocastra), NG2 (rabbit IgG; Chemicon), β-actin (mouse IgG; Sigma), Grb2 (rabbit IgG; Abcam), Fyn (mouse IgG; BD Biosciences), Src Family Kinase phosphorylated-tyrosine 418 (rabbit IgG; Calbiochem), phosphorylated Akt Ser473 (rabbit IgG; Cell Signaling), Akt (rabbit IgG; Cell Signaling) , phosphorylated ERK1/2 (T202/Y204) (rabbit IgG; R&D Systems), p44/42 MAP Kinase (total ERK1/2) (rabbit IgG; Cell Signaling), IRS1 (rabbit IgG; Abcam).The following antibodies were used in immunocyochemistry: α-DG (mouse IgM, clone IIH6; Upstate Biotechnologies); MBP (rat IgG; Serotec), FLAG peptide (mouse IgG; Sigma), IRS1 (rabbit IgG; Abcam), Grb2 (rabbit IgG; Abcam), FITC-conjugated goat anti-mouse IgM (Sigma) or Texas Red-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch), and FITC-, Texas Red-, or CY5-conjugated donkey IgG against mouse, rabbit or rat IgG.

Proteins: Human recombinant IGF-1 (PeproTech) was used at 50 or 100ng/mL. Unless otherwise indicated, plates or chamber slides were coated for 1 hour with 2.5ug/mL PDL (Sigma) at 37^0C in 7.5% CO_2. This PDL coating was followed by washes and 4 hour incubation with 10ug/mL laminin-2 (Millipore) under the same conditions. Recombinant laminin protein, rE3, which is comprised of the laminin α2 subunit LG domains 4 and 5 (rLG4/5), was purified as described previously (Li et al., 2005) using FLAG and heparin affinity column chromatography

CHAPTER 3 – Dystroglycan is expressed in oligodendrocytes and plays a role in mediating their terminal differentiation

Summary

Developmental abnormalities of myelination are observed in the brains of laminin-deficient humans and mice. The mechanisms by which these defects occur remain unknown. It has been proposed that, given their central role in mediating extracellular matrix interactions, integrin receptors are likely to play a role. However, it is a non-integrin extracellular matrix receptor, dystroglycan, which provides the key linkage between the dystrophin-glycoprotein complex (DGC) and laminin in skeletal muscle basal lamina such that disruption of this bridge results in muscular dystrophy. In addition, the loss of dystroglycan from Schwann cells causes myelin instability and disorganization of the nodes of Ranvier. Prior to present studies, it was unknown whether dystroglycan played a role during central nervous system (CNS) myelination. Here we report that oligodendrocytes, express and utilize dystroglycan receptors to regulate myelin formation and that expression correlates with differentiation. In the absence of normal dystroglycan expression, primary oligodendrocytes showed substantial deficits in their ability to differentiate and to produce normal levels of myelin-specific proteins. Together these results indicate that oligodendrocytes express the non-integrin laminin receptor, dystroglycan and use dystroglycan to mediate

laminin-induced differentiation.

Introduction

Extrinsic regulatory molecules such as growth factors are important for oligodendrocyte development during central nervous system (CNS) myelination (chapter 1). While less is known about the extrinsic regulation of oligodendrocyte development by extracellular matrix (ECM) molecules, at least one class of ECM, the laminin family of secreted glycoproteins, is required for normal CNS myelination (Colognato, ffrench-Constant et al. 2005). The LAMA2 gene encodes for the laminin-α2 subunit; mutations in this gene cause a severe form of muscular dystrophy termed MDC1A (congenital muscular dystrophy type 1A). This laminin-deficient dystrophy is characterized by accompanying developmental defects in white matter that are thought to reflect a failure of normal myelination (Jones, Morgan et al. 2001). Laminin α2 deficiency also causes delays in oligodendrocyte differentiation (Relucio, Tzvetanova et al. in press) and abnormal myelination in mice (Chun, Rasband et al. 2003). However, while a link exists between laminin expression and myelination in the CNS, the molecular mechanisms that underlie this requirement remain unclear.

Previous work showing increased cell death in newly-formed oligodendrocytes in the developing brains of mice lacking the laminin receptor α6β1 integrin has implicated integrins in regulating the interactions between laminins and oligodendrocytes (Colognato, Baron et al. 2002). However it remains unknown

whether laminins interact with oligodendrocytes solely through this integrin receptor. Evidence for multiple laminin receptors exists in the developing peripheral nervous system, where laminins are also required for normal myelination (reviewed in (Colognato, ffrench-Constant et al. 2005)). While β1 integrins are required for normal radial sorting of axons and Schwann cells (Feltri, Graus Porta et al. 2002), laminin-deficient Schwann cells have additional defects in cell survival, proliferation, and the ability to form normal myelin (Madrid, Jaros et al. 1975; Sunada, Edgar et al. 1995; Matsumura, Yamada et al. 1997; Yu, Ling et al. 2001; Chen and Strickland 2003; Occhi, Zambroni et al. 2005; Yang, Bierman et al. 2005). Several other laminin receptors were therefore proposed to regulate Schwann cell development, including the integrin α6β4 and dystroglycan (Yamada, Shimizu et al. 1994; Saito, Masaki et al. 1999; Sherman, Fabrizi et al. 2001; Feltri, Graus Porta et al. 2002; Previtali, Nodari et al. 2003; Saito, Moore et al. 2003). Studies in which mice were engineered to lack dystroglycan in Schwann cells showed that the PNS requires dystroglycan to achieve normal myelination (Saito, Moore et al. 2003; Occhi, Zambroni et al. 2005). Unlike integrin-null Schwann cells, the majority of dystroglycan-null Schwann cells are able to perform radial sorting of axons and to myelinate but have abnormalities in myelin ensheathment and node organization that cause myelin instability and neuropathy. The phenotype of the laminin α2-deficient PNS therefore reflects a loss of both integrin and dystroglycan signaling with each receptor playing distinct roles in the different stages of myelination.

In contrast to the PNS, it is unknown currently whether other laminin receptors play a role in CNS myelination as, to date, only the α6β1 integrin laminin-binding receptor has been identified in the oligodendrocyte lineage (Milner and Ffrench-Constant 1994; Buttery and Ffrench-Constant 1999). Here, by demonstrating binding and retention of a recombinant form of the laminin-2 (rE3) which contain the dystroglycan binding domain (LG4 and LG5) but lack the integrin binding domain (LG2 and LG3), we provide evidence for the presence of non-integrin receptors on oligodendrocyte surfaces. Double immunostaining for MBP and dystroglycan reveals that oligodendrocytes do indeed express dystroglycan on their surfaces. Maintaining cells in conditions conducive to differentiation (in plates pre-coated with laminin-2 or in low-serum media treated with exogenous insulin-like growth factor-1) caused significant enhancement of dystroglycan expression suggesting a correlation between dystroglycan expression and oligodendrocyte differentiation. Using small interfering RNA (siRNA) to knock down dystroglycan expression in oligodendrocytes resulted in significantly reduced levels of the differentiation marker proteins CNP and MBP compared to samples transfected with control siRNA. Taken together, these findings reveal that a non-integrin laminin receptor, dystroglycan, is expressed in oligodendrocytes and that dystroglycan expression correlates with and is needed to drive appropriate terminal differentiation. Such results suggest that dystroglycan may play a role in mediating laminin-2-induced myelinogenesis.

Results

Recombinant laminin (rE3) containing dystroglycan-binding sites but not integrin-binding sites, binds to the surface of differentiated oligodendrocytes--As a preliminary experiment to probe for dystroglycan or other non-integrin receptors in oligodendrocytes, we examined the ability of differentiated primary oligodendrocyte cultures to bind recombinant laminin protein. This flag tagged laminin protein, rE3, was designed to mimic E3, a well-characterized proteolytic fragment of laminin that is generated via elastase cleavage of the laminin α-chain C-terminal globular domain (Timpl, Johansson et al. 1983)(Fig 3.1A). This recombinant protein, which does not contain any integrin binding sites has been shown to exhibit similar dystroglycan binding capacity as that in intact laminin. Consequently, it has been used previously to investigate interactions between laminin and dystroglycan in muscle cells (Li, Harrison et al. 2002; Smirnov, McDearmon et al. 2002; Li, Liquari et al. 2005). However, it must be noted that this region of laminin can also bind to a variety of other cell-surface proteoglycans and therefore serves only as preliminary screen and not an exclusive measure of dystroglycan-receptor availability. Oligodendrocyte progenitor cells (OPCs) were isolated from mixed glial cultures as described in the methods section and freshly isolated cells (OPCs) were plated on poly-D-lysine (PDL) coated dishes and incubated in Sato's medium containing 0.5% serum, a condition known to induce differentiation. These cells were incubated with, or without, 10ug/ml rE3 in Sato's medium for one, three or five days. Prior to detergent lysis for Western blot analysis or fixation for immunocytochemistry, cells were washed extensively to remove unbound rE3. Because rE3 contains a

FLAG-epitope tag, detection using FLAG antibodies can be achieved by Western blotting and immunocytochemistry (Li, Harrison et al. 2002; Smirnov, McDearmon et al. 2002; Li, Liquari et al. 2005). Cell lysates were therefore evaluated by Western blotting for the presence of rE3 using FLAG antibodies (Fig 3.1A) and total protein load was monitored using actin antibodies. A progressive increase in rE3 binding was observed over five days of differentiation (Fig 3.1B). At day five of differentiation, the increase in rE3 capture compared with that of day 1 was increased significantly (n=3, P=0.0495; Fig 3.1B), rE3 binding to cells was also confirmed using indirect immunofluorescence with FLAG antibodies; a representative field of differentiated oligodendrocytes with bound rE3 is shown adjacent to a control field (Fig 3.1C). Together, these results indicate that differentiating oligodendrocytes are likely to express one or more non-integrin laminin receptors.

Oligodendrocytes express dystroglycan---Given that rE3 bound to and was retained by differentiated oligodendrocytes (Fig 3.1), and, that dystroglycan is expressed in Schwann cells where it is necessary for PNS myelination (Saito, Moore et al. 2003; Occhi, Zambroni et al. 2005), we deemed it logical that dystroglycan could be expressed in oligodendrocytes where it may serve as a second laminin receptor. To determine if dystroglycan was expressed on the surface of oligodendrocytes, we performed double immunocytochemistry with α-dystroglycan antibodies and MBP antibodies. Co-immunoreactivity of MBP and dystroglycan was found in both oligodendrocyte cell bodies and processes,

demonstrating that dystroglycan is expressed in differentiated oligodendrocytes (Fig 3.2).

Dystroglycan expression in oligodendrocytes correlates with differentiation---To further characterize dystroglycan in oligodendrocytes, we assessed dystroglycan protein levels under various cell culture conditions. To assess dystroglycan during oligodendrocyte development, cells were differentiated for either one day or four days and then evaluated for the presence of dystroglycan by immunoblotting cell lysates (Fig. 3.3A). To assess the effects of laminin on dystroglycan expression (laminin has been shown previously to positively regulate oligodendrocyte differentiation in vitro (Buttery and ffrench-Constant 1999)), cells were differentiated for four days in plates pre-coated with either laminin or poly-D-lysine (PDL), a non-ECM substrate. Lysates from respective samples were then evaluated for the presence of dystroglycan by immunoblotting (Fig. 3.4A). Finally, to assess the influence of exogenous IGF-1 on dystroglycan expression, another condition demonstrated to positively regulate differentiation of oligodendrocytes (McMorris, Mozell et al. 1993; D'Ercole, Ye et al. 2002; Ye, Li et al. 2002; Hsieh, Aimone et al. 2004), cells were treated with or without 50ng/mL IGF-1, incubated for 3 days in low serum media and evaluated for the presence of dystroglycan and MBP by immunoblotting lysates (Fig 3.5A).

We found that α-dystroglycan and β-dystroglycan were elevated (approximately 100%) in oligodendrocytes differentiated for 4 days compared to cells differentiated for 1 day. This difference was statistically significant for β-

dystroglycan (p=0.0494) but was not significant for α-dystroglycan levels (figure 3.3B). It should be noted, however, that α-dystroglycan antibodies recognize unique carbohydrates attached to the α-subunit and thus do not necessarily reflect core protein levels per se. These findings further demonstrate that dystroglycan is expressed in differentiated oligodendrocytes, and is suggestive of a positive correlation between dystroglycan expression and oligodendrocyte differentiation. Consistent with this, samples differentiated on laminin contained higher levels of dystroglycan than samples differentiated on PDL (Fig 3.4). Densitometry results for β- dystroglycan show a 34% increase in relative blotting intensity (figure 3.4B). Moreover, compared to untreated controls, IGF-1 treated samples contained significantly higher levels of MBP and β-Dystroglycan. Densitometry results show 14% and 20% increases in relative blotting intensity for MBP and β-Dystroglycan, respectively (Fig 3.5, B and C). These results confirm earlier reports that IGF-1 promotes differentiation in oligodendrocytes while providing further evidence of a correlation between dystroglycan expression and oligodendrocyte differentiation.

Finally, we do observe a small percentage of cells in our oligodendrocytes that are glial fibrillary acid protein (GFAP)-positive astrocytes (approximately 2-5%), but this percentage of cells does not change in our differentiation conditions that contain only 0.5% serum. To rule out that changes in dystroglycan levels could be attributed to changes in astrocyte dystroglycan, we purified astrocytes and tested whether astrocyte dystroglycan levels were detectable and also whether they changed under our differentiation conditions. As previously

reported, we confirmed that astrocytes express dystroglycan, but unlike with oligodendrocytes, astrocyte dystroglycan levels did not change during the four-day differentiation window (not shown).

Dystroglycan is necessary for proper differentiation in oligodendrocytes—To better address the role of dystroglycan during oligodendrocyte differentiation, our next approach was to transfect OPCs with siRNAs designed to target and degrade rat dystroglycan mRNA. At approximately 16 hours post-transfection, OPCs were switched to differentiation medium and differentiated for either 2 or 4 days (Fig 3.6). Knockdown of dystroglycan protein was achieved by day two and knockdown was reasonably well-maintained by day four (Fig 3.6 B). A representative field of cell-surface α-dystroglycan immunocytochemistry (Fig 3.6A, anti-dystroglycan signal is green) is shown for oligodendrocytes differentiated for four days following control or dystroglycan-siRNA transfection. Cell lysates from control and dystroglycan siRNA-treated oligodendrocytes were evaluated by Western blotting to monitor proteins that reflect the differentiation status of oligodendrocytes (Figure 3.7). At day two, only the two major isoforms of MBP (17.2 and 18.5 kDa) were detectable using longer exposure times (Fig. 3.7A, blot labeled as MBP'). We observed a substantial decrease in the level of MBP in cells treated with dystroglycan siRNA (n=3, 44.5+/- 14.3% relative to control cultures). We also evaluated the expression of 2', 3'-cyclic nucleotide 3'phosphodiesterase (CNP), an oligodendrocyte differentiation marker that is expressed earlier than MBP (Figure 3.7, A-C). As with MBP, we observed

decreased levels of CNP protein in dystroglycan siRNA treated oligodendrocytes relative to control cultures (66.0+/-15.1% with p= 0.0173 at day 2 and 71.8+/-14.0% with p=0.0068 at day 4). In contrast, a protein associated with early lineage oligodendrocytes, the cell surface proteoglycan NG2, was found to have no significant change in expression (93.7+/-49.3% at day 2, and 114.4+/-48.2% at day 4). Relative protein levels for all densitometric analysis were normalized to actin protein levels.

Discussion:

The dysmyelination observed in laminin α2-deficient mice, and potentially in humans, points to a role for laminins in myelination. The underlying mechanisms, however, remain unknown. Several lines of evidence implicate signaling via the integrin receptor α6β1 in regulating interactions between laminins and oligodendrocytes. First, increased cell death is observed in newly-formed oligodendrocytes in the developing brain of mice with a constitutive knock-out of the α6-subunit (Colognato, Baron et al. 2002) or a conditional knockout in oligodendroglia of the β1-subunit (Benninger, Colognato et al. 2006). Second, survival of oligodendrocytes grown in culture is reduced in the presence of antibodies that block oligodendrocyte α6β1 receptors (Corley, Ladiwala et al. 2001; Colognato, Baron et al. 2002; Frost, Zhou et al. 2009). Third, β1-integrin blocking antibodies have been shown to reduce myelin membrane sheet formation in cultured oligodendrocytes (Buttery and ffrench-Constant 1999; Relvas, Setzu et al. 2001; Olsen and Ffrench-Constant 2005). Fourth, expression of dominant negative β1-integrin in oligodendrocytes

perturbs remyelination in injured spinal cord (Relvas, Setzu et al. 2001) and disrupts myelination in the developing spinal cord and optic nerve (Lee, de Repentigny et al. 2006). Finally, several integrin-associated signaling molecules, including Integrin Linked Kinase (ILK) and Fyn, have been shown to be activated downstream of laminins in oligodendrocytes (Chun, Rasband et al. 2003; Colognato and ffrench-Constant 2004; Liang, Draghi et al. 2004). And, at least one of these signaling effector molecules, the Src family kinase Fyn, is required for normal CNS myelination (Umemori, Sato et al. 1994; Umemori, Kadowaki et al. 1999; Sperber, Boyle-Walsh et al. 2001; Sperber and McMorris 2001).

Despite the evidence that laminins transmit signals to oligodendrocytes using integrin receptors, it remains unclear to what extent the dysmyelination associated with laminin deficiency is caused by the loss of integrin signaling. The α6-integrin null mouse dies at birth due to severe skin blistering and thus only initial stages of myelination have been evaluated in the developing brain stem and spinal cord at embryonic day 18.5 (Georges-Labouesse, Messaddeq et al. 1996; Colognato, Baron et al. 2002). However, mice engineered to lack the integrin β1-subunit in oligodendrocytes were found to form normal appearing myelin in the brain and spinal cord (Benninger, Colognato et al. 2006). Another study examined the development of CNS myelination in the presence of a dominant-negative β1-integrin receptor and found that myelination was perturbed in the spinal cord and optic nerve, but not in the corpus callosum (Lee, de Repentigny et al. 2006). And, a third more recent

study found that, when β1-integrins were removed at the neural stem cell stage in the developing CNS, the resulting brain and spinal cord did indeed myelinate but generated thinner myelin as evidenced by g-ratio (Barros, Nguyen et al. 2009). On the other hand, in laminin deficient mice, myelination defects have been reported in the corpus callosum, optic nerve, and cerebellum, but absent in the spinal cord (Chun, Rasband et al. 2003; Relucio, Tzvetanova et al. in press). These laminin-associated defects include both thinner myelin i.e. dysmyelination but also include hypomyelination i.e. substantially increased levels of unmyelinated axons. Thus, laminin deficient mice, β1−integrin deficient mice, and β1−integrin compromised mice have different myelination phenotypes, pointing to additional oligodendrocyte laminins receptors that could contribute to myelination. Our current study shows that dystroglycan likely represents one of these additional receptors.

Here we utilized primary oligodendrocyte cultures to characterize the expression and role of dystroglycan in oligodendrocytes. By first demonstrating a strong affinity of flag-tagged rE3 (recombinant laminin consisting exclusively of purified, dystroglycan binding, LG3 and LG4 domains) for differentiated oligodendrocytes, we've provided the first evidence of non-integrin receptors in oligodendrocytes. This was confirmed by Immunocytochemistry demonstrating co-localization of dystroglycan and MBP in cells that were differentiated for 4 days. Immunoblotting was subsequently utilized to confirm the expression of dystroglycan in differentiated oligodendrocytes. Pre-coating plates with laminin which has been previously demonstrated to positively regulate oligodendrocyte

differentiation in vitro (Buttery and ffrench-Constant 1999) resulted in significant increases of dystroglycan expression compared to plating cells on poly-D-lysine (PDL) a standard non-ECM containing substrata used in growing primary cultures. Furthermore, treatment of samples with the pro-differentiation growth factor, IGF-1 enhanced levels of both MBP and dystroglycan. This confirms the growth factor as a positive regulator of differentiation in primary oligodendrocyte cultures as well as providing additional data to support a correlation between dystroglycan expression and oligodendrocyte differentiation. Finally, we were able to show that selective ablation of dystroglycan results in significantly reduced levels of the oligodendrocyte stage-specific marker proteins, CNP and MBP, suggesting that dystroglycan expression is necessary for proper terminal differentiation of oligodendrocytes.

Combining our current data with what is known about laminin/integrin interactions together suggests that the regulation of oligodendrocyte development by laminin is mediated by both integrins and dystroglycan. In support of this, other cell types have also been shown to utilize both integrin and dystroglycan receptors in mediating interactions with laminin extracellular matrices. In skeletal muscle and peripheral nerve, the disruption of each receptor type creates a distinct set of abnormalities that represent a subset of those caused by removal of the laminin ligand (reviewed (Feltri and Wrabetz 2005; Jimenez-Mallebrera, Brown et al. 2005)). In peripheral nerves, laminin removal causes a severe combination of amyelination and dysmyelination. However, the removal of Schwann cell β1-integrin causes a less severe failure in radial sorting

that may impede normal myelination (Feltri, Graus Porta et al. 2002) while the removal of Schwann cell dystroglycan has only minimal effects on radial sorting, yet results in disorganized myelin and improper nodal architecture that is susceptible to degeneration (Saito, Moore et al. 2003). In skeletal muscle a different hierarchy emerges: dystroglycan removal has the more severe consequence for muscle function while removal of the primary laminin-binding integrin, α7β1, causes subtle deficits (Mayer, Saher et al. 1997; Cohn, Mayer et al. 1999). A further complication of these studies is the fact that, in the absence of one receptor type, other receptor types can be dysregulated (Cohn, Mayer et al. 1999; Cote, Moukhles et al. 1999; Cote, Moukhles et al. 2002; Moghadaszadeh, Albrechtsen et al. 2003). This occurs in skeletal muscle in which integrin receptors are upregulated in the absence of dystroglycan. In the current study, we found that the interactions between laminin and oligodendrocytes resulted in an increase in the amount of dystroglycan protein that was expressed (Fig 3.4). So far, it is not known how laminin alters dystroglycan protein expression. One potential scenario is that early laminin/integrin interactions in oligodendrocytes trigger an increase in dystroglycan, which is then poised to play a role in more differentiated myelinating cells. In such a model, integrins have a more specific role in survival and process outgrowth earlier in development while dystroglycan is up-regulated in more mature oligodendrocytes where it is needed to drive terminal differentiation (Fig 3.8). In chapter 4 we explore the mechanism by which

interaction between lamimin and dystroglycan mediates differentiation in oligodendrocytes.

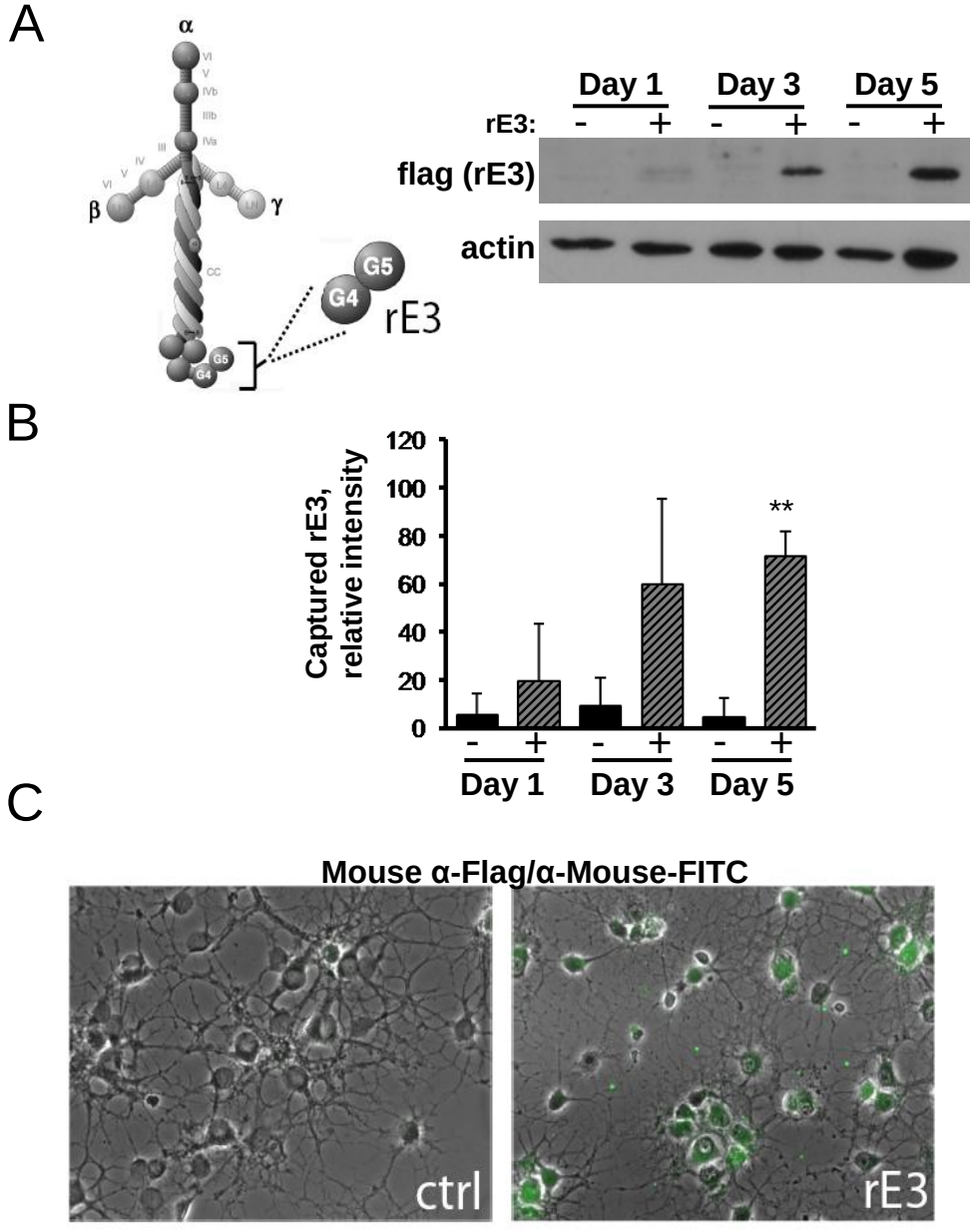


Figure 3.1 **Recombinant laminin (rE3) containing dystroglycan-binding sites but not integrin-binding sites, binds to the surface of differentiated oligodendrocytes. (A)** rE3 is a recombinant laminin subunit. rE3 has been shown previously to contain dystroglycan binding (LG4 and LG5), but not integrin binding (LG1 and LG2) sites. Oligodendrocytes were differentiated for indicated times in the presence or absence of 10ug/ml rE3 (flag tagged), washed to remove unbound protein and lysed. Lysates were probed to detect rE3 (anti-flag antibody) and actin (loading control) **(B)** Densitometry to determine the average relative intensity of the captured rE3 signal (n=3, **P<0.01, error bars represent s.d.) **(C)** FLAG antibodies followed by FITC-conjugated secondary antibodies were used to detect cell-surface binding of rE3 to differentiated oligodendrocytes. Control (ctrl) image depicts FLAG immunofluorescence on cells without added rE3. Representative merged phase and fluorescent micrographs are shown.

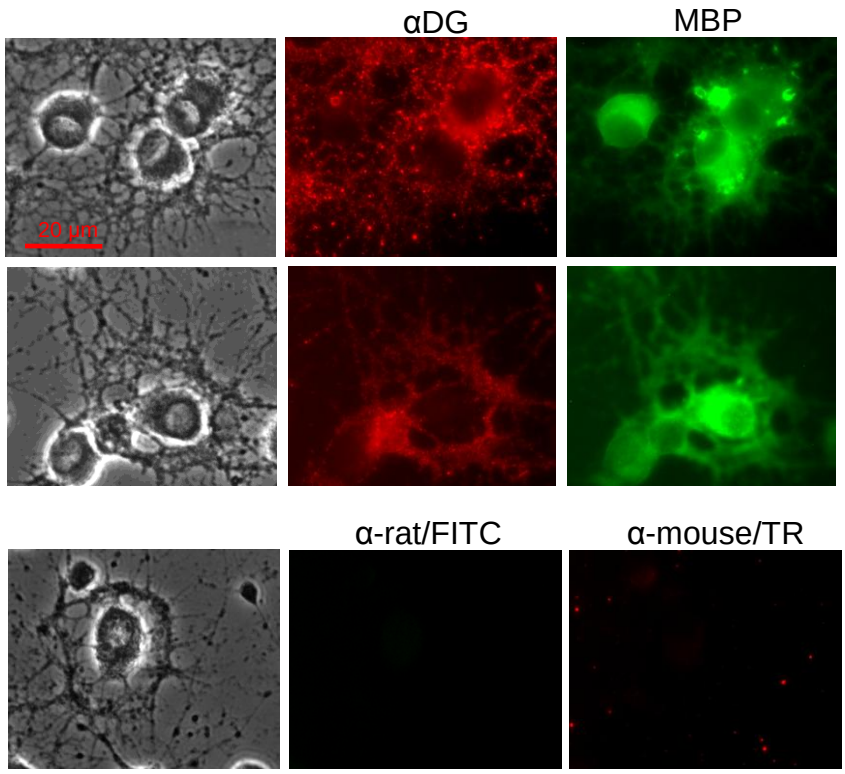
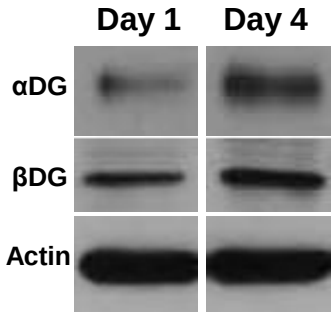


Figure 3.2 **Dystroglycan is expressed in oligodendrocytes.** Co-expression of α-Dystroglycan (αDG) and MBP (late stage oligodendrocyte marker) in oligodendrocytes differentiated for 3 days (top two rows). Control images showing staining resulting from secondary antibodies alone (bottom row). Scale bar: 20 µm.

A



B

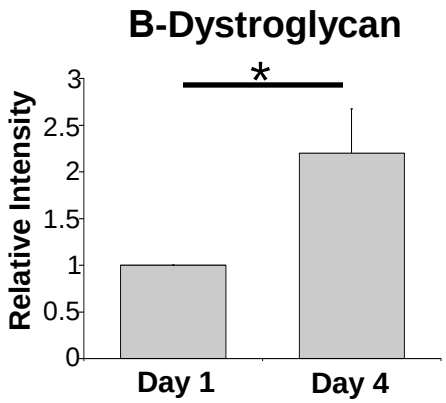
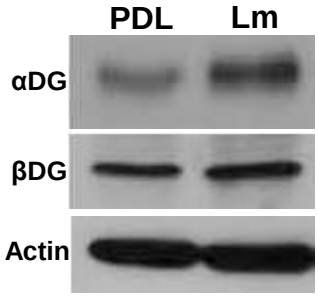


Figure 3.3 **Dystroglycan expression in oligodendrocytes correlates with their differentiation. (A)** Relative dystroglycan expression in oligodendrocytes differentiated for 1 or 4 days on laminin (Lm). Western blots were probed with antibodies specific for α-dystroglycan (α-DG), β-dystroglycan (β-DG) or actin (loading control). **(B)** Densitometry to determine relative β-DG expression. The average fold increase relative to expression at day 1 (n=3, *P<0.05, error bars represent SEM).

A



B

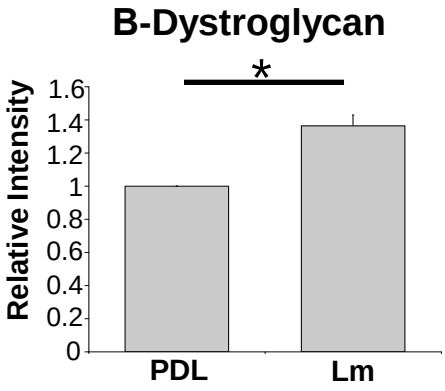


Fig 3.4 **Oligodendrocytes differentiated on laminin show increased levels of dystroglycan. (A)** Relative dystroglycan expression in oligodendrocytes differentiated for 4 days on poly-D-lysine (PDL) or laminin (Lm). Western blots were probed with antibodies specific for α-dystroglycan (α-DG), β-dystroglycan (β-DG) or actin (loading control). **(B)** Densitometry to determine relative β-DG expression. The average fold increase relative to expression on PDL (n=3, *P<0.05, error bars represent SEM).

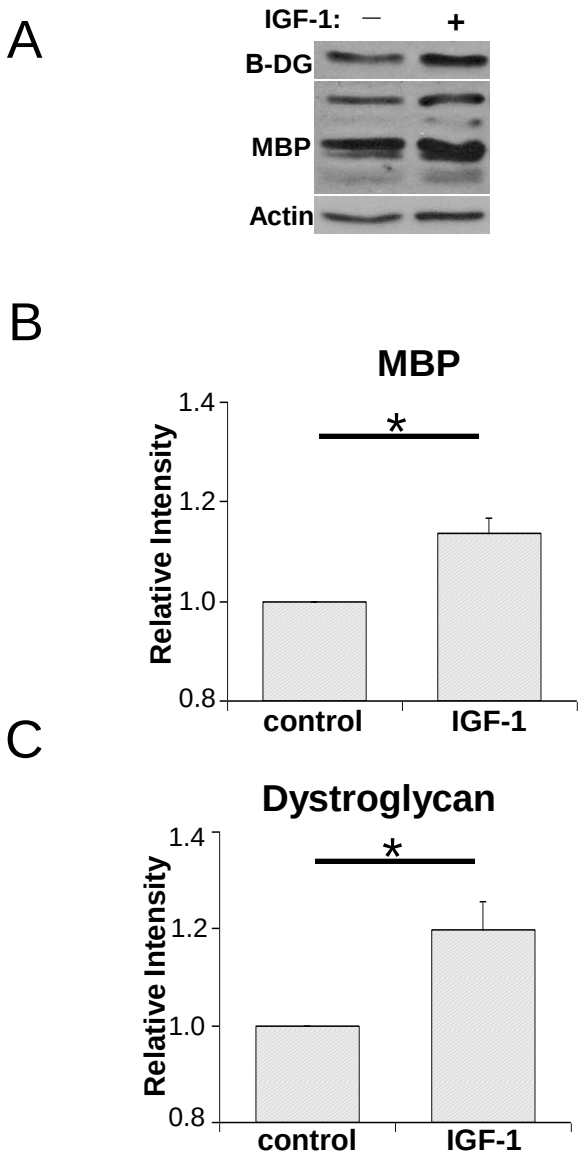


Fig 3.5 **Oligodendrocytes treated with exogenous IGF-1 show increased levels of myelin protein and dystroglycan production. (A)** Relative dystroglycan and MBP (late stage oligodendrocyte marker) expression in oligodendrocytes treated with 50ng/mL of exogenous IGF-1 and differentiated for 3 days. Western blots were probed with antibodies specific for β-dystroglycan (β-DG) and myelin basic protein (MBP). Blots were reprobed with antibodies against actin as a loading control. A representative western blot is shown. **(B)** Densitometry to determine relative MBP expression in sample treated with exogenous IGF-1. The average fold increase relative to untreated sample is shown (n=3,*P<0.05, error bars represent SEM) **(C)** Densitometry to determine relative β-DG expression in sample treated with exogenous IGF-1. The average fold increase relative to untreated sample is shown (n=3,*P<0.05, error bars represent SEM)

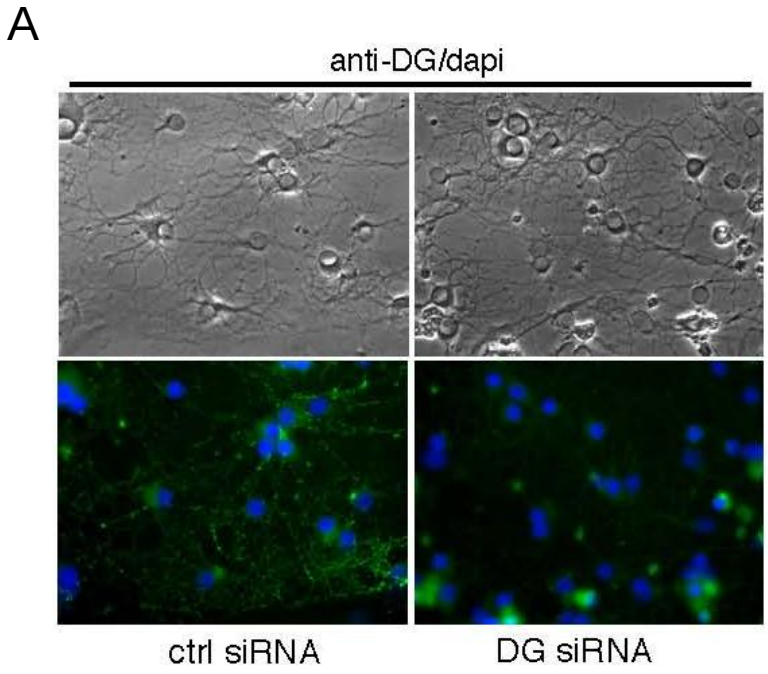


Fig 3.6 **Deletion of dystroglycan expression using siRNA (A)** Dystroglycan expression is reduced in oligodendrocytes differentiated for four days following transfection with siRNA designed to target dystroglycan mRNA (DAG-1 siRNA) compared with control siRNA (ctrl siRNA). Representative fields of cells visualized with α-dystroglycan antibodies (anti-DG) are shown. **(B)** Immunoblots using lysates obtained from cells transfected with control (ctrl) or dystroglycan (DG) siRNA following 2 or 4 days differentiation. Blotts were probed with antibodies to detect α-dystroglycan, β-dystroglycan and actin (loading control)

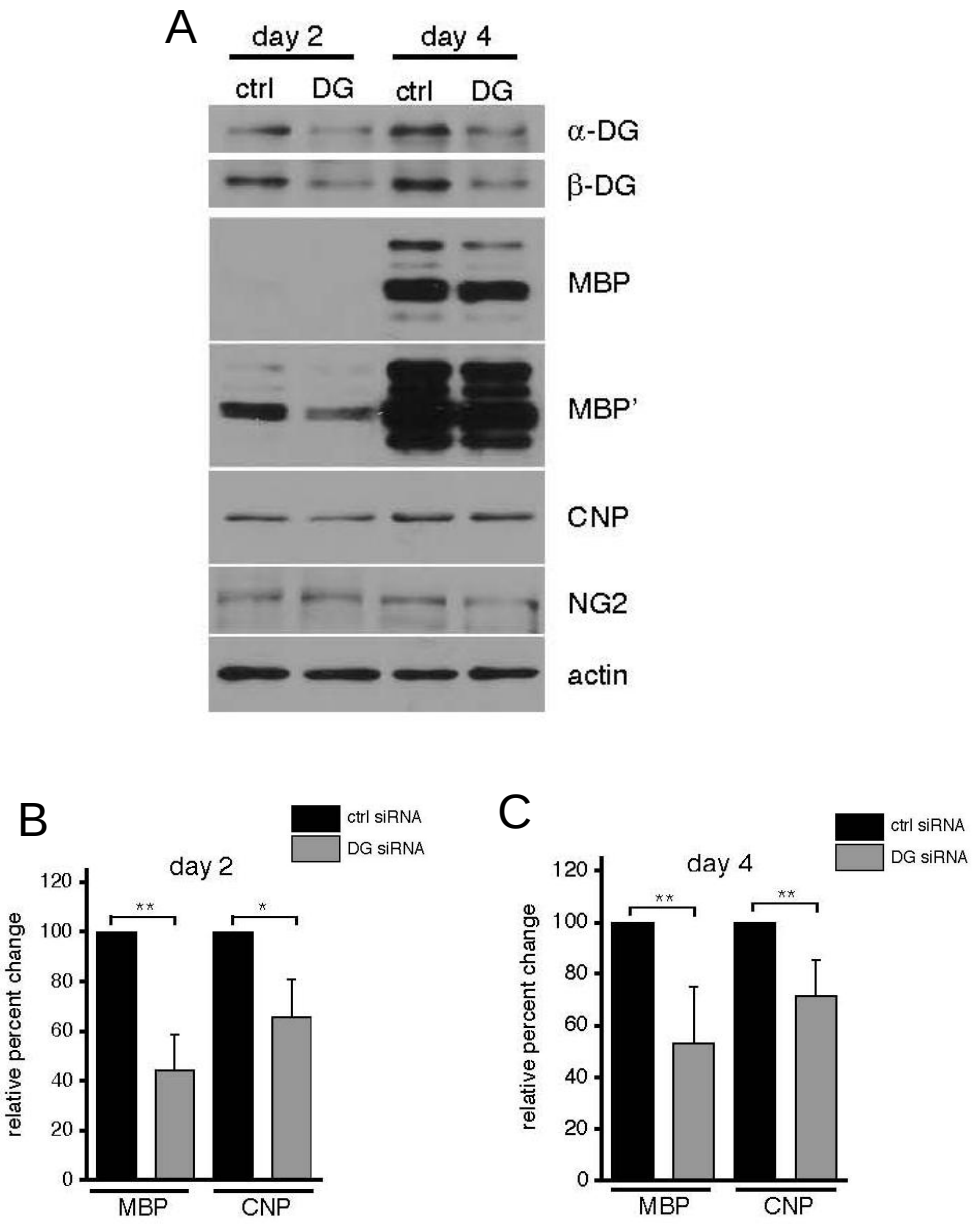


Fig 3.7 **Dystroglycan is required for differentiation of oligodendrocytes (A)** Immunoblots using lysates obtained from cells transfected with control (ctrl) or dystroglycan (DG) siRNA following 2 or 4 days of differentiation. Blotts were probed with antibodies to detect MBP (MBP' shows a longer exposure to visualize the MBP at day 2), CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase, mid-stage oligodendrocyte marker), NG2 (oligodendrocyte progenitor marker) and actin (loading control). **(B,C)** Average expression, relative to control siRNA at 100%, for MBP or CNP at day 2 (B; n=3; *P<0.05 and **P<0.01) or day 4 (C; n=4;**P<0.01) (error bars represent s.d.).

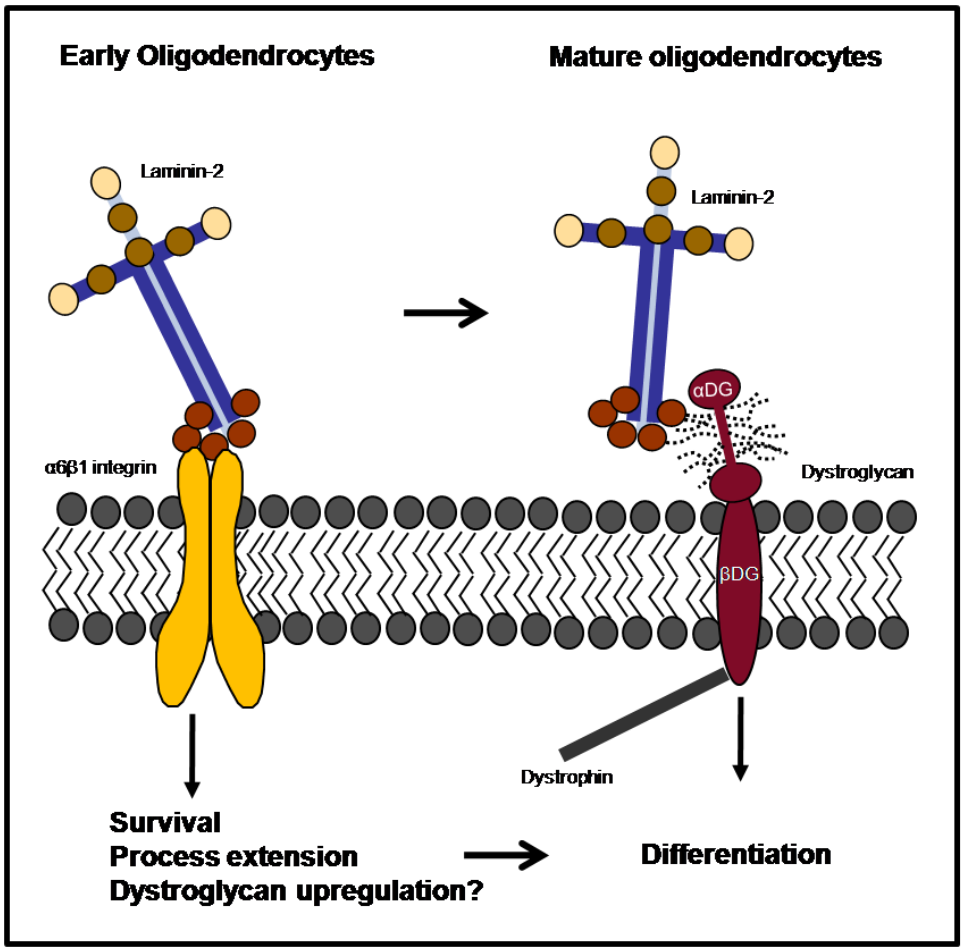


Fig 3.8 **Model for potential integrin and dystroglycan hierarchy.** In newly formed oligodendrocytes, laminin-2 interacts preferentially with integrins through which it regulates survival and process extension while possibly inducing dystroglycan expression. During the later stages of oligodendrocyte development, laminin-2 interacts with dystroglycan to positively regulate differentiation

CHAPTER 4 – Dystroglycan modulates insulin-like growth factor-1 signaling to promote oligodendrocyte differentiation

Summary

The adhesion receptor dystroglycan positively regulates terminal differentiation of oligodendrocytes. However the mechanism by which this occurs remains unclear. Using primary oligodendrocyte cultures, we identified and examined a functional connection between dystroglycan and insulin-like growth factor-1 (IGF-1) signaling. Consistent with previous reports, treatment with exogenous IGF-1 caused an increase in MBP protein that was preceded by activation of PI3K (AKT) and MAPK (ERK) signaling pathways. The extracellular matrix protein laminin was furthermore shown to potentiate the effect of IGF-1 on oligodendrocyte differentiation. Depletion of the laminin receptor dystroglycan using siRNA, however, blocked the ability of IGF-1 to promote oligodendrocyte differentiation in cells grown on laminin, suggesting a role for dystroglycan in IGF-1-mediated signal transduction. Indeed, loss of dystroglycan led to a reduction in the ability of IGF-1 to activate MAPK, but not PI3K, signaling pathways. Pharmacological inhibition of MAPK signaling also prevented IGF-1-induced increases in MBP, indicating that MAPK signaling was necessary to drive IGF-1-mediated enhancement of oligodendrocyte differentiation. Using immunoprecipitation, we found that dystroglycan, the adaptor protein Grb2, and insulin receptor substrate-1 (IRS1), were associated in a protein complex. Taken together, our results suggest that the positive regulatory effects of laminin on

oligodendrocyte differentiation are attributed, at least in part, to dystroglycan's ability to promote IGF-1-induced activation of MAPK signaling.

Introduction

The majority of cells in the brain lack a detectable cell-associated extracellular matrix (ECM), or basement membrane. However, basement membrane proteins such as laminins have been identified outside of traditional basement membrane structures e.g. in the adult subventricular zone (SVZ) in blood vessel-associated "fractone" structures, and, in the developing brain associated with future white matter axon tracts (Colognato, Baron et al. 2002; Mercier, Kitasako et al. 2003; Tavazoie, Van der Veken et al. 2008). Laminins that contain the α2-subunit are furthermore proposed to regulate CNS development as children born with laminin α2 deficiencies have white matter abnormalities, agyrias, and hypoplasias (Jones, Morgan et al. 2001). In vitro, laminins have been shown to enhance the differentiation of oligodendrocytes (Buttery and ffrench-Constant 1999). Laminin-deficient mice have been shown to have impaired myelinogenesis (Chun, Rasband et al. 2003) as well as delayed oligodendrocyte differentiation (Relucio, Tzvetanova et al. in press), suggesting that laminins may regulate oligodendrogenesis.

While the precise role of laminins during CNS myelination is not fully understood, examination of oligodendroglial laminin receptors (α6β1 integrin and the transmembrane glycoprotein dystroglycan) has provided insight into laminins potential influence on oligodendrogenesis. α6β1 integrin plays a role in

mediating growth factor-induced signaling through activation of PI3K/AKT (Colognato, Baron et al. 2002; Colognato, Ramachandrappa et al. 2004; Barros, Nguyen et al. 2009). Yet, while oligodendrocytes with deleted or disrupted β1 integrin exhibit minor CNS myelin abnormalities (Lee, de Repentigny et al. 2006; Barros, Nguyen et al. 2009; Camara, Wang et al. 2009) and cell survival deficits (Benninger, Colognato et al. 2006), animals with compromised integrin function have relatively normal oligodendrocyte development, in contrast to the oligodendrogenesis abnormalities observed in laminin-deficient animals (Chun, Rasband et al. 2003; Relucio, Tzvetanova et al. in press). Dystroglycan, on the other hand, has been shown to promote the differentiation of cultured oligodendrocytes (Colognato, Galvin et al. 2007). In contrast to α6β1 integrin, however, dystroglycan does not appear to affect oligodendrocyte survival, suggesting that the two receptors have distinct roles during oligodendrocyte development. Here, we aim to elucidate signaling mechanism(s) responsible for dystroglycan-mediated differentiation of oligodendrocytes.

In muscle and other tissues, dystroglycan is a critical component of the dystrophin-glycoprotein complex that links the cytoskeleton with the ECM (Sunada, Edgar et al. 1995) In addition, evidence suggests that dystroglycan may influence intracellular signaling through its association with adaptor molecules such as Grb2 (Russo, Di Stasio et al. 2000). In both muscle and the brain, Grb2 has been shown to bind to dystroglycan (Yang, Bierman et al. 2005). Grb2 contains SH2 and SH3 domains enabling it to bind a number of signaling molecules e.g. association between Grb2 and the guanine exchange factor SOS

(son of sevenless) mediates activation of MAPK/ERK signaling through RAS/RAF (Skolnik, Lee et al. 1993). Grb2 has also been shown to be critical in the propagation of IGF-1 signaling via its association with insulin receptor substrate-1 (IRS-1) (Holgado-Madruga, Emlet et al. 1996). Given that dystroglycan and the IGF-1-signaling cascade both associate with Grb2 and promote differentiation in oligodendrocytes, we examined a potential functional connection between dystroglycan, IGF-1 signaling, and oligodendrocyte differentiation. We observed that laminin's ability to enhance IGF-mediated effects on oligodendrocyte differentiation depended on dystroglycan, suggesting a functional connection between oligodendrocyte dystroglycan signal transduction and IGF-1 receptor signaling. Maximal activation of MAPK signaling by IGF-1, necessary for IGF-1-mediated enhancement of oligodendrocyte differentiation, was also found to be dystroglycan-dependent. We furthermore identified a protein complex in differentiating oligodendrocytes containing dystroglycan, Grb2, and IRS-1, and propose that this complex is a critical regulatory element that integrates extrinsic input from laminins and IGF-1 during oligodendrogenesis.

Results:

Laminin potentiates the ability of insulin-like growth factor-1 (IGF-1) to promote oligodendrocyte differentiation--IGF-1 has been shown previously to positively-regulate oligodendrocyte differentiation (McMorris, Mozell et al. 1993; D'Ercole,

Ye et al. 2002; Ye, Li et al. 2002; Hsieh, Aimone et al. 2004)(Fig 3.5). Since the extracellular matrix protein laminin has been shown previously to enhance oligodendrocyte growth factor receptor signaling downstream of neuregulin-1 and PDGF, we next examined whether laminin could potentiate the pro-differentiation effects of IGF-1 (Fig. 4.1). Cells were differentiated for 3 days on PDL or laminin substrates in the presence of IGF-1 and followed by CNP and MBP immunocytochemistry to visualize oligodendrocytes (CNP) and mature oligodendrocytes (MBP) (Fig 4.1, A and B). Oligodendrocytes differentiated with laminin plus IGF showed significantly increased percentages of MBP-positive oligodendrocytes (Fig. 4.1D; 21.4±1.3% on Lm versus 14.4±1.5% on PDL, n=3, p=0.0438) relative to cells differentiated in the presence of IGF alone. An additional indication of oligodendrocyte differentiation is an increase in cell area, mediated by process extension/branching and plasma membrane expansion. Here, we observed that IGF-mediated increases in mean area covered by CNP(+) (Fig 4.1E; 484.1±176.4% on Lm versus 335.6±155.0% on PDL, n=3, p=0.0268) or MBP(+) cells (Fig 4F; 108.9±12.8% on Lm versus 70.0±17.0% on PDL, n=3, p=0.0429) increased significantly when cells were grown on laminin. Together, these data suggested that IGF-mediated effects on oligodendrocyte differentiation were amplified by the presence of laminin.

Dystroglycan is required for laminin-mediated enhancement of oligodendrocyte differentiation by IGF-1--To investigate whether the laminin receptor dystroglycan was important for laminin-mediated enhancement of IGF-1, we compared IGF-1-

induced differentiation on laminin for 3 days in cells containing normal levels of dystroglycan (transfected with control siRNA) with that in cells containing reduced levels of dystroglycan (transfected with DAG-1 siRNA) (Fig. 4.2). First, we obtained protein lysates from control or dystroglycan-deficient cells to confirm effective knock-down of dystroglycan protein. As in our previous study (Colognato, Galvin et al. 2007) we obtained significant depletion of oligodendrocyte dystroglycan using a pool or 4 siRNAs to rat dystroglycan mRNA (Fig 4.2A). Cell lysates were also evaluated for the levels of oligodendrocyte lineage stage-specific proteins, NG2 (OPC), CNP (oligodendrocyte), and MBP (mature oligodendrocyte) (Fig 4.2A). As expected, control cells differentiated with IGF-1 showed a significant increase in total MBP protein content relative to untreated control cells (Fig 4.2B; 126.4±2.2%, n=3, p=0.0084). In contrast, dystroglycan-deficient cells did not increase MBP protein levels in the presence of added IGF-1 (Fig 4.2B; 79.7±6.9% of untreated control cells in the absence of IGF-1 versus 90.1±1.1% of untreated control cells in the presence of IGF-1, n=3, p=0.2975). The exon2-containing 21.5 kDa MBP isoform is believed to play a role in the regulating the onset of myelin synthesis and is highly associated with active myelination (Capello, Voskuhl et al. 1997). Densitometry was therefore performed selectively on the 21.5 kDa MBP isoform to determine whether dystroglycan loss preferentially affected this key isoform (Fig 4.2C). Control cells differentiated with IGF-1 showed a significant increase in the MBP protein content relative to untreated control cells (Fig. 4.2, B and C); 184.7±30.6%, n=3, p=0.0368). In contrast, dystroglycan-deficient cells did not show increased levels

of the 21.5 kDa MBP isoform when treated with IGF-1 (Fig. 3C; 76.7±22.7% of untreated control cells in the absence of IGF-1 versus 94.3±15.3% of untreated control cells in the presence of IGF-1, n=3, p=0.2782). In comparison with MBP, however, the loss of dystroglycan had little effect on the levels of NG2 or CNP, indicating that the requirement for dystroglycan in promoting laminin's ability to potentiate the effects of IGF-1 was restricted to later stages of oligodendrogenesis i.e. acquisition of myelin proteins (Fig 4.2A).

As oligodendrocytes expand the space covered by their processes and membrane during their maturation, an additional read-out for oligodendrocyte differentiation is an increase in the area covered by MBP-positive cells. The MBP-positive cell regions were visualized using immunocytochemistry of control or dystroglycan-depleted cells that were differentiated on laminin in the presence or absence of IGF-1 (Fig 4.3A). Mean MBP-immunoreactive areas, as a function of total cells per field, were significantly increased in control cells that were differentiated in the presence of IGF-1 (Fig 4.3, A and B; 99.9±4.9 μm2 with IGF-1 versus 58.5±2.3 μm2 without IGF-1, n=4, p=0.0043). In contrast, dystroglycan-deficient cells showed less MBP-positive area and even showed a slight decrease in MBP-positive area upon added IGF-1 (Fig 4.3, A and B; 48.7±1.3 μm2 without IGF-1 versus 30.1±1.7 μm2 with IGF-1, n=4, p=0.0177). And, when comparing both cell types in the presence of IGF-1, dystroglycan-deficient cells showed significantly less MBP-positive area compared to control cells (Fig 4.3B; n=4, p=0.0007). Together these data indicated that the ability of IGF-1 to

enhance the differentiation of oligodendrocytes grown on laminin was dependent on the presence of dystroglycan.

IGF-stimulation of MAPK/ERK1/2, but not PI3K/Akt, is promoted by dystroglycan- - MAPK, PI3K and SFK signaling cascades are key intrinsic regulators of oligodendrocyte development (Colognato and ffrench-Constant 2004; Liang, Draghi et al. 2004; Baron, Colognato et al. 2005). IGF-1 has previously been shown to promote both MAPK and PI3K signaling in oligodendrocytes (Bibollet-Bahena and Almazan 2009). To confirm and further characterize the signaling events activated by IGF-1, oligodendrocytes grown on laminin were serum starved for 2 hours, followed by treatment with 50 ng/ml IGF-1 for the indicated time points (Fig 4.4). Blots indicated that during the time course of IGF-1 stimulation, both ERK1/2 and Akt phosphorylation increased, peaking at approximately 20 minutes post-stimulation, and then decreased to near baseline levels by 1 hour. In contrast, Fyn, an SFK implicated in oligodendrocyte development (Sperber, Boyle-Walsh et al. 2001; Liang, Draghi et al. 2004; Relucio, Tzvetanova et al. in press), did not show substantial changes in phosphorylation during the IGF-1 time course (Fig. 4.4). To determine whether dystroglycan was necessary for IGF-1 mediated activation of ERK1/2, control and dystroglycan-deficient cells were differentiated on laminin, serum-starved as above, and then treated with 50 ng/ml IGF-1 for 20 minutes (Fig 4.5A). Control cells showed a significant increase in ERK1/2 phosphorylation (relative to total ERK1/2 protein) following IGF-1 treatment (Fig 4.5B; 157.2±15.8% of untreated

cells, n=4, p=0.0214). In contrast, dystroglycan-deficient cells showed significantly less relative ERK1/2 phosphorylation in the absence of IGF-1 (Fig 4.5B; 45.7±10.7%, n=4, p=0.0038) as well as when treated with IGF-1 (Fig 4.5B; 113.2±8.5%, n=4, p=0.0319). However, dystroglycan-deficient cells remained responsive to IGF-1 stimulation such that deficient cells showed a significant increase with IGF-1 (Fig 4.5B; n=4, p=0.0041). Thus, dystroglycan was required for a maximal ERK1/2 phosphorylation response, yet dystroglycan-deficient cells remained able to respond to IGF-1 and induce ERK1/2 phosphorylation.

As previously stated, primary oligodendrocyte cultures obtained using mechanical dissociation and differential adhesion typically contain small amounts of contaminating astrocytes (approximately 3%-5%) which, like oligodendrocytes, express dystroglycan. Therefore, it is conceivable that dystroglycan-dependent, IGF-1-mediated activation of ERK1/2 signaling could be occurring in contaminating astrocytes as well as in oligodendrocytes. To examine this possibility, we treated purified astrocyte cultures as described above (control and dystroglycan-deficient cells were differentiated on laminin, serum-starved, and then treated with or without 50 ng/ml IGF-1 for 20 minutes). While ERK1/2 signaling was activated by IGF-1 and dystroglycan expression was knocked down by DAG-1 siRNA, the activation of ERK1/2 signaling in astrocytes does not appear to be dependent on dystroglycan expression as there was no difference in band intensity between control and dystroglycan-deficient astrocytes (Fig 4.5C). Hence, in oligodendrocyte cultures, dystroglycan dependent ERK1/2

signaling is likely a function of oligodendrocyte properties and not due to contaminating astrocytes.

In contrast to ERK1/2 activation, IGF-1-induced AKT phosphorylation was not significantly affected by the loss of dystroglycan protein (Fig. 4.6, A and B). In other words, AKt phosphorylation in response to IGF-1 remained unchanged in the presence or absence of dystroglycan. Untreated control cells were therefore not significantly different from dystroglycan-deficient cells (Fig 4.6B; 121.2±22.4%, n=3, p=0.6834), and IGF-1 stimulated control cells were therefore not significantly different form IGF-1 stimulated dystroglycan-deficient cells (Fig 4.6B; 260.5±21.9% in control cells versus 252.6±49.1% in dystroglycan-deficient cells, n=3, p=0.5149). Together, these data suggest that dystroglycan-laminin interactions are required for enhancement of ERK1/2, but not Akt, phosphorylation in response to IGF-1. This finding is consistent with our previous studies reporting that the enhancement of PDGF-mediated oligodendrocyte survival by laminin did not require dystroglycan but instead involved the activation of PI3K/Akt signaling through α6β1 integrin (Colognato, Galvin et al. 2007).

We noted that ERK1/2 phosphorylation was lower in dystroglycan-deficient cells in the absence of exogenous IGF-1 (Fig 4.5, A and B) and wondered whether IGF-1 that was synthesized by oligodendrocytes was a significant contributor. Indeed, IGF-1 synthesized by oligodendrocytes has previously been reported to promote autocrine signaling (Pfeiffer, Warrington et al. 1993). Here we used the IGF-1 inhibitor Tyrphostin 1-OMe-AG (Tyrphostin) to test whether

blockade of IGF-1 autocrine effects could further dampen oligodendrocyte differentiation and/or MAPK signaling (Fig 4.7). Treatment with 10uM tyrphostin caused a reduction in ERK1/2 phosphorylation and in MBP protein levels, when compared to cells treated with vehicle control (Fig 4.7, A and B). These data confirm that, in primary oligodendrocyte cultures, autocrine IGF-1 signaling activates MAPK/ERK signaling and promotes differentiation.

IGF-1-mediated differentiation is mediated by MAPK signaling--The ability of IGF-1 to enhance oligodendrocyte differentiation has been found to require MAPK signaling (Baron, Metz et al. 2000; Palacios, Sanchez-Franco et al. 2005; Bibollet-Bahena and Almazan 2009). To confirm that IGF-1-mediated effects on oligodendrocyte differentiation were also MAPK-pathway-dependent when cells were grown on laminin, we compared cells treated for 3 days with MEK1/2 inhibitor (PD98059) or vehicle control (Fig 4.8). As expected, oligodendrocytes grown on laminin and treated with IGF-1 for 3 days showed significantly increased levels of MBP protein relative to cells without added IGF-1 (Fig 4.8, A and B; 125.6$\pm$2.8 of untreated cells, n=4, p=0.296). In contrast, those that were differentiated in the presence of PD98059 showed no change in MBP levels upon IGF-1 treatment (81.4$\pm$10.0% without IGF-1 and 85.0$\pm$9.2% with IGF-1, n=4, p=0.9372). As in Fig 4.2, relative MBP levels were determined as a percentage of that found in control cells without added IGF-1. These data confirm and extend previous reports that IGF-1-mediated effects on oligodendrocyte differentiation rely on an intact MAPK signaling cascade; in the current study we additionally

demonstrate that combined IGF-1/laminin effects on differentiation are also reliant on MAPK transduction.

Oligodendrocyte dystroglycan associates with Grb2 and IRS1--The adaptor protein Grb2, important in many contexts for appropriate MAPK signal transduction, has been reported to associate with dystroglycan in fibroblasts through interaction of its SH3 domains with the C-terminal region of beta dystroglycan (Spence, Dhillon et al. 2004). In addition, the IGF-1 signaling protein, IRS1, has been shown to interact with Grb2 through interaction of its SH2 domain (Holgado-Madruga, Emlet et al. 1996). Together these reports suggested that Grb2 may play a role in linking the dystrophin-glycoprotein complex with the IGF-1 signaling pathway. To test this hypothesis we first used immunocytochemistry to determine whether dystroglycan, Grb-2 and IRS1 were co-expressed in primary oligodendrocytes cultures (Fig 4.9, A and B). Co-immunocytochemistry for IRS1 with MBP and dystroglycan (Fig 4.9A), as wells as for Grb2 with MBP and dystroglycan (Fig 4.9B), confirms the presence of GRB2 and IRS1 in differentiated oligodendrocytes. Next, we asked whether, using co-immunoprecipitation, we could identify Grb2, dystroglycan, and/or IRS-1 together in a protein complex (Fig 4.9C). Indeed, immunoprecipiations using the Grb2 antibody revealed both dystroglycan and IRS-1 in the isolated complexes, while the IRS1 antibody immunoprecipitated dystroglycan and Grb2 (Fig 4.9C). These data suggested that dystrgoglycan is able to influence IGF-1-mediated

signal transduction through its association with Grb2 and thus with IRS-1, an important component of the IGF-1 receptor signaling apparatus.

Discussion

In the current study we investigated the downstream mechanism(s) by which the extracellular matrix (ECM) receptor, dystroglycan, is able to promote oligodendrocyte maturation. We identified a functional link between the IGF-1 receptor signaling pathway and oligodendrocyte dystroglycan. Previous studies have shown that ECM-binding integrins potentiate oligodendroglial responses to a variety of growth factors, including PDGF and neuregulin (Frost, Buttery et al. 1999; Colognato, Baron et al. 2002; Frost, Zhou et al. 2009). However, the current study provides the first evidence that laminin-dystroglycan interactions are also capable of potentiating growth factor signaling during oligodendrogenesis.

As a key regulator of myelinogenesis, IGF-1 has been shown to promote the generation, proliferation, survival, and differentiation of oligodendrocytes (McMorris, Smith et al. 1986; Barres, Schmid et al. 1993; Jiang, Frederick et al. 2001; Hsieh, Aimone et al. 2004). Studies in transgenic mice overexpressing IGF-1 have furthermore revealed that excess IGF-1 results in increased myelin thickness as well as an increase in the percentage of myelinated axons (D'Ercole, Ye et al. 2002). IGF-1-null mice, as well as in mice that over-express IGFBP-1, an IGF-1 binding protein that interferes with endogenous IGF-1 signaling, both show a decreased percentage of myelinated axons (Ye, Li et al.

2002; Ye, Li et al. 2002). IGF-1 signaling has also been implicated in remyelination, as genetic disruption of the IGF-1 receptor (IGF1R) leads to impaired myelin repair following recovery from cuprizone-mediated demyelination (Mason, Jones et al. 2000; Mason, Xuan et al. 2003).

Dystroglycan has previously been shown by our group to promote the maturation of oligodendrocytes (Chapter 3)(Colognato, Galvin et al. 2007), yet the underlying signaling mechanism(s) necessary for this role have not been elucidated. Dystroglycan protein complexes, of both signaling and structural varieties, have been identified, however, in a variety of cells, including other glial cell types. In Schwann cells, dystroglycan is required for proper clustering of sodium channels, formation of microvilli, and folding of myelin (Occhi, Zambroni et al. 2005). Several dystroglycan interacting proteins have been identified in Schwann cells including sarcoglycans, sarcospan, α-dystrobrevin 1, Dystrophin-related protein 2, DP116 (an alternatively spiced form of dystrophin) and α1-syntrophin (Saito, Moore et al. 2003) In astrocytes, dystroglycan has been shown to complex with, and mediate clustering of, the sodium channel Kir 4.1 and the water channel aquaporin 4 (AQP4) (Guadagno and Moukhles 2004). To date, most information regarding dystroglycans role as a component of signal transduction cascades has been gleaned from studies of fibroblasts, where dystroglycan has been shown to bind extracellular regulated kinase (ERK) and its upstream activator, mitogen activated protein kinase kinase 2 (MEK2) (Spence et. al. (2004)).

Here, we set out to elucidate the potential signaling mechanisms involved in dystroglycan-mediated oligodendrocyte differentiation. In chapter 3 we confirmed previous reports that IGF-1 promotes oligodendrocyte differentiation (Fig 3.5). Interestingly, in the current study, we found that the dystroglycan ligand, laminin, was able to potentiate these effects (Fig 4.1). Subsequently, we also found that this laminin effect was dystroglycan-dependent (sensitive to the depletion of dystroglycan using small interfering RNAs) (Fig 4.2 and 4.3).

IGF-1 signaling is initiated by the binding of IGF-1 to its receptor tyrosine kinase, IGF1R. Activation of the receptor is marked by conformational changes and the phosphorylation of tyrosines along the cytoplasmic beta subunit (Gronborg, Wulff et al. 1993). Some of these phosphorylated sites serve as docking sites for insulin receptor substrates (IRS1-IRS4), a group of molecules that mediate IGF-1 signaling downstream of IGF1R. Activated IRS's can activate both MAPK and PI3K signaling through the recruitment of, and association with certain adaptor and signaling proteins. Indeed, the adapter protein, growth factor receptor-bound protein-2 (Grb2) is known to bind to activated IRS1 via its SH2 domain (Holgado-Madruga, Emlet et al. 1996). In addition, Grb2, through one of its SH3 domains, has been reported to associate with the proline-rich region of β-dystroglycan (Russo, Di Stasio et al. 2000). Here, we confirm previously reported expression of IRS1 and Grb2 in oligodendrocytes (Freude, Leeser et al. 2008)(Fig 4.8), and through immunoprecipitation, demonstrate that Grb2 and IRS1 are found associated in a protein complex that includes dystroglycan (Fig 4.9). Surprisingly, the addition of exogenous IGF-1 did not yield detectable

increases in the amount of proteins immunopreciptated (data not shown). It is possible that endogenous IGF-1 (the presence of which was confirmed in Fig 4.7) may have been sufficient to induce maximum binding between IRS1, Grb2 and dystroglycan. However, this hypothesis could not be substantiated as efforts to ablate autocrine signaling with IGF-1 inhibitor (Tyrphostin 1-OMe-AG) caused a dramatic reduction in cell number and subsequent protein yield that rendered immunoprecipitation data unreliable. Hence, it remains unclear whether or not the association between IRS1, Grb2 and dystroglycan was ligand dependent. Nonetheless, the current findings provide novel evidence of an association between dystroglycan and the IGF-1 signaling cascade in oligodendrocytes; such an association could be the structural basis for observed functional relationships. For instance, in Fig 4.5, we report that IGF-1-induced activation of ERK1/2 is dystroglycan dependent. Perhaps intact dystroglycan enables maximal ERK1/2 activation via IGF-1 by fostering efficient recruitment of Grb2 by IRS1. In support of this, over-expression of Grb2 has been reported to enhance insulin-induced MAPK signaling through Ras and result in increased association among Grb2, Sos and IRS1 (Skolnik, Lee et al. 1993).

In addition to a well documented mitogenic role in oligodendrocyte progenitors (Jiang, Frederick et al. 2001), MAPK/ERK signaling has been shown to drive differentiation and process extension in maturing oligodendrocytes (Althaus, Hempel et al. 1997; Stariha, Kikuchi et al. 1997; Baron, Metz et al. 2000). Furthermore, the ablation of Raf-2, a key upstream activator of MAPK/ERK signaling in neural precursor cells, revealed that MAPK signaling is necessary for

appropriate CNS myelination (Galabova-Kovacs, Catalanotti et al. 2008). Consistent with this role for MAPK/ERK signaling in promoting differentiation, we found that treatment of primary oligodendrocyte cultures with MAPK/ERK inhibitor (PD98059) impaired IGF-1-enhanced MBP expression after 3 days in low serum media (Fig 4.7). These data suggest that IGF-1 induced increases in ERK1/2 signaling (Fig 4.4 and 4.5), at least partially underlie IGF-1-induced increases in oligodendrocyte differentiation (Fig 3.5 and 4.5). Perhaps the ability of IGF-1 to optimally stimulate MAPK signaling and, by extension, promote oligodendrocyte differentiation, is modulated by dystroglycan/laminin interactions through structural association with the IGF-1 signaling cascade via Grb2 and IRS1 (Fig 4.10).

Taken together, these data suggest that laminin's ability to modulate growth factor signaling is not limited to its interactions with integrin receptors, and instead may be dependent on the precise repertoire of laminin receptors that are available and/or engaged. Further investigation will be required to determine whether additional mechanisms exist whereby dystroglycan is able to promote oligodendrocyte differentiation, and whether IGF-1 and dystroglycan similarly cooperate either during developmental myelination or during myelin repair following injury.

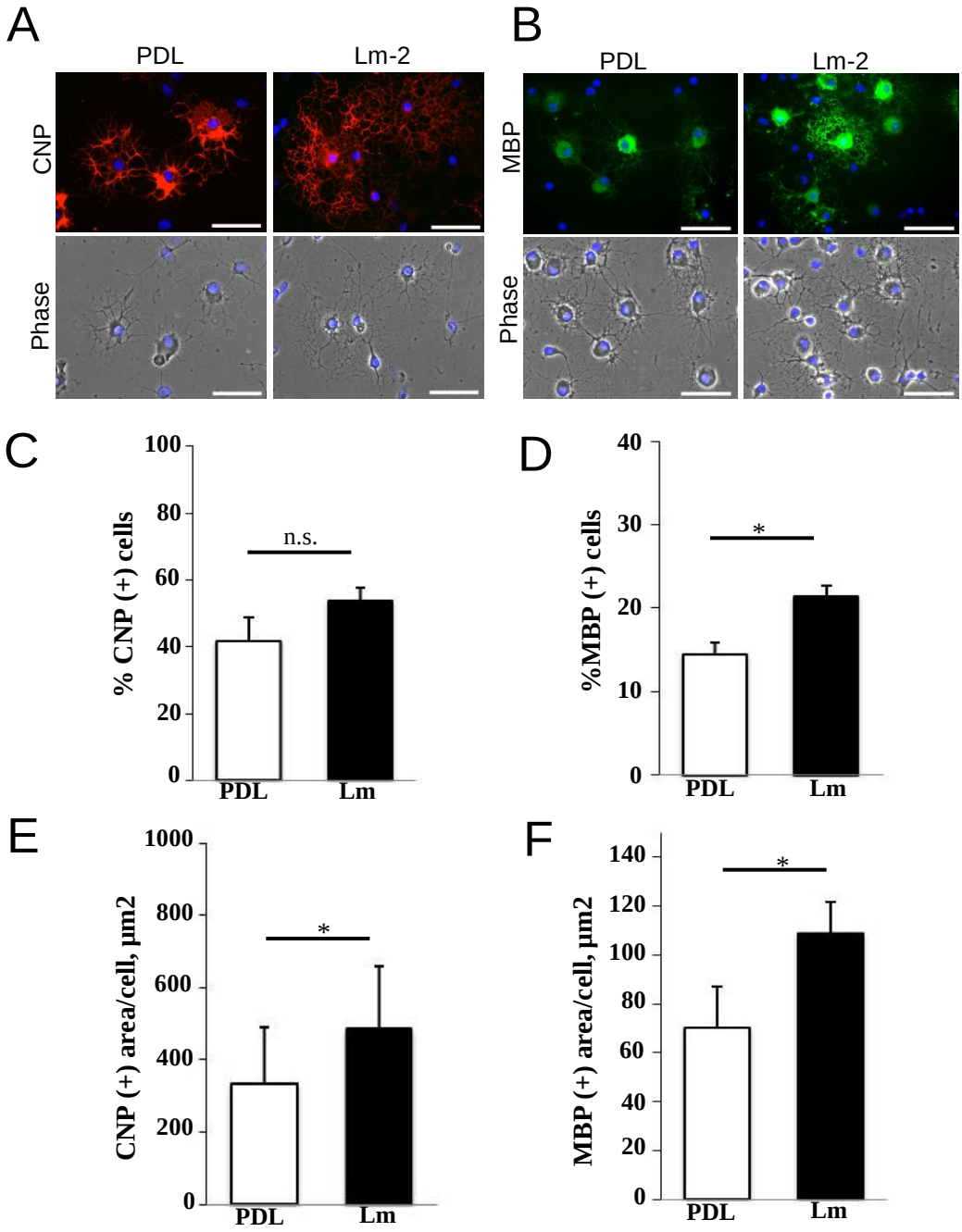


Fig 4.1 **Laminin potentiates IGF-mediated effects on oligodendrocyte differentiation.** Oligodendrocytes were differentiated on PDL or laminin substrates for 3 days in the presence of 50 ng/ml IGF-1. **(A)** Differentiated oligodendrocytes were evaluated using CNP immunocytochemistry. Representative micrographs of CNP immunoreactivity (red) and DAPI nuclear stain (blue) are shown. Scale bar equals 50µm. **(B)** Differentiated oligodendrocytes were evaluated using MBP immunocytochemistry. Representative micrographs of MBP immunoreactivity (green) and DAPI nuclear stain (blue) are shown. Scale bar equals 50µm. **(C)** Bars represent the mean percentage (± sem) of CNP(+) cells after differentiation on PDL or laminin (Lm). **(D)** Bars represent the mean percentage (± sem) of MBP(+) cells after differentiation on PDL or laminin (Lm). **(E)** Bars represent the mean (± sem) relative change in CNP(+) oligodendrocyte cell area after differentiation on PDL or laminin (Lm). Mean areas are normalized to mean area of control cells on PDL. **(F)** Bars represent the mean (± sem) relative change in MBP(+) oligodendrocyte cell area after differentiation on PDL or laminin (Lm). Mean areas are normalized to mean area of control cells on PDL (*p<0.05).

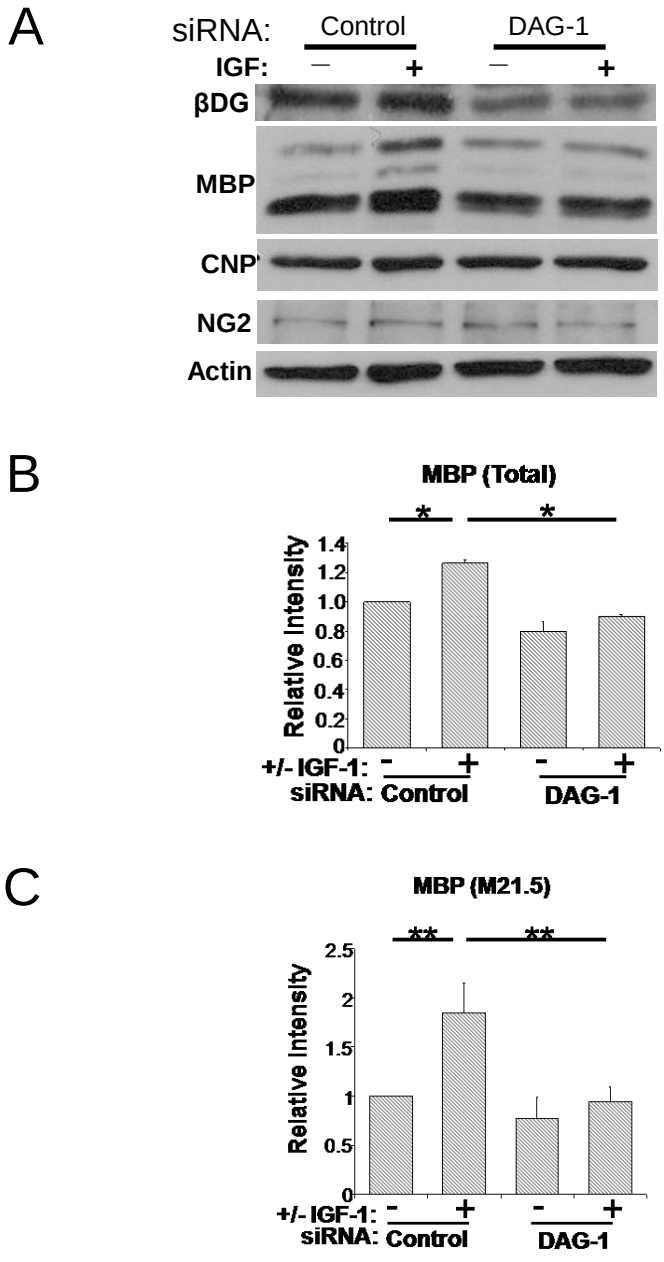
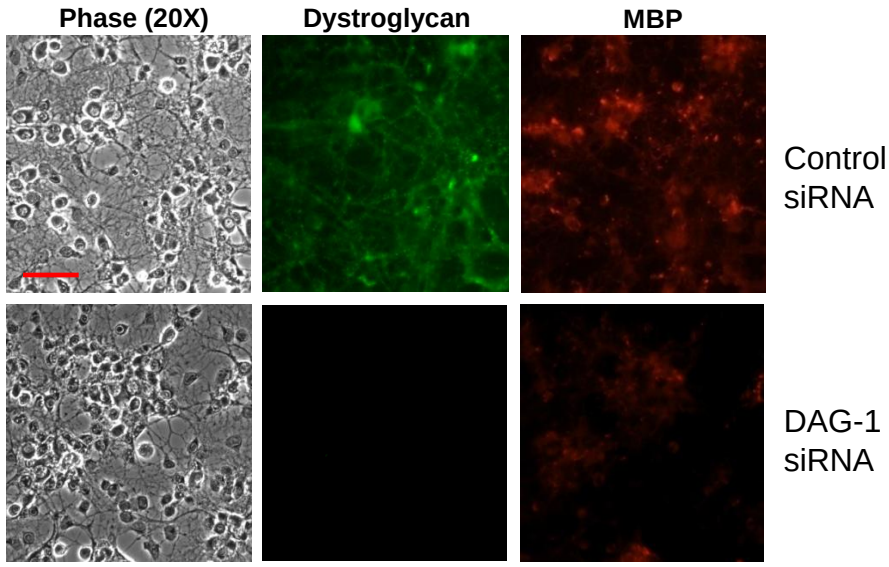


Fig 4.2 **Dystroglycan siRNA reverses laminin/IGF-1-induced increases in myelin protein production. (A)** Immunoblots using lysates obtained from cells differentiated for 3 days on laminin following transfection with control or dystroglycan (DAG-1) siRNA and treated with or without 50ng/mL IGF-1. Blots were probed with antibodies to detect β-dystroglycan, MBP, CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase, mid-stage oligodendrocyte marker), NG2 (early stage oligodendrocyte marker) or actin (loading control). **(B,C)** Densitometry to determine MBP expression (total (B) or 21.5 kDa isoform (C)) relative to untreated sample transfected with control siRNA (n=4,*P<0.05, error bars represent SEM)

A



B

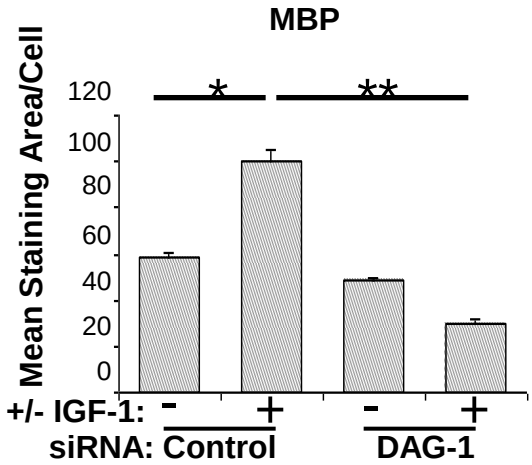


Fig 4.3 **Dystroglycan siRNA reverses laminin/IGF-1-induced increases in myelin protein production. (A)**) Representative fields of cells visualized with MBP and β-DG antibodies. Displayed samples were plated on laminin, treated with IGF-1 following transfection with DAG-1 or control siRNA. Scale bar: 50 µm. **(B)** Quantitation of MBP (staining area per cell) as described in methods section (n=3,*P<0.05, **P<0.01, error bars represent SEM)

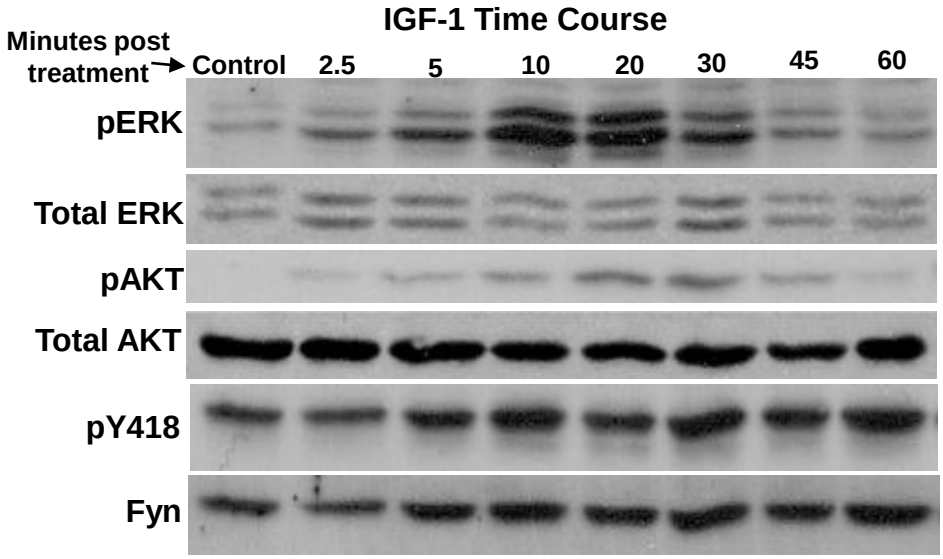


Fig 4.4 **IGF-1 activates MAPK/ERK and PI3K/AKT but not SFK signaling cascades in oligodendrocytes (A)** Immunoblots from Oligodendrocyte progenitors lysed at indicated time points following treatment with 100ng IGF-1. Blots were probed with antibodies to detect pERK (phosphorylated extracellular signal-regulated kinase, a marker of MAPK activation), total ERK (loading control for pERK), pAKT (phosphorylated AKT, a marker of PI3K signal activation), total AKT (loading control for pAKT), pY418 (phosphorylated tyrosine-418 of srk-family kinases (SFK's), marker of SFK activation and Fyn (loading control for pY418)

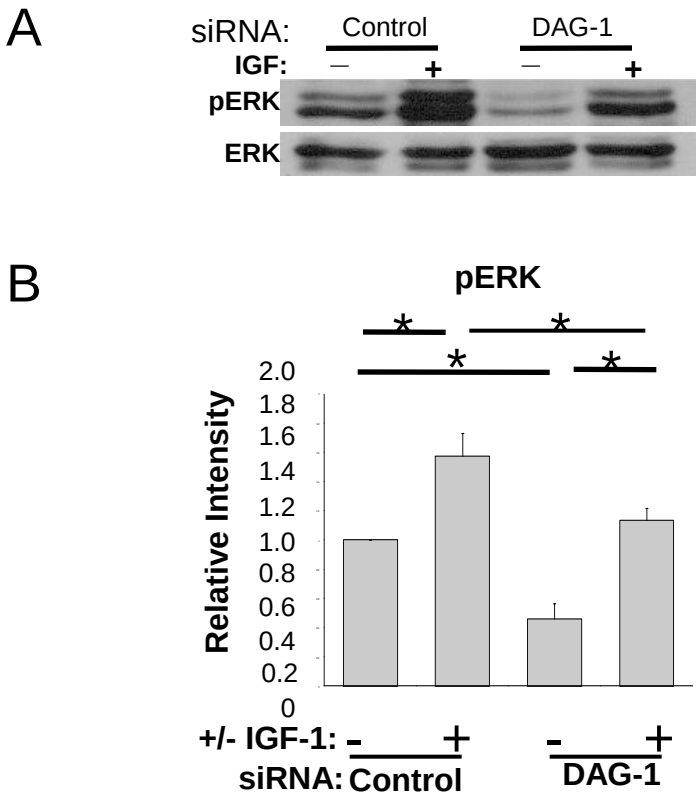
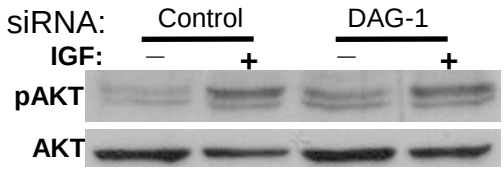


Fig 4.5 **IGF-1-induced activation of MAPK/ERK signaling in oligodendrocytes, but not astrocytes, requires dystroglycan (A)** Immunoblots using lysates obtained from oligodendrocytes transfected with control or dystroglycan (DAG-1) siRNA and treated with or without 100ng/mL IGF-1 following a 2-hour incubation in serum-free media. Cells were lysed 20 minutes following treatment with IGF-1. Blots were probed with antibodies to detect pERK and ERK (loading control) **(B)** Densitometry to determine pERK levels relative to untreated (no exogenous IGF-1) control siRNA (n=4,*P<0.05, error bars represent SEM) **(C)** Immunoblots using lysates obtained from purified astrocyte cultures treated as described in (A). Blots were probed with antibodies to detect β-DG, pERK and ERK (loading control) relative to untreated (no exogenous IGF-1) control siRNA (n=4,*P<0.05, error bars represent SEM

A



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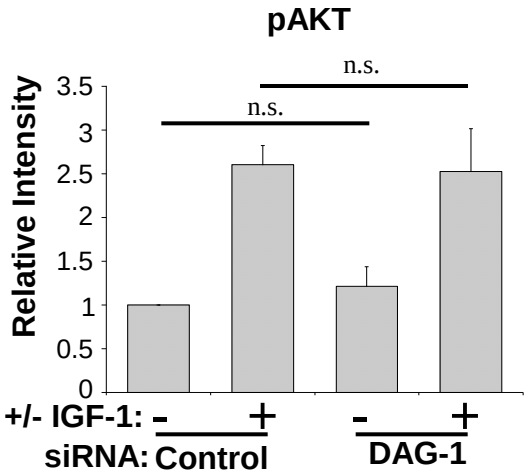


Fig 4.6 **IGF-1-induced activation of PI3K/AKT signaling does not require dystroglycan (A)** Immunoblots using lysates prepared as described in B. Blots were probed with antibodies to detect pAKT and AKT (loading control) **(B)** Densitometry to determine pAKT levels relative to untreated (no exogenous IGF-1) control siRNA (n=4,*P<0.05, error bars represent SEM)

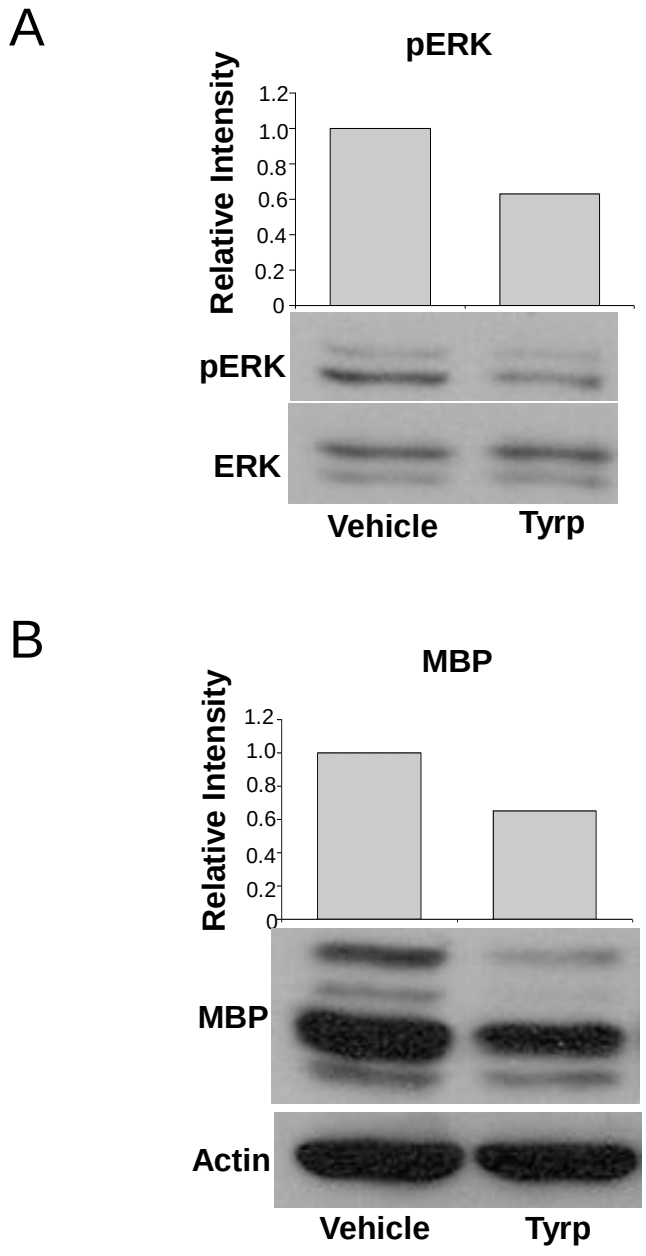
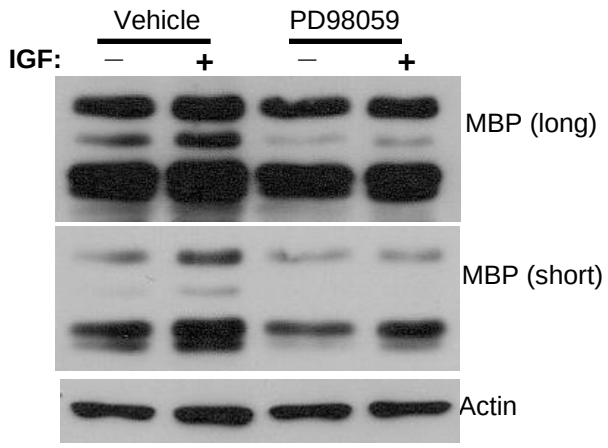


Figure 4.7 **Autocrine IGF-1 signaling is present in primary oligodendrocyte cultures. (A)** Immunoblots and accompanying Densitometry plot from OPC's treated with 10uM of the IGF1R inhibitor, tyrphostin1-OMe-AG or equivalent volume of vehicle (75% ethanol, 25% DMSO) following a 2 hour incubation in serum-free media. Cells were lysed 30 minutes following treatment. Blots were probed with antibodies to detect pERK and total ERK (loading control). Densitometry to determine pERK levels relative to vehicle control (n=1) **(B)** Immunoblots and accompanying densitometry plot from oligodendrocytes treated with 10uM of the IGF1R inhibitor, tyrphostin1-OMe-AG or vehicle (75% ethanol, 25% DMSO) following 3 days of differentiation. Blots were probed with antibodies to detect MBP and actin (loading control). Densitometry to determine MBP levels relative to vehicle control (n=1).

A



B

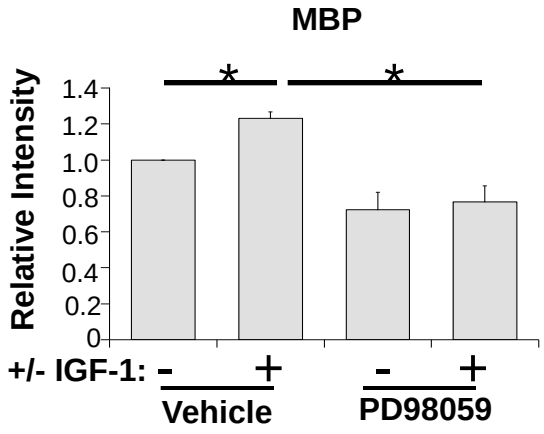


Fig 4.8 **MAPK/ERK signaling is required for IGF-1-induced differentiation of oligodendrocytes. (A)** Immunoblots of oligodendrocytes differentiated for 3 days following treatment with 50ng/ml IGF-1 and 10uM PD98059 (MAPK/ERK signal inhibitor) or equivalent volume of vehicle (75% ethanol, 25% DMSO). Blots were probed with antibodies to MBP and actin (loading control). A representative western blot is shown displaying short and long exposure times for MBP. **(B)** Densitometry to determine MBP levels relative to untreated vehicle control sample. PD98059 abolishes IGF-1-induced enhancement of MBP expression (n=3,*P<0.05, error bars represent SEM)

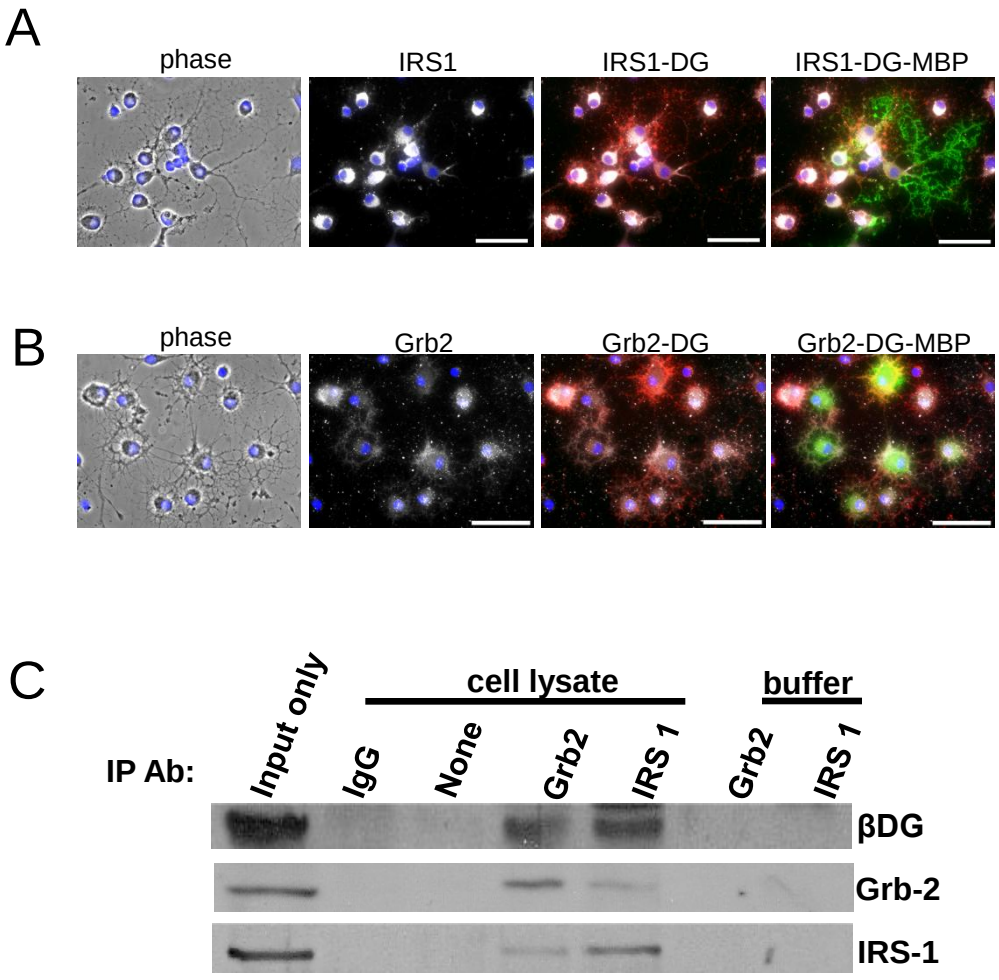


Fig 4.9 **Oligodendrocytes express Grb2 and IRS1 which are found in a protein complex with dystroglycan (A)** Oligodendrocytes were differentiated for three days on laminin followed by immunocytochemistry to visualize IRS1 (white) in conjunction with dystroglycan (red). Co-expression of the mature oligodendrocyte protein, MBP (green), was used to confirm oligodendrocyte identity. Scale bar: 50 μm. **(B)** Oligodendrocytes were differentiated for three days on laminin followed by immunocytochemistry to visualize Grb2 (white) in conjunction with dystroglycan (red). Co-expression of the mature oligodendrocyte protein, MBP (green), was used to confirm oligodendrocyte identity. Scale bar: 50 μm. **(C)** Immunoprecipitation from lysate of oligodendrocytes differentiated for 3 days (input) using antibodies against Grb2 or IRS1 in conjunction with Protein A/G beads. Grb2 antibody pulled down Grb-2, β-dystroglycan and IRS1. IRS1 antibody pulled down IRS1, β-dystroglycan and Grb-2. Samples devoid of antibody or treated with the equivalent amount of control antibody (rabbit IgG) did not yield detectable levels of Grb-2, β-dystroglycan or IRS1. Control samples (buffer) containing equivalent amounts of antibody (Grb-2 or IRS1) but devoid of lysate were included to demonstrate observed bands were not an artifact of non-specific antibody binding.

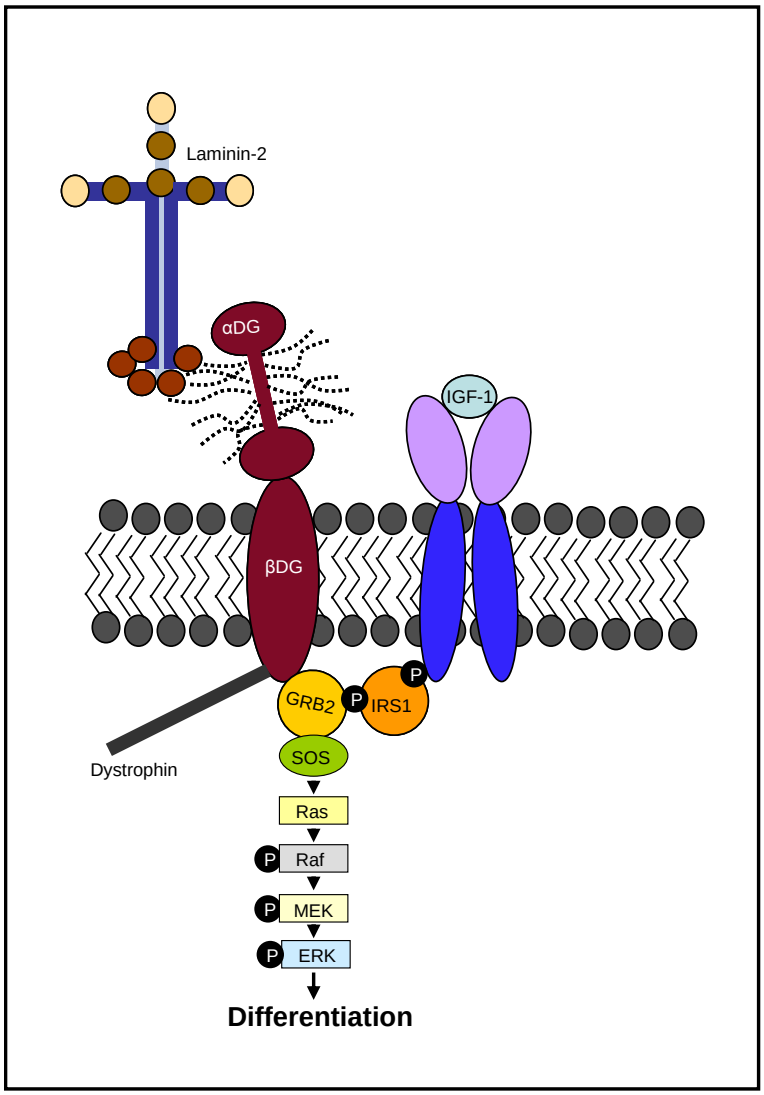


Fig 4.10 **Model for potential mechanism of dystroglycan-mediated differentiation in oligodendrocytes** The binding of Grb2 to β-dystroglycan places it in a position that favors efficient recruitment by IRS1 upon activation of the IGF-1 signaling cascade. MAPK signaling and subsequent differentiation can then be activated through Ras/Raf via association of Grb2 with Sos (son of sevenless).

CHAPTER 5 – Conclusions, future directions and potential applications

Summary of conclusions:

The present findings provide further insight to our understanding of CNS myelinogenesis and the regulatory roles of the adhesion receptor, dystroglycan. First, we revealed that mature oligodendrocytes express dystroglycan, a non-integrin laminin receptor (chapter 3). While other groups have reported robust expression of dystroglycan in Schwann cells, the myelin synthesizing cells of the PNS (Occhi, Zambroni et al. 2005), prior to current findings, α6β1 integrin has been the only laminin-2 receptor identified and studied in oligodendrocytes. Second, we've provided evidence that the expression of dystroglycan is necessary for optimal differentiation of oligodendrocytes (chapter 3). This discovery is pertinent to two major areas of study: (1) it implicates dystroglycan as a novel intrinsic regulator of differentiation in oligodendrocytes and; (2) it suggests a developmental role for dystroglycan that, prior to current findings, has not been reported in other cell types. Finally, we've identified a previously undocumented functional linkage between dystroglycan and the IGF-1 signaling cascade in oligodendrocytes (chapter 4). Together, these findings provide a foundation for extensive future study and may have relevance in better understanding and treating various myelin-associated diseases (discussed ahead).

Future directions:

In vitro studies: This dissertation work provides several examples of oligodendrocyte primary cultures being effectively utilized to examine the function of dystroglycan in oligodendrocytes. Here, I will discuss how this study model may be further employed to build upon current data and to address some as of yet unanswered questions regarding laminin/dystroglycan interactions in oligodendrocytes.

In figure 3.4 we provided evidence that Laminin-2 enhances dystroglycan expression in oligodendrocytes. While there are studies demonstrating laminin-induced up-regulation of integrin receptors (Rooney, Gurpur et al. 2009), no other examples of laminin-induced dystroglycan expression have been reported. Since in oligodendrocytes, integrin expression precedes dystroglycan expression, it is tempting to assume that laminin-induced dystroglycan expression is mediated by interaction with α6β1 integrin. This hypothesis could be examined, therefore, by analyzing dystroglycan expression in cells differentiated on laminin and treated with or without integrin blocking antibody (Ha2/5). This antibody disrupts the ability of laminin to bind to its integrin receptor thereby preventing transmission of laminin/integrin signaling into oligodendrocytes. Alternatively, we could analyze dystroglycan expression following knock down of either the α6 or β1 integrin subunits. Our previously optimized siRNA protocol could be utilized to achieve this.

In chapter 4, we've shown that knock-down of dystroglycan reduces the ability of primary oligodendrocytes to differentiate in response to exogenous IGF-1 (Fig 4.2 and 4.3). However, we have not yet explored the effects of over-expressing dystroglycan in oligodendrocytes. Since dystroglycan is needed for IGF-1-mediated differentiation, perhaps increasing dystroglycan expression could enhance the capacity of oligodendrocytes to differentiate and even synthesize myelin in response to IGF-1. If so, it may be possible to induce myelin synthesis in vivo by pharmacologically increasing dystroglycan expression in oligodendrocytes. As a primary step toward addressing this intriguing possibility, we are currently utilizing various expression vectors to optimize over-expression of dystroglycan in primary oligodendrocyte cultures.

In chapter 4, we proposed a model wherein IGF-1-induced ERK1/2 activation and subsequent differentiation may be attributed to a physical association between dystroglycan, the adapter protein Grb2 and the intracellular IGF1R adapter protein, IRS1 (Fig 4.10). One way this model could be more formally tested is by examining IGF-1-induced ERK1/2 signaling and differentiation following knock-down of Grb2 or IRS1. Having effectively optimized the procedure for using siRNA to ablate dystroglycan expression (Fig 3.6), it is reasonable to assume that the same procedure could be used effectively for knocking down expression of Grb2 or IRS1. In congruence with our model, I predict that knocking down expression of Grb2 or IRS1 will disrupt IGF-1-induced ERK1/2 activation and differentiation in a manner and degree similar to that observed from knocking down dystroglycan expression (Fig 4.2, 4.3 and 4.5).

One potential pitfall of this strategy is that conclusions could be marred by the collateral effects of knocking down these proteins. Deletion of IRS1 has been shown to cause up-regulation of IRS2 and IRS4 which are both expressed in oligodendrocytes (Freude, Leeser et al. 2008) and can functionally compensate for IRS1 (Ye, Li et al. 2002). In addition, the effects of knocking down Grb2 would not likely be limited to IGF-1 signaling as it is known to interact with other signaling cascades (Lowenstein, Daly et al. 1992). Perhaps a more refined approach to testing our model (Fig 4.10) would be to disrupt the interaction between dystroglycan and Grb2. By eliminating or disabling the proline-rich region of β-dystroglycan, which binds one of two SH3 domains on Grb2 (Russo, Di Stasio et al. 2000), we could prevent Grb2 binding to dystroglycan in a way that's minimally disruptive to extraneous signaling pathways. One way this could be achieved is by knocking down dystroglycan expression and subsequently over-expressing it in a truncated form that is devoid of the proline-rich, Grb2 binding domain.

In chapter 4 it was proposed that by binding to dystroglycan, Grb2 may be spatially primed for efficient recruitment and activation by IRS1. One way this could be tested is by determining how interaction between Grb2 and IRS1 is affected by dystroglycan expression. If our hypothesis is correct, binding between IRS1 and Grb2 should be disrupted in dystroglycan deficient cells. This would involve combining immunoprecipitation with siRNA knock-down of dystroglycan. There is little question that we have the technical capacity to perform such procedures as both methods have been optimized in the present

studies. However, performing reliable immunoprecipitations requires at least 500µL of highly concentrated lysates and nucleofection procedures cause substantial cell loss. Therefore, we estimate that performing this experiment would require approximately 5 x 10^7 cells or 25 rat pups. Although, while such a large scale experiment would require considerable planning, it would certainly be feasible. Alternatively, we could explore further optimization of immunoprecipitation procedures to permit us to start with fewer oligodendrocytes.

Here, we've identified a functional link between dystroglycan and IGF-1 signaling in oligodendrocytes but not astrocytes (chapter 4). Though, it remains to be determined if such a linkage is present in other cell types. By applying the techniques we've used to investigate dystroglycan in primary oligodendrocytes (siRNA knockdown, immunoprecipitation etc), we should be able to determine if dystroglycan-dependent IGF-1 signaling occurs in other dystroglycan-expressing cells types such as muscle and Schwann cells.

In vivo studies: In vivo models are perhaps the most reliable means of assessing the role of specific proteins in living mammals. Consequently, we're currently considering various conditional dystroglycan knock-out models for use in subsequent investigation of dystroglycan in oligodendrocytes. With a healthy colony of DAG-1 floxed mice and various colonies of mice expressing Cre recombinase at different promoters (CNP, NG2 and nestin), we have the capacity to examine dystroglycan knock-out in various types of cells in the central nervous system. As discussed in chapter 1, CNP, in the CNS, is a marker of early

oligodendrocytes, and NG2 is a marker of oligodendrocyte precursors (OPCs). Nestin is an intermediate filament protein expressed in neural precursor cells. Therefore, our available colonies will enable us to knock out dystroglycan expression from early oligodendrocytes, OPCs or throughout the CNS. Subsequently, phenotypes can be compared between mutants and wild type littermates. Perhaps the most precise and revealing method for assessing oligodendrocyte-associated phenotypes, is the use of electron microscopy (EM) to examine CNS myelin ultrastructure and degree of myelin wrapping.

We also have access to other animal models that may be used to test conclusions made from in vitro studies. For instance, we demonstrated increased levels of dystroglycan in oligodendrocytes differentiated on laminin compared to those differentiated on poly-D-lysine (Fig 3.4). To assess whether or not this effect occurs in vivo, we could evaluate dystroglycan levels in the oligodendrocytes of laminin-α2 deficient (dy/dy) or knock-out (LAMA2 null) mice. If such an effect is indeed observed, β1 integrin conditional knock-out mice, could then be used to determine if is integrin-mediated.

Disease relevance:

Multiple Sclerosis: One of the clinical hallmarks of multiple sclerosis (MS) is areas of demyelination (lesions) that contain oligodendrocytes in a state of incomplete differentiation. The inability of these cells to differentiate and

synthesize myelin has been linked to the impairment of remyelination of these regions (Wolswijk 1998). In this regard, while laminin/dystroglycan interactions are unlikely to have direct effects on the pathology of MS, they could be exploited as targets through which, remyelination may be pharmacologically activated. As discussed in the previous section, since dystroglycan plays a critical role in IGF-1-mediated differentiation, it may be possible to induce myelin synthesis by pharmacologically increasing dystroglycan expression in cells present in MS lesions. Figure 3.4 provides evidence that laminin enhances dystroglycan expression in oligodendrocytes. Figure 4.1 provides evidence that laminin/dystroglycan interactions potentiate IGF-1 mediated differentiation. Perhaps, therapeutic administration of small peptides or molecular compounds designed to mimic the receptor binding LG domains of laminin-2 could be used to facilitate myelin repair following MS relapses. Ordinarily such large molecules would not be expected to penetrate the blood brain barrier. However, because the blood brain barrier is substantially compromised during MS relapses (McQuaid, Cunnea et al. 2009), larger molecules, such as those the size likely needed to mimic laminin LG domains, could potentially get through.

Congenital muscular dystrophies: Mutations in the dystroglycan-expressing DAG-1 gene result in prenatal death due to structural disruptions of a critical embryonic membrane (Williamson, Henry et al. 1997). However, evidence of myelin deficiency is found in many of the congenital muscular dystrophies (CMDs), specifically those in which there is compromised association between laminin-2 and dystroglycan. Type 1A congenital muscular dystrophy (MDC1A) is

caused by a mutation in the LAMA2 gene which encodes the laminin-α2 subunit. In addition to a severe dystrophic phenotype (progressive muscle degeneration), patients afflicted with this disease exhibit abnormal white matter patterns in MRIs that may be indicative of myelin deficits. (Jones, Morgan et al. 2001). Other human congenital muscular dystrophies are caused by mutations in glycosyltransferases that modify the unusual O-linked carbohydrate moieties that are found on dystroglycan (Schachter, Vajsar et al. 2004; Cohn 2005). The loss of these carbohydrate modifications has been shown to disrupt dystroglycan binding to several ligands including laminins (Kim, Hayashi et al. 2004; Kanagawa, Michele et al. 2005; Patnaik and Stanley 2005; Saito, Blank et al. 2005). Like laminin deficiencies that cause MCD1A, these dystrophies also cause developmental brain abnormalities and include MDC1C (*myd* in mice), FCMD (Fukuyama's muscular dystrophy), Muscle-Eye-Brain Disease (MEB), and Walker-Warburg Syndrome (WWS). In MDC1C, mutations in the LARGE gene, a putative O-linked glycosyl transferase, have been found to cause aberrant white matter development, indicating a potential alteration in CNS myelination (Longman, Brockington et al. 2003). Given that LARGE mutations cause both aberrant dystroglycan function and abnormal white matter, it may be that the dysregulation of oligodendrocyte dystroglycan contributes to the LARGE phenotype. Our finding that oligodendrocytes express dystroglycan and use it to mediate IGF-1-induced differentiation offers new insight into these dystroglycanopathies that cause brain dysmyelination, as well as into the mechanism that underlies CNS myelin abnormalities caused by laminin

deficiencies.

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