

Identification of dystroglycan as a second laminin receptor in oligodendrocytes, with a role in myelination

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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 (ECM) interactions, integrin receptors are likely to be involved. However, it is a non-integrin ECM receptor, dystroglycan, that 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. To date, it is unknown whether dystroglycan plays a role during central nervous system (CNS) myelination. Here, we report that the myelinating glia of the CNS, oligodendrocytes, express and use dystroglycan receptors to regulate myelin formation. 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. After blocking the function of dystroglycan receptors, oligodendrocytes failed both to produce complex myelin membrane sheets and to initiate myelinating segments when co-cultured with dorsal root ganglion neurons. By contrast, enhanced oligodendrocyte survival in response to the ECM, in conjunction with growth factors, was dependent on interactions with beta-1 integrins and did not require dystroglycan. Together, these results indicate that laminins are likely to regulate CNS myelination by interacting with both integrin receptors and dystroglycan receptors, and that oligodendrocyte dystroglycan receptors may have a specific role in regulating terminal stages of myelination, such as myelin membrane production, growth, or stability.

KEY WORDS: Dystroglycan, Oligodendrocyte, Laminin, Myelin, Integrin, DRG, Rat

INTRODUCTION

Extrinsic regulatory molecules, such as growth factors, are important for oligodendrocyte development during central nervous system (CNS) myelination. Although less is known about the extrinsic regulation of oligodendrocyte development by extracellular matrix (ECM) molecules, at least one class of ECM molecule, the laminin family of secreted glycoproteins, is required for normal CNS myelination (reviewed in Colognato et al., 2005). The *LAMA2* gene encodes the laminin $\alpha 2$ 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 its accompanying developmental defects in white matter that reflect a failure of normal myelination (Jones et al., 2001). Laminin $\alpha 2$ deficiency also causes abnormal CNS myelination in mice, where regions of amyelination as well as of thinner myelin have been observed (Chun et al., 2003). However, although 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 $\alpha 6\beta 1$ -integrin has implicated integrins in regulating the interactions between laminins and oligodendrocytes (Colognato et al., 2002). However, it remains unknown whether

laminins interact with oligodendrocytes solely via this integrin receptor. Evidence for multiple laminin receptors exists in the developing peripheral nervous system (PNS), where laminins are also required for normal myelination (reviewed in Colognato et al., 2005). Although $\beta 1$ -integrins are required for normal radial sorting of axons and Schwann cells (Feltri et al., 2002), laminin-deficient Schwann cells have additional defects in cell survival, proliferation and in the ability to form normal myelin (Chen and Strickland, 2003; Madrid et al., 1975; Matsumura et al., 1997; Occhi et al., 2005; Sunada et al., 1995; Yang et al., 2005; Yu et al., 2005). Several other laminin receptors were therefore proposed to regulate Schwann cell development, including the integrin $\alpha 6\beta 4$ and α -dystroglycan (Feltri et al., 1994; Previtali et al., 2003; Saito et al., 1999; Saito et al., 2003; Sherman et al., 2001; Yamada et al., 1994), and studies in which mice were engineered to lack dystroglycan in Schwann cells showed that the PNS requires dystroglycan to achieve normal myelination (Occhi et al., 2005; Saito et al., 2003). 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 $\alpha 2$ -deficient PNS therefore reflects a loss of both integrin signaling 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 $\alpha 6\beta 1$ -integrin laminin-binding receptor has been identified in the oligodendrocyte lineage (Buttery and ffrench-Constant, 1999; Milner and ffrench-Constant, 1994). In the current study, we therefore investigated: first, whether the myelinating glia of the CNS, oligodendrocytes, express other laminin receptors, in particular dystroglycan; second, whether dystroglycan mediates

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interactions between laminin and oligodendrocytes; and third, whether dystroglycan plays a role in laminin-regulation of oligodendrocyte survival or differentiation. We show that oligodendrocytes express dystroglycan and present evidence that, as in the PNS, different laminin receptors are required at different developmental stages. Interactions between laminins and integrins amplify the survival effects of soluble growth factors, whereas interactions between laminins and dystroglycan contribute to the formation of myelin membrane. These data provide the first evidence that non-integrin receptors may play a role in mediating the effects of laminin on CNS myelination.

MATERIALS AND METHODS

Cell culture

Dissociated rat neonatal cortices were cultured (37°C, 7.5% CO₂) in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) on poly-D-lysine (PDL)-coated flasks. Medium was changed every 3-4 days. By days 10-14, mixed glial cultures were obtained that consisted of oligodendrocyte precursor cells and microglia on an astrocyte monolayer. Purified oligodendrocyte precursor cells were isolated from mixed glial cultures using a modification of the mechanical dissociation and differential adhesion method described by McCarthy and de Vellis (Colognato et al., 2004; McCarthy and de Vellis, 1980). For immunocytochemistry and survival assays, purified oligodendrocyte precursor cells (OPCs) were added to PDL or laminin-coated eight-well chamber slides in a modified Sato's medium, as described previously (Colognato et al., 2004). To evaluate protein expression, cells were grown on Nunclon tissue-culture dishes coated with PDL or laminin. For coating, ligands were incubated at 10 µg/ml on dishes or wells for 4 hours at 37°C. Following coating, surfaces were blocked with 10 µg/ml heat-inactivated bovine serum albumin (BSA) for 30 minutes at 37°C and washed with PBS.

Protein analysis

Cells were lysed at 0°C in 1% Triton-X-100, 0.1% SDS, 10 mM Tris (pH 7.4), 5 mM EDTA, and 150 mM NaCl that contained a cocktail of protease and phosphatase inhibitors (Calbiochem). Cell lysates were scraped, transferred to microfuge tubes and incubated on ice for 15 minutes, then centrifuged at 18,000 g to remove insoluble material. Protein concentration was determined (detergent compatible protein assay, BioRad) and lysates were boiled for 5 minutes in Laemmli solubilizing buffer (LSB), 3% βME. Proteins were separated by SDS-PAGE using 10% or 12% acrylamide minigels (0.75 mm thick) and blotted onto 0.45 µm nitrocellulose. Membranes were blocked in Tris buffered saline with 0.1% Tween20 (TBS-T) that contained 4% BSA (blocking buffer) for 1 hour, followed by incubation with primary antibodies in blocking buffer overnight at 4°C. Alternatively, some immunoblotting required a block with 1% non-fat dried milk. Membranes were washed in TBS-T, incubated for 1 hour in HRP-conjugated secondary antibodies (Amersham) diluted 1:3000 in blocking buffer, washed in TBS-T and developed using enhanced chemiluminescence (Amersham). Experiments were performed a minimum of three times and representative blots are depicted.

Survival assays

Eight-well chamber slides were coated for 4 hours at 37°C with 5 µg/ml PDL or 10 µg/ml laminin. Each well was seeded with 20,000 oligodendrocyte progenitors suspended in Sato's medium. At 1 hour post-attachment, soluble platelet-derived growth factor (PDGF) was added and the cells were differentiated for 4 days. PDGF was used at 0.0, 0.1, 1.0 or 10.0 ng/ml. Immunostaining using rabbit anti-galactocerebroside (anti-GalC) antibodies was used to identify newly formed oligodendrocytes (GalC⁺). TUNEL assay using indirect immunofluorescence was used to visualize nicked DNA according to the manufacturer's instructions (ApopTag). In each well, a minimum of 100 GalC-positive cells were scored as TUNEL-positive or -negative. Cell survival was defined as the percentage of TUNEL-negative cells in the GalC-positive population. To compare different experiments, the percent change in cell survival above or below the internal control (survival on PDL with no treatment or growth factors) was

calculated. Experiments were performed a minimum of three times and the mean percent changes and standard deviations were calculated. Statistical significance was determined using the Student's paired *t*-test.

Immunocytochemistry

To visualize dystroglycan (DG) expression, live cells grown in eight-well Permanox chamber slides were incubated with 15 µg/ml IIIH6 anti-DG antibody diluted in differentiation medium. After 45 minutes at room temperature, cells were washed three times with medium and fixed using 100% methanol at -20°C for 5 minutes. Cells were next washed four times in PBS and incubated with FITC-conjugated goat anti-mouse IgM (Sigma) or Texas Red donkey anti-mouse IgM (Jackson ImmunoResearch) for 1 hour. Finally, cells were washed four times with PBS, incubated for 10 minutes with 10 µg/ml Hoechst (DAPI) and mounted in Fluoromount G (Southern Biotech). To perform double immunofluorescence to visualize both DG and myelin basic protein (MBP), cells were processed as above for anti-DG live labeling, but labeled additionally with anti-MBP diluted in PBS with 10% goat serum for 1 hour following fixation. Then, secondary antibodies for both antibodies were incubated together. For single MBP immunostaining, cells were fixed with methanol as above, washed four times with PBS, blocked for 1 hour in PBS containing 10% goat or donkey serum (block buffer) and incubated with MBP antibodies diluted in block buffer. Cells were then incubated for 1 hour in FITC- or TRITC-conjugated secondary antibodies diluted in block buffer. For GalC immunostaining, cells were incubated with rabbit anti-GalC live, as described in the survival assay. Finally, all immunocytochemistry finished with a 10 minute incubation in 10 µg/ml Hoechst (DAPI) in PBS to visualize nuclei.

Myelinating co-cultures

This culture system was a modification of that previously described by Chan et al. (Chan et al., 2004), and is described in more detail elsewhere (Wang et al., 2007). Briefly, dorsal root ganglions (DRGs) were dissected from E14-E16 rats and were dissociated with Papain (1.2 U/ml; Worthington), L-cysteine (0.24 mg/ml, Sigma) and DNase I (40 µg/ml, Sigma) at 37°C for 45 minutes. The dissociated cells were plated onto 22 mm coverslips coated with poly-D-lysine (10 µg/ml; Sigma) followed by growth factor reduced Matrigel (1:40 dilution, BD Bioscience) at a density of 5 × 10⁵ cells/ml. Neurons were grown for 2 weeks in DMEM (Sigma) with 10% fetal bovine serum (Gibco), in the presence of nerve growth factor (NGF; 100 ng/ml; Serotec). To remove contaminating fibroblasts and glial cells, the cultures were pulsed three times with 5-fluorodeoxyuridine (10 µM, Sigma) for 2 days each time. OPCs prepared as above from P0 rats were seeded onto coverslips with purified DRGs at a density of 5 × 10⁴ cells/coverslip. The medium was changed to 50:50 DMEM:Neurobasal (Gibco) with Sato, B27 supplement (Gibco), NGF (100 ng/ml), N-acetyl cysteine (5 µg/ml, Sigma) and D-biotin (10 ng/ml). Blocking anti-DG antibodies were added at the same time and co-cultures were maintained for 14 days, with medium and antibodies changed every 3 days. For analysis, cultures were fixed with 4% paraformaldehyde, and then permeabilized and blocked in 50% normal goat serum (Sigma) with 0.4% Triton X-100 (Sigma). The cultures were then incubated with primary antibodies for 2 hours at room temperature: anti-MBP antibody (1:100) to visualize myelin formation and anti-neurofilament 200 antibody (Sigma; 1:1000) to visualize neurites. The cultures were washed in PBS and incubated with Alexa fluor-conjugated secondary antibody (488 or 586) for 1 hour at room temperature. Neurite density was determined by comparing the area of neurites to the total area of the field. Best-fit and statistical analysis on myelinating/total OL plotted versus neurite density were performed by One-way ANOVA analysis using Prism software (GraphPad) as described (Wang et al., 2007).

Microscopy and image acquisition

Slides were visualized using either a Zeiss Axioplan upright fluorescence microscope fitted with a 10× eyepiece magnification using 20× (0.5 N.A.) and 40× (0.75 N.A.) objectives, or, using a Zeiss Axioplan inverted fluorescence microscope fitted with a 10× eyepiece magnification using 10× (0.3 N.A.), 20× (0.5 N.A.) and 40× objectives. Images were acquired using a Hamamatsu C4742-95 digital camera and OpenLab imaging software (upright microscope) or using a Zeiss AxioCam MRM digital camera and Zeiss Axiovision imaging software (inverted microscope).

Analysis of myelin membrane morphology

Oligodendrocytes, differentiated for 2 or 4 days in Sato's medium containing 0.5% FCS (differentiation medium), were evaluated for the expression of MBP using immunocytochemistry. Myelin membrane complexity was graded according to morphological characteristics and to the degree of myelin membrane formation, as described previously (Colognato et al., 2004). In brief, categories 1-3 describe MBP-positive cells with increasing degrees of MBP-positive processes (1=MBP-positive primary processes or MBP-positive cell body; 2=MBP-positive primary and secondary processes; 3=MBP-positive primary, secondary and tertiary processes that are extensively branched but lack visible myelin membrane). Categories 4-6 describe branched MBP-positive cells that, in addition, have low (4; cells with <25% myelin membrane coverage in branched areas), medium (5; ~25-75% myelin membrane coverage in branched areas) or high (6; >75%-to-complete myelin membrane coverage in branched areas) amounts of myelin membrane.

Reagents

Antibodies

The following antibodies were used for immunocytochemistry: rabbit polyclonal IgG against GalC (Sigma); rabbit polyclonal IgG against α -DG (Upstate); mouse monoclonal IgM against α -DG (clone IIH6, a generous gift from K. Campbell, University of Iowa, IA and HHMI, MD); and rat monoclonal IgG against MBP (Serotec). FITC- or TRITC-conjugated donkey antibodies against rabbit, mouse and rat IgG were used as secondary antibodies (Jackson ImmunoResearch). The following antibodies were used for western blotting: mouse monoclonal IgG against β -DG (Novocastra); rabbit polyclonal IgG against α -DG (Upstate); rabbit polyclonal IgG against NG2 (a generous gift from J. Levine, Stony Brook University, NY); mouse monoclonal IgG against β -actin (Sigma); mouse monoclonal IgG antibody against the laminin α 2 subunit (clone 5H2, Chemicon); rabbit polyclonal IgG antibodies against laminin-1 (Sigma); polyclonal IgG antibodies against FLAG peptide (Sigma); and mouse monoclonal IgG against 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Sigma). The following antibodies were used for blocking receptors in cultured cells: hamster monoclonal IgM against β 1-integrin (clone Ha2/5, Pharmingen).

Proteins

Human recombinant PDGF-A was used at 0.1, 1.0 or 10.0 ng/ml (PeproTech). PDL (Sigma) was used at 5 μ g/ml and human placental laminin, a mixture composed primarily of laminins-2 and -4 that contain the laminin α 2 subunit (Chemicon), was used at 10 μ g/ml. Recombinant laminin protein, rE3, which is comprised of the laminin α 1 subunit LG domains 4 and 5 (rLG4/5), was purified as described previously (Li et al., 2005) using FLAG and heparin affinity column chromatography.

siRNA transfection

Pools of four siRNA duplexes designed to target rat DG mRNA were used to deplete oligodendrocyte DG. Two different control siRNA pools were used: siRNA against Lamin A/C (also known as Lamin A, LMNA – Mouse Genome Informatics) or siRNA non-targeting pool for rat (siCONTROL). siRNAs were synthesized by Dharmacon and were transfected into OPCs using the Nucleofector electroporation system with the rat oligodendrocyte transfection reagent as per the manufacturer's instructions (Amaxa). Cells were seeded directly onto dishes or chamber slides and changed to differentiation medium (Sato + 0.5% FCS) 16 hours later. Fluorescent siGLO siRNA (Dharmacon) was used to determine siRNA-delivery efficiency (~90%).

RESULTS

To determine whether oligodendrocytes express laminin receptors other than the previously characterized integrins, we tested whether differentiating oligodendrocytes were able to capture and retain a recombinant laminin protein on the cell surface. This laminin protein, designated 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 et al., 1983) (Fig. 1A). rE3 has been used extensively to study

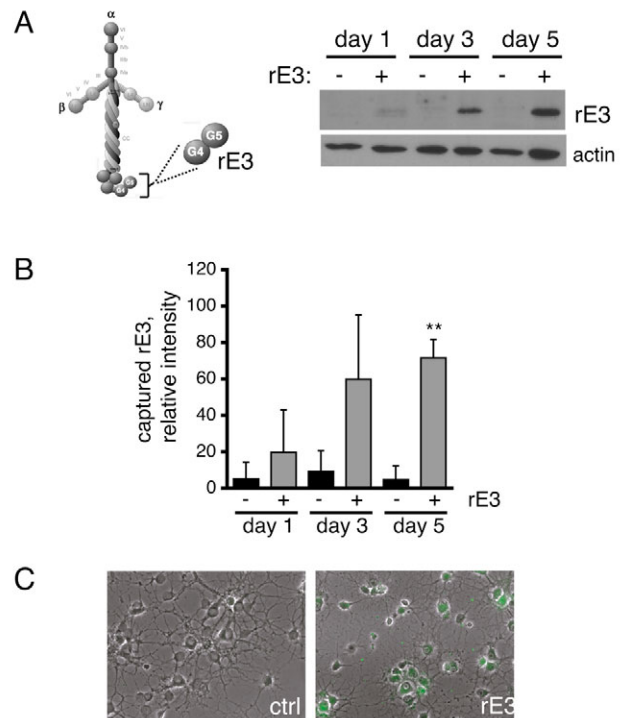


Fig. 1. Recombinant laminin that contains dystroglycan-binding, but not integrin-binding, sites binds to the surface of differentiated oligodendrocytes. (A) rE3 is a recombinant laminin protein that comprises the last two LG domains of the laminin α -subunit. rE3 has been shown previously to contain dystroglycan-binding, but not integrin-binding, sites. Oligodendrocytes were differentiated for the indicated times in the presence or absence of 10 μ g/ml rE3, washed to remove unbound protein and lysed. Western blots of lysates were performed to detect the presence of rE3. Blots were reprobbed with actin antibodies as a 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.

laminin-dystroglycan interactions in muscle cells because it does not contain any integrin-binding sites but has been shown to be equivalent to intact laminin in terms of its ability to bind to dystroglycan (DG) (Li et al., 2002; Li et al., 2005; Smirnov et al., 2002). However, this region of laminin can also bind to a variety of cell-surface proteoglycans, so binding of rE3 is not an exclusive measure of DG-receptor availability. To obtain differentiating oligodendrocytes, oligodendrocyte progenitor cells (OPCs) were isolated from mixed glial cultures obtained from newborn rat cerebral cortices. These freshly isolated OPCs were plated on poly-D-lysine (PDL) or laminin-coated dishes and grown in Sato's medium containing 0.5% serum, a condition known to induce differentiation over 3-5 days. These cells were incubated with 10 μ g/ml rE3 in Sato's medium for 1, 3 or 5 days. Prior to detergent lysis, cells were washed extensively to remove unbound rE3. rE3 has been engineered to contain a FLAG-epitope tag, allowing detection using FLAG antibodies (Li et al., 2002; Li et al., 2005; Smirnov et al., 2002). Cell lysates were therefore evaluated by western blot for

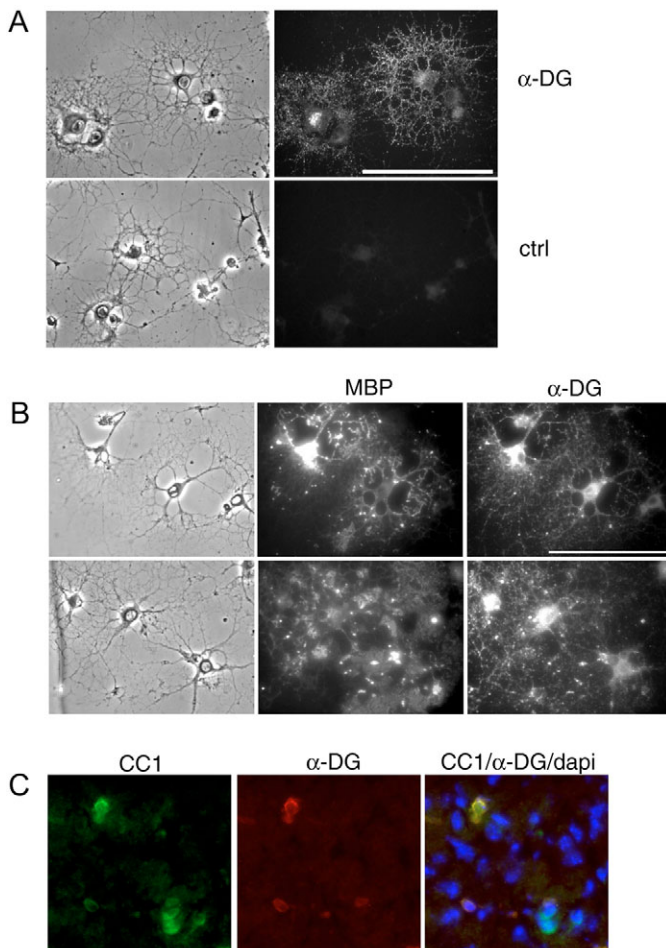


Fig. 2. Oligodendrocytes express dystroglycan. (A) α -dystroglycan (α -DG) expression in primary oligodendrocytes. Secondary antibody alone is shown in control image (ctrl). Scale bar: 50 μ m. (B) α -DG and MBP co-expression in oligodendrocytes. Two different fields are shown. Scale bar: 100 μ m. (C) Co-expression of oligodendrocyte marker (CC1) and α -DG in corpus callosum from a postnatal-day 8 mouse brain.

the presence of rE3 using FLAG antibodies (Fig. 1A) and total-protein load was monitored using actin antibodies. Relatively little rE3 was captured by oligodendrocytes following 1 day of differentiation; however, rE3-capture increased by approximately three- and four-fold, respectively, by day 3 and day 5 of differentiation (Fig. 1B). At day 5 of differentiation, the increase in rE3 capture compared with that of day 1 was increased significantly ($n=3$, $P=0.0495$). rE3 binding to cells was also confirmed using indirect immunofluorescence with FLAG antibodies – a representative field of differentiated oligodendrocytes decorated with rE3 is shown adjacent to a control field in Fig. 1C. A significant increase ($P=0.0429$) in relative mean fluorescent intensity was observed in rE3-treated cultures (32.6 ± 16.3 , $n=4$) compared with control BSA-treated cultures (10.2 ± 6.3 , $n=4$). Together, these results indicate that differentiating oligodendrocytes are likely to express one or more non-integrin laminin receptors.

In light of the work on Schwann cells showing a key role for DG in PNS myelination (Occhi et al., 2005; Saito et al., 2003), we reasoned that this receptor was a likely candidate for an additional

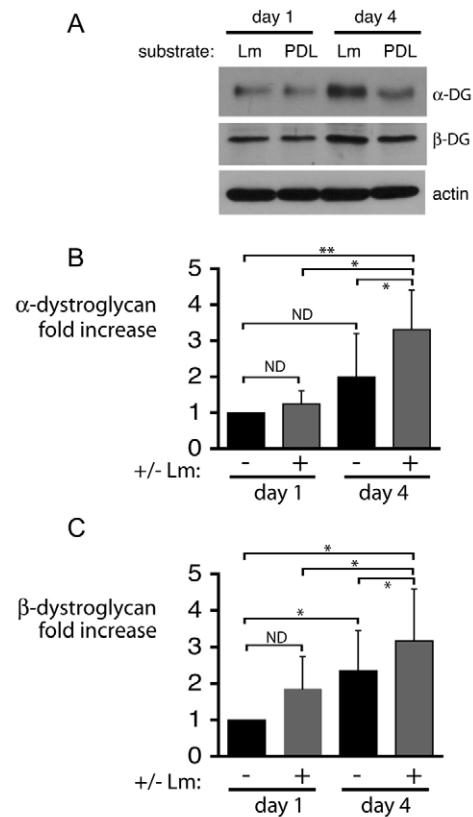


Fig. 3. Oligodendrocytes differentiated on laminin show increased levels of dystroglycan. (A) Relative dystroglycan expression in oligodendrocytes differentiated for 1 or 4 days on poly-D-lysine (PDL) or laminin (Lm). Western blots were probed with antibodies specific for α -dystroglycan (α -DG) or β -dystroglycan (β -DG). Blots were re-probed with antibodies against actin as a loading control. A representative western blot is shown. (B) Densitometry to determine relative α -DG expression. The average fold increase relative to expression at day 1 on PDL is shown ($n=3$, $*P<0.05$ and $**P<0.01$, error bars represent s.d.). (C) Densitometry to determine relative β -DG expression. The average fold increase relative to expression at day 1 on PDL is shown ($n=3$, $*P<0.05$, error bars represent s.d.). ND, no significant difference.

laminin receptor. To determine whether oligodendrocytes expressed DG, immunofluorescence and immunoblotting were performed on oligodendrocytes and oligodendrocyte lysates (Figs 2, 3). We used indirect immunocytochemistry to visualize the presence of DG in oligodendrocytes obtained after differentiation for 4 days (Fig. 2A). DG is a single-gene product of the *Dagl* gene that is cleaved post-translationally into a transmembrane β -DG subunit and an extracellular α -DG subunit, which remains associated with its β -DG partner. α -DG antibodies were used in order to perform the immunocytochemistry on live oligodendrocytes. Using α -DG antibodies, we were able to determine that α -DG was expressed on cells with characteristic oligodendrocyte morphology and was found on the cell surface, as predicted for an ECM receptor. To confirm the identity of these cells as oligodendrocytes, we performed double immunocytochemistry with α -DG antibodies and myelin basic protein (MBP) antibodies (Fig. 2B). We observed α -DG immunoreactivity both in cell bodies and in cell processes in differentiated oligodendrocytes that were co-stained with antibodies against MBP. We also examined myelinating tracts at post-natal day

8 to determine whether oligodendrocytes expressed DG *in vivo*. We observed α -DG-immunopositive cells that co-labeled with the oligodendrocytes-lineage antibody CC1 (Fig. 2C).

Next, we examined whether DG protein levels were altered during oligodendrocyte development. Cells were differentiated for either 1 or 4 days and were then evaluated for the presence of DG by immunoblotting cell lysates (Fig. 3). On poly-D-lysine (PDL) substrates, which do not provide ligands for cell surface ECM receptors, we found that α -DG and β -DG were elevated (approximately twofold) in oligodendrocytes differentiated for 4 days compared with cells differentiated for 1 day. This difference was significant statistically for β -DG ($P=0.0494$), but was not significant for α -DG levels. In cells differentiated on laminin, however, DG levels (both α and β) were approximately threefold higher than at day 1, indicating that laminin signaling may enhance the upregulation of DG expression. DG levels in cells differentiated for 4 days on laminin showed significant increases over DG levels in cells at day 1 of differentiation on either PDL ($P=0.0076$ for α -DG and $P=0.0187$ for β -DG) or on laminin itself ($P=0.0201$ for α -DG and $P=0.0377$ for β -DG). β -DG levels were higher at day 4 on laminin than at day 4 on PDL (mean 3.1-fold increase compared with day 1 versus 2.36-fold increase compared with day 1, respectively, $P=0.0343$) and α -DG levels were higher at day 4 on laminin than at day 4 on PDL (mean 1.99-fold increase compared with day 1 versus 3.32-fold increase compared with day 1, respectively, $P=0.0101$). It should be noted, however, that α -DG antibodies, at least in part, recognize DG carbohydrate epitopes. Therefore, changes in α -DG reactivity could reflect changes in DG post-translational modifications as well as changes in DG protein levels. Finally, we did observe a small percentage of cells in our oligodendrocytes that were GFAP-positive astrocytes (~2-5%), but this percentage of cells did not change in our differentiation conditions that contained only 0.5% serum. These astrocytes also expressed DG but, unlike in oligodendrocytes, DG levels did not change during the 4 day differentiation window (data not shown).

Having demonstrated that DG is present on the cell surface of newly formed oligodendrocytes, we examined next the function of DG receptors during oligodendrocyte development. Previous work has implicated laminins in three distinct phases of oligodendrocyte development: differentiation, survival of newly formed oligodendrocytes, and myelination (reviewed by Colognato et al., 2005). We first tested whether laminin-DG interactions were able to influence oligodendrocyte differentiation. Our previous studies had shown that antibodies against the β 1-integrin subunit had no effect on the ability of oligodendrocytes to express MBP, a marker for myelin-forming oligodendrocytes (Relvas et al., 2001). In the present study, we added DG-blocking antibodies to cultures differentiated on PDL or laminin and counted the percentage of cells that expressed MBP (Fig. 4A). As expected, percentages of MBP-positive cells were higher after 4 days of differentiation (78.7 \pm 3.4% on PDL and 86.0 \pm 1.5% on laminin) than after 2 days of differentiation (29.3 \pm 5.6% on PDL and 35 \pm 4.1% on laminin). Although the small elevation in MBP expression in oligodendrocytes differentiated on laminin compared with on PDL was significant at day 4 (78.7 \pm 3.4% on PDL versus 86.0 \pm 1.5% on laminin, $n=4$, $P=0.0062$), there was no statistical difference between these results and those in which we included DG-blocking antibodies (27.8 \pm 1.3% on PDL at day 2, 30.9 \pm 4.2% on laminin at day 2, 74.9 \pm 8.1% on PDL at day 4 and 85.1 \pm 1.2% on laminin at day 4). This finding indicated that disruption of DG interactions using blocking antibodies does not interfere with the ability to initiate MBP protein expression in differentiating oligodendrocytes.

Immunocytochemistry using antibodies to another marker of oligodendrocyte differentiation, galactocerebroside (GalC), was used to confirm this finding. We observed similar numbers of GalC-positive cells following treatment with anti-DG or control antibodies (Fig. 4B). At day 2 of differentiation on laminin, cultures treated with control antibodies contained 48.3 \pm 4.3% GalC-positive cells and cultures treated with anti-DG contained 52.9 \pm 4.7% GalC-positive cells ($n=4$). By day 4, almost 95% of cells were GalC-positive in all conditions (data not shown).

To evaluate differentiation further, we next asked whether DG-blocking antibodies influenced the ability of MBP-expressing oligodendrocytes to develop complex myelin membrane sheets (Fig. 4C-E). In contrast to MBP-expression studies, our previous work had shown that myelin membrane sheet formation was inhibited in the presence of blocking antibodies against laminin-binding integrins (Relvas et al., 2001; Buttery and French-Constant, 1999). In the present study, we differentiated OPCs for 2 or 4 days in the presence of DG antibodies or control antibodies. Then, we performed MBP immunocytochemistry and assigned all MBP-positive cells to one of six different categories that reflected increasing branching complexity and myelin membrane sheet formation, as described previously (Fig. 4E) (Colognato et al., 2004). Categories 1-3 were designated to cells that exhibited increasing degrees of branching, but no myelin membrane (see Materials and methods). Categories 4-6 were designated to cells that, in addition, contained myelin membrane sheets at low (4), medium (5) or high (6) levels. At day 2, few cells had myelin membrane sheets and, thus, the majority of MBP-positive cells were found in categories 1-3 [in agreement with our previous study (Colognato et al., 2004)]. The addition of DG-blocking antibodies had no effect on myelin membrane sheet formation at day 2 (Fig. 4C,E). By day 4, however, cells grown in the presence of DG-blocking antibodies had significantly fewer complex myelin membrane sheets than did control cells (Fig. 4D,E). This change altered the category distribution such that more cells were found in categories 1-3 and fewer cells were found in categories 4-6, compared with cells grown in the absence of DG-blocking antibodies (Fig. 4E). Thus, we concluded that, although DG-blocking antibodies did not reduce the ability of cells to express MBP, they did reduce the ability of oligodendrocytes to either extend or to maintain MBP-positive myelin membrane sheets. It should be noted that process branching of MBP-positive cells, reflected by cells that were assigned to categories 1-3, did not appear to be perturbed by the addition of anti-DG antibodies.

To address better the role of DG during oligodendrocyte differentiation, our next approach was to transfect OPCs with siRNAs designed to target and degrade rat DG mRNA. At approximately 16 hours post-transfection, OPCs were switched to differentiation medium and differentiated for either 2 or 4 days (Fig. 5). Knockdown of DG protein was achieved by day 2 (Fig. 5B), and knockdown was reasonably well-maintained by day 4 (Fig. 5A,B). Representative fields of cell-surface α -DG immunocytochemistry (Fig. 5A, anti-DG, green) is shown for oligodendrocytes differentiated for 4 days following control- or DG-siRNA transfection. Cell lysates from control- and DG-siRNA-treated oligodendrocytes were evaluated by western blotting to monitor proteins that reflect the differentiation status of oligodendrocytes (Fig. 5B-D). At day 2, only the two major isoforms of MBP (17.2 and 18.5 kDa) were detectable using longer exposure times (Fig. 5B, blot labeled as MBP'). We observed a substantial decrease in the level of MBP expression in cells treated with DG siRNA ($n=3$, 44.5 \pm 14.3% relative to control cultures,

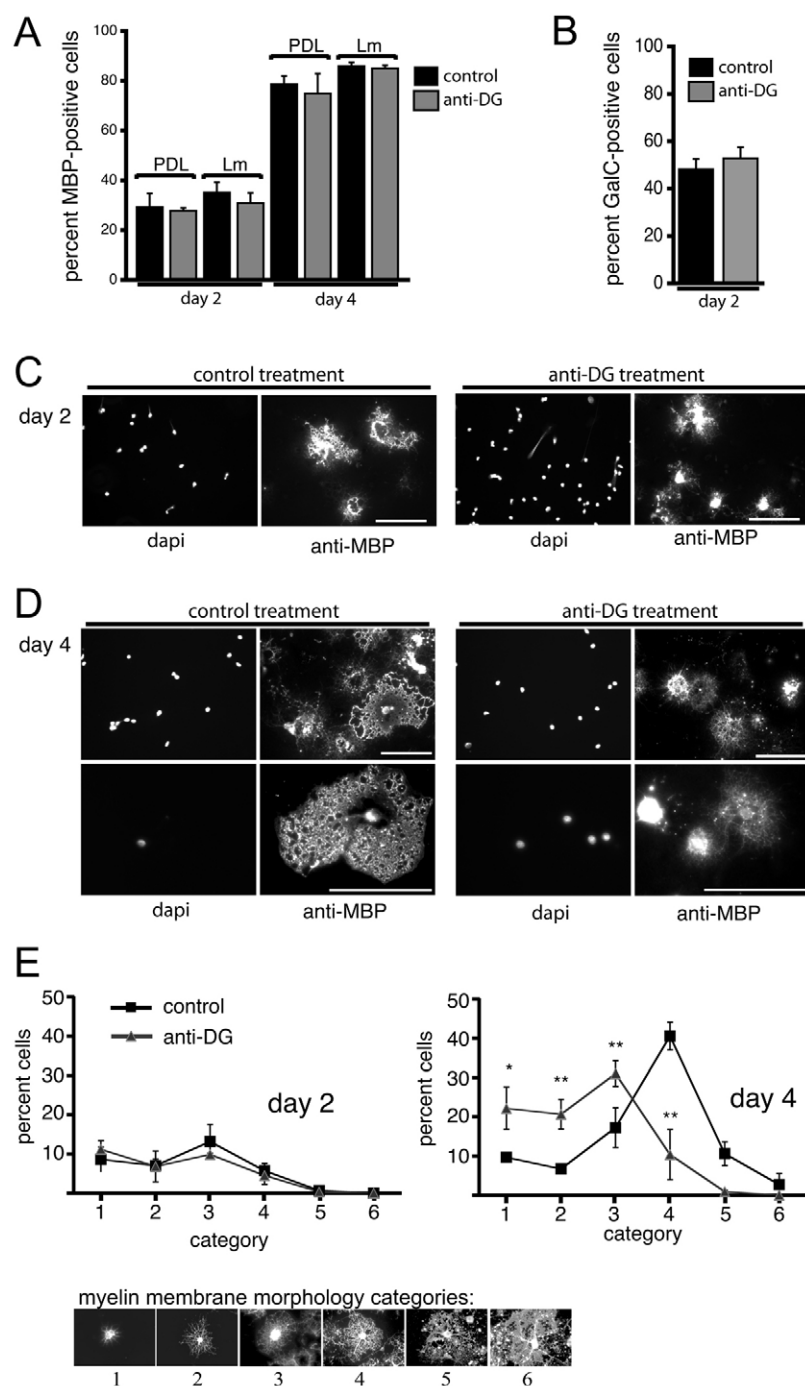


Fig. 4. Myelin membrane sheet formation is reduced by dystroglycan-blocking antibodies.

(A) Oligodendrocytes were differentiated for 2 or 4 days on PDL or laminin (Lm) in the presence of dystroglycan-blocking antibodies (anti-DG) or control antibodies. Indirect immunofluorescence was used to detect myelin basic protein (MBP) expression. The percentage of MBP-positive cells was determined using DAPI nuclear stain to obtain total cell counts per field. In the presence of blocking antibodies, no change in the percentage of MBP-positive cells was observed at either time point or on either substrate. (B) The average percentage of cells differentiated on laminin that express galactocerebroside C (GalC) following treatment with dystroglycan-blocking antibodies (anti-DG) or control antibodies. No significant change was found. (C) Typical morphology at day 2. Oligodendrocytes were differentiated for 2 days on laminin in the presence of dystroglycan-blocking or control antibodies. MBP immunofluorescence was used to visualize differentiated cells. Nuclei were detected using DAPI nuclear stain. (D) Typical morphology at day 4. Oligodendrocytes were differentiated for 4 days in the presence of dystroglycan (DG) or control antibodies. MBP immunofluorescence was used to visualize differentiated cells. Nuclei were detected using DAPI nuclear stain. (E) Myelin membrane sheet complexity is reduced by DG-blocking antibodies. A morphology classification scheme (bottom) was used to evaluate the degree of myelin membrane in MBP-positive cells. Examples of MBP-expressing cells: stages 1, 2 and 3 show increasing levels of process outgrowth and branching, without myelin membrane, whereas stages 4, 5 and 6 show increasing levels of complexity and myelin membrane. The percentage of cells within each category is shown for oligodendrocytes differentiated for 2 or 4 days in the presence or absence of DG-blocking antibodies (control, black squares; anti-DG, gray triangles). In the presence of DG-blocking antibodies, no difference in morphology profile is observed at day 2; however, at day 4, a significant shift away from more-complex myelin membrane structures is observed (* $P < 0.05$, ** $P < 0.01$, error bars represent s.d.). Scale bars: 100 μm .

$P = 0.0026$). This was in contrast to treatment with DG-blocking antibodies, in which we saw reduced myelin membrane sheet formation but no change in MBP expression. At day 4, MBP expression in DG-siRNA cultures remained decreased compared with control-siRNA cultures ($n = 4$, $53.1 \pm 22.2\%$, $P = 0.0056$). We also evaluated the expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), an oligodendrocyte-differentiation marker that is expressed earlier than MBP (Fig. 5B-D). As with MBP, we observed decreased CNP protein in DG-siRNA-treated oligodendrocytes relative to control cultures ($66.0 \pm 15.1\%$ with $P = 0.0173$ at day 2 and $71.8 \pm 14.0\%$ with $P = 0.0068$ at day 4). By contrast, a protein that is associated with early lineage oligodendrocytes, the cell-surface proteoglycan NG2 (also known

as Cspg4 – Mouse Genome Informatics), was found to have no significant change in expression ($93.7 \pm 49.3\%$ at day 2, $n = 3$, and $114.4 \pm 48.2\%$ at day 4, $n = 4$). Relative protein levels for all densitometric analysis were normalized to actin protein levels. In addition to the three proteins discussed above (MBP, CNP and NG2), cell lysates were evaluated for the expression of p27, which has been shown previously to contribute to the regulation of *Mbp* gene expression in oligodendrocytes. DG-siRNA-treated oligodendrocytes showed a reduction in p27 levels ($72.1 \pm 48.7\%$ at day 2 and $76.7 \pm 22.9\%$ at day 4, $n = 3$); however, this reduction was not significant by Student's *t*-test. We also performed immunocytochemical analysis to evaluate the percentage of oligodendrocytes that were MBP-positive (Fig. 5E). No significant

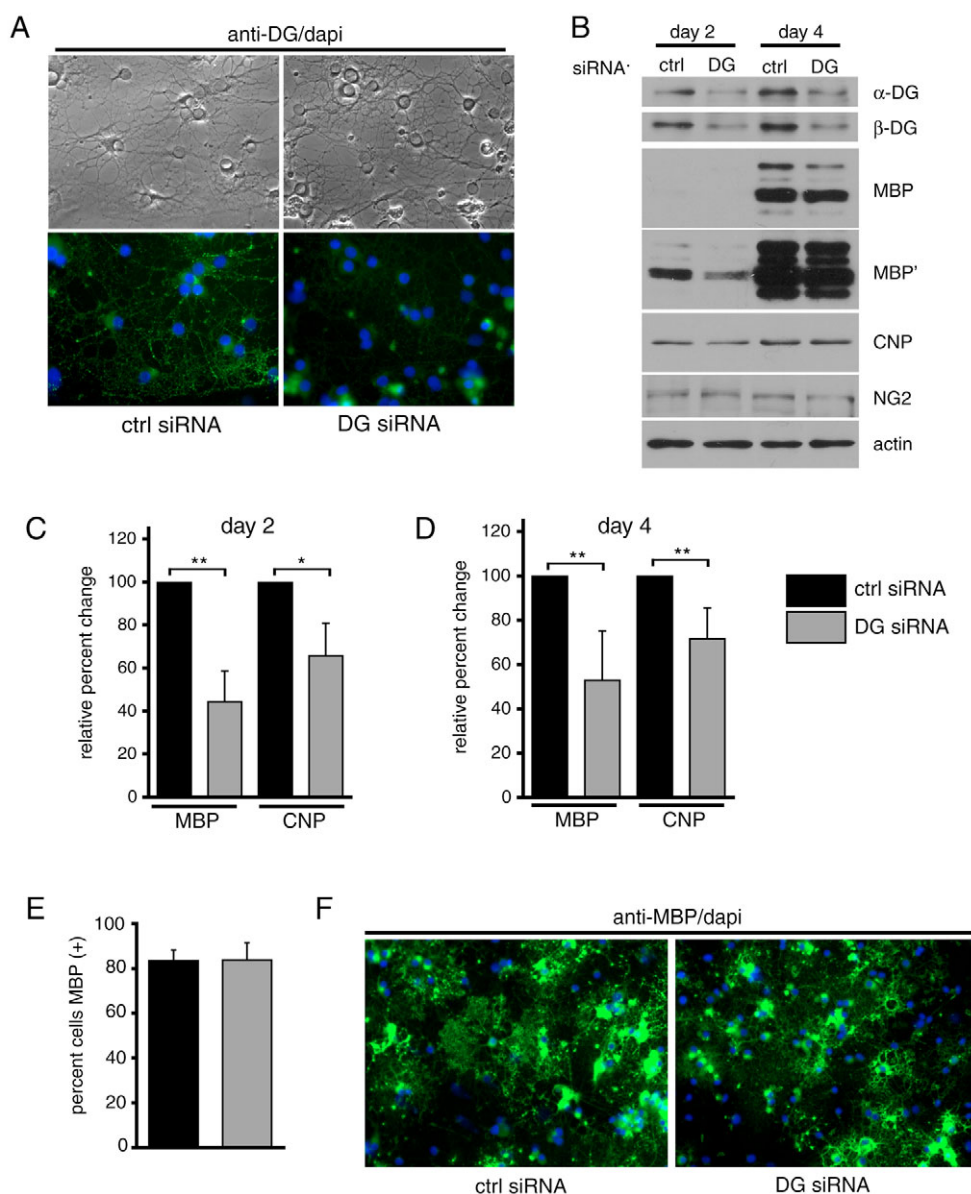


Fig. 5. Dystroglycan siRNA causes a decrease in oligodendrocyte differentiation and myelin protein production.

(A) Dystroglycan expression is reduced in oligodendrocytes differentiated following transfection with siRNA designed to target dystroglycan mRNA (DG 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. Blots were probed with antibodies to detect α -dystroglycan, β -dystroglycan, MBP (late-stage oligodendrocyte marker; MBP' shows a longer exposure to visualize the MBP at day 2), CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase, mid-stage oligodendrocyte marker), NG2 (early stage oligodendrocyte marker) or actin (loading control). **(C,D)** Average expression, relative to control siRNA at 100%, for MBP or CNP at day 2 (C; $n=3$; * $P<0.05$ and ** $P<0.01$) or day 4 (D; $n=4$; ** $P<0.01$) (error bars represent s.d.). Black bars depict control siRNA and gray bars depict dystroglycan siRNA. **(E)** Average percentage of cells, treated with either control (ctrl) or dystroglycan siRNA, that express MBP, as determined by immunocytochemistry ($n=4$, error bars represent s.d.). **(F)** Representative fields of MBP immunocytochemistry as described in E.

difference was observed between control ($85.9\pm 5.6\%$) and DG-siRNA-treated cultures ($84.0\pm 7.7\%$) differentiated on laminin for 4 days ($n=4$). A similar analysis was performed using GalC immunoreactivity. In DG-siRNA oligodendrocytes differentiated for 2 days on laminin, no significant difference was observed in the percentage of cells that expressed GalC ($31.7\pm 11.9\%$ in control siRNA compared to $28.4\pm 1.4\%$ in DG siRNA). Thus, although the level of MBP expression was reduced, a normal proportion of cells were able to express some MBP in the absence of normal DG expression. It should also be mentioned that a small percentage of cells in these cortical oligodendrocyte preparations were astrocytes (after 4 days in differentiation medium $4.4\pm 0.6\%$ of cells were GFAP-positive). To determine whether DG siRNA had an effect on the proportion of contaminating astrocytes, we performed GFAP immunoblotting of lysates obtained from control- and DG-siRNA-treated cells. We found that DG siRNA did not cause a significant change in GFAP expression at either 2 or 4 days in differentiation medium ($105.3\pm 5.8\%$ of control at day 2 and $103.6\pm 11.2\%$ of control at day 4).

Next, we asked whether DG plays a role in oligodendrocyte survival, as we have shown previously for the $\alpha 6\beta 1$ -integrin laminin receptor both in vitro and in the developing brain (Baron et al., 2005; Benninger et al., 2006; Colognato et al., 2002; Colognato et al., 2004; Frost et al., 1999). We monitored cell death using indirect immunofluorescent TUNEL in oligodendrocyte cultures differentiated following control- or DG-siRNA treatment (Fig. 6A). However, after incubating the cells on laminin for 2 days in differentiation medium, we did not observe any significant change in the percentage of TUNEL-positive cells in DG-siRNA-treated cultures ($5.6\pm 3.0\%$, $n=3$) compared to control-siRNA-treated cultures ($6.7\pm 5.1\%$, $n=3$). Next, we performed survival assays on laminin or on PDL substrates in the presence of increasing concentrations of the oligodendrocyte survival factor platelet-derived growth factor (PDGF). PDGF has been shown, both in vitro and in vivo, to enhance the ability of newly formed oligodendrocytes to survive (Barres et al., 1993; Calver et al., 1998). Laminin enhances this response by increasing survival in response to low, physiological doses of PDGF that are insufficient to enhance

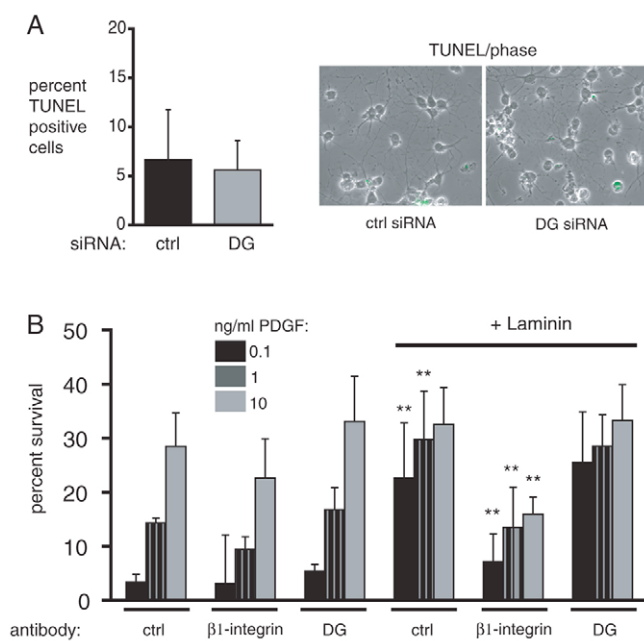


Fig. 6. Enhancement of survival by laminin requires β 1-integrins, but not dystroglycan. (A) Indirect immunofluorescent TUNEL to detect cell death in oligodendrocytes transfected with control (ctrl) or dystroglycan (DG) siRNA. Bar graph depicts the average percentage of TUNEL-positive cells relative to total cell population (no significant difference, $n=3$, error bars represent s.d.). (B) Oligodendrocyte progenitors were differentiated on poly-D-lysine (PDL) or laminin for 4 days with increasing amounts of soluble growth factor PDGF. Integrin- and dystroglycan-blocking antibodies, or control antibodies, at 10 μ g/ml were added as indicated. Survival was evaluated using TUNEL on the GalC-positive cell population (newly formed oligodendrocytes). Laminin caused a significant shift in the PDGF dose-response such that survival was increased in response to 0.1 and 1.0 ng/ml PDGF compared with cells grown on PDL (** $P<0.01$, error bars represent s.d.). Antibodies against β 1-integrin, but not dystroglycan, blocked the ability of laminin to amplify PDGF-mediated survival at all concentrations (** $P<0.01$, error bars represent s.d.).

survival of newly formed oligodendrocytes grown on non-laminin substrates such as PDL or fibronectin (Cognato et al., 2002; Cognato et al., 2004). This is in contrast to our typical differentiation conditions, which contain 0.5% fetal bovine serum and maintain survival levels at a relatively high level (Fig. 6A). Here, we performed the more-stringent serum-free assays to measure the survival of newly formed oligodendrocytes after 4 days in culture in the presence or absence of particular laminin-receptor blocking antibodies. We performed sequential immunocytochemistry to visualize both TUNEL labeling (to measure survival) and GalC immunoreactivity (to label newly formed oligodendrocytes). This additional GalC step was necessary in order to ensure that, while using conditions of increasing PDGF concentrations that promote different degrees of differentiation, we would only evaluate the survival of the subset of cells that had differentiated. We confirmed our previous findings (Cognato et al., 2002) that newly formed oligodendrocytes grown on laminin have an enhanced survival response to low doses of PDGF (Fig. 6C; $P=0.0017$ for 0.1 ng/ml and $P=0.0098$ for 1.0 ng/ml). Also, as seen previously, the use of blocking antibodies against the β 1-integrin subunit significantly reduced survival of oligodendrocytes grown on laminin ($P=0.0054$

at 0.1 ng/ml PDGF, $P=0.0065$ at 1.0 ng/ml PDGF and $P=0.006$ at 10 ng/ml PDGF) (Fig. 6B) (Cognato et al., 2002; Cognato et al., 2004). By contrast, the use of blocking antibodies against α -DG had no effect on the ability of laminin to enhance PDGF-mediated survival. Neither the anti-integrin nor the anti-DG antibodies significantly reduced the survival of oligodendrocytes grown on PDL. These results indicated that, although oligodendrocytes express two different laminin receptors, integrins are likely to be the preferential receptor to mediate the effects of laminin on cell survival in newly formed oligodendrocytes.

Finally we examined the final stage of differentiation – the formation of a myelin sheath (Fig. 7). To do this, we took advantage of the ability of oligodendrocytes to form nascent myelinating structures when co-cultured with dorsal root ganglion (DRG) neurons. DRG neurons obtained from embryonic day (E) 14–16 rats were cultured for 2 weeks on 22 mm diameter coverslips (see Materials and methods). At 2 weeks, DRG cultures had developed a dense bed of neurites and were seeded subsequently with OPCs at a density of 5×10^4 cells per coverslip. To first ascertain whether DRG cultures contained appropriate laminin ligands for oligodendrocyte DG receptors, we performed immunocytochemistry using laminin antibodies on DRG cultures (Fig. 7A,B) and immunoblotting of lysates obtained from DRG co-cultures (Fig. 7C). Some, but not all, DRG neurites were immunoreactive for antibodies against the laminin α 2 subunit (the laminin subunit that is mutated in congenital muscular dystrophies with CNS myelination abnormalities). Laminin α 2 immunoreactivity was more pronounced in solo DRG neurite cultures (Fig. 7A) compared with DRG neurites co-cultured with oligodendrocytes (Fig. 7B). It is possible, however, that this qualitative difference may simply reflect a change in epitope access. It was not possible to evaluate laminin-1 immunoreactivity because DRG cultures were grown on Matrigel, which is highly enriched in laminin-1. Control experiments were performed to determine that laminin α 2 antibodies did not react with Matrigel laminin-1 (not shown). Immunoblotting was performed to evaluate both laminin α 2 and laminin-1 expression in DRG neurite co-cultures in comparison to cultures of only astrocytes or oligodendrocytes (Fig. 7C). Using a monoclonal antibody against laminin α 2, we observed substantial expression in astrocyte cultures (Fig. 7C; A), lower expression in DRG cultures and little to no expression in oligodendrocyte cultures (Fig. 7C; OL). Astrocytes and oligodendrocyte cultures were grown for 4 days in differentiation medium (Sato + 0.5% FCS) and DRG cultures were grown for 14 days as described (see Materials and methods). Using polyclonal antibodies against laminin-1 (this antibody can react with the laminin α 1, β 1 and γ 1 subunits), in astrocyte lysates, we observed a band at approximately 200 kDa (where β 1 and γ 1 are predicted to migrate) but no band at the larger size of 400 kDa (where α 1 is predicted to migrate). In DRG cultures, we observed a wide band that appeared to migrate slightly slower than the β 1 and/or γ 1 band of astrocyte cultures, as well as a larger laminin α 1-subunit band that was approximately 400 kDa. Because DRG neurons are grown on Matrigel, these laminin α 1, β 1 and γ 1 proteins are likely to be comprised, at least in part, of Matrigel laminin. It should be noted that DRG laminin α 1 and β 1 and/or γ 1 bands migrated slower and were fuzzier. This appearance is characteristic of laminin-1 purified from the EHS tumor (from which Matrigel is purified) due to EHS-laminin-1 being more heavily glycosylated than laminins from normal tissue.

To evaluate the role of DG during oligodendrocyte myelination of DRG neurites, OPCs were premixed with DG-blocking or control antibodies at the time of co-culture. Co-cultures were then

differentiated for 14 days, with fresh medium and antibodies changed at 3-day intervals. At the end of 14 days co-culture, cultures were fixed and indirect immunofluorescence was performed using

MBP antibodies to detect mature oligodendrocytes, and neurofilament (NF) antibodies to detect DRG neurites (Fig. 7D). We found no difference in the numbers of MBP-positive cells per field

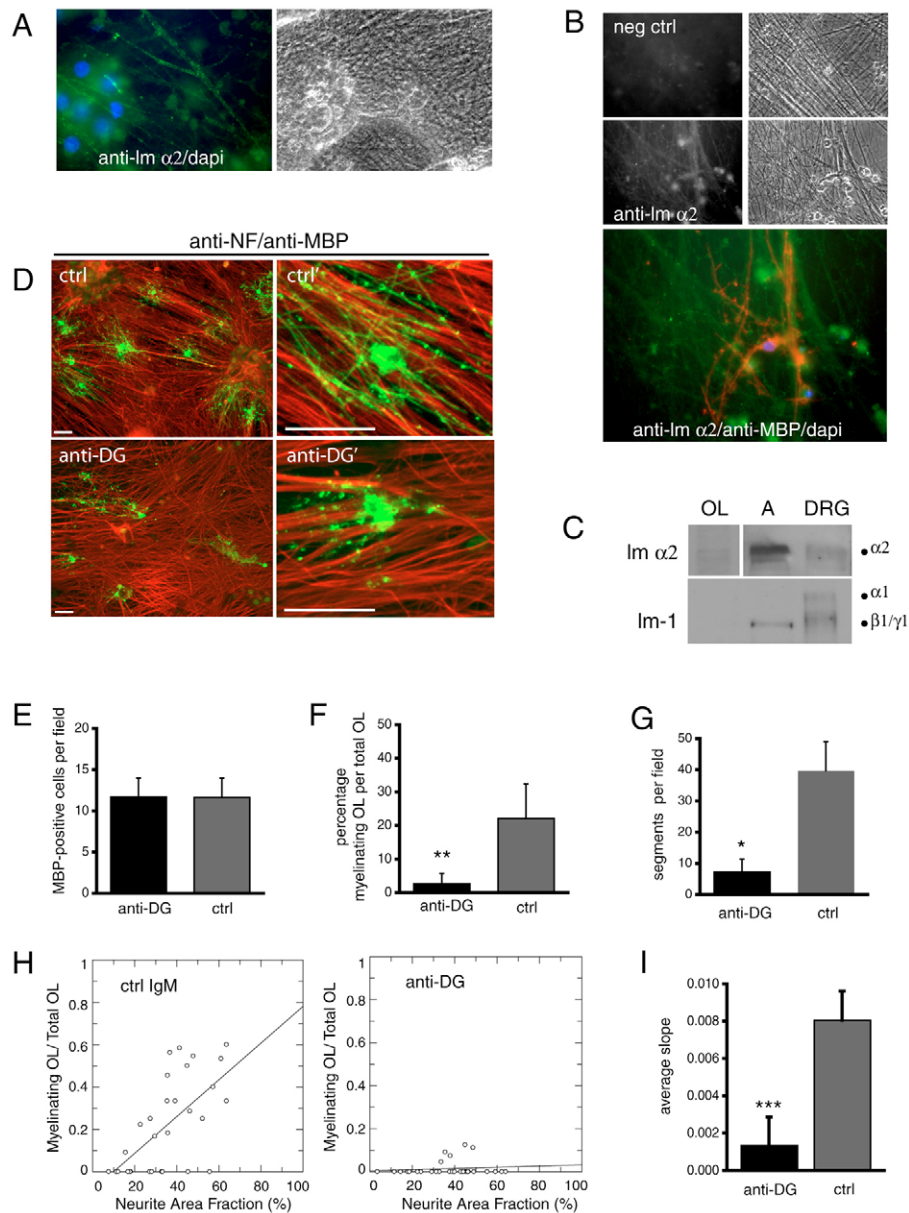


Fig. 7. Myelination in oligodendrocyte-neuron co-cultures is perturbed by dystroglycan-blocking antibodies. (A) Rat dorsal root ganglion (DRG) neuron cultures, which contain laminin $\alpha 2$. Immunocytochemistry was used to detect laminin $\alpha 2$ (green) and nuclei (DAPI; blue). (B) Rat DRG neurons co-cultured with oligodendrocytes. Myelinating oligodendrocytes in rat DRG neuron co-cultures are found in association with laminin $\alpha 2$. Double immunocytochemistry was used to detect laminin $\alpha 2$ (green) and myelin basic protein (MBP; red). Nuclei were visualized using DAPI (blue). (C) Western blots to detect laminin proteins. Lysates obtained from oligodendrocytes (OL), astrocytes (A) or DRG neuron cultures were immunoblotted with a monoclonal antibody against the laminin $\alpha 2$ subunit (Im $\alpha 2$) or a polyclonal antibody against laminin-1 (Im-1). Note that laminin-1 antibodies detect the laminin $\alpha 1$, $\beta 1$ and $\gamma 1$ subunits. (D) Rat DRG neurons were seeded with oligodendrocyte progenitors in the presence of dystroglycan-blocking or control antibodies. At 2 weeks, differentiated oligodendrocytes were visualized using MBP immunofluorescence (green) in conjunction with neurofilament (NF) immunofluorescence (red) to visualize the underlying neurite network. (E) The average number of MBP-positive cells per field was scored in cultures treated with control and dystroglycan-blocking antibodies. No significant difference was observed ($n=4$, error bars represent s.d.). (F) The average percentage of myelinating MBP-positive oligodendrocytes (OL) was scored in dystroglycan-blocking- and control-antibody conditions. Dystroglycan-blocking antibodies significantly reduced the percentage of myelinating oligodendrocytes compared with control antibodies ($n=4$, $**P<0.01$, error bars represent s.d.). (G) The average number of myelinating segments per field was scored in cultures treated with control and dystroglycan-blocking antibodies. Dystroglycan-blocking antibodies significantly reduced the number of segments per field compared with control antibodies ($n=4$, $**P<0.01$, error bars represent s.d.). (H) Representative plots depicting the correlation between the myelinating oligodendrocyte (OL) to total oligodendrocyte (OL) ratio and neurite density (neurite area fraction, %). (I) Average slope of plots depicted in H ($n=4$, $***P<0.001$, error bars represent s.d.). Scale bars: 100 μm .

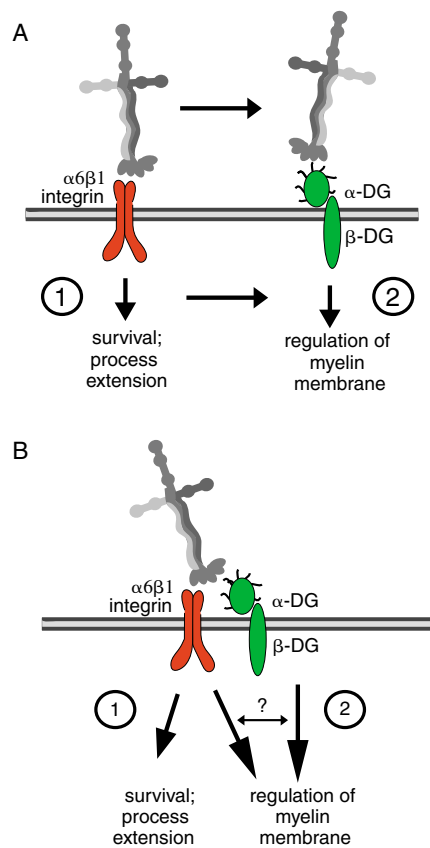


Fig. 8. Model of potential integrin and dystroglycan hierarchies.

(A) In a sequential model, laminins interact preferentially with integrins in newly formed oligodendrocytes (1) during the regulation of survival and process outgrowth, and preferentially with dystroglycan during the later stages (2), such as myelin membrane formation. (B) In a parallel model, laminins interact with both integrins and dystroglycan to promote myelin membrane formation, but receptor crosstalk or signaling changes can alter the relative contribution of each receptor during distinct oligodendrocyte developmental stages.

in control- and DG-blocking-antibody-treated co-cultures (Fig. 7E), and no change in the level of MBP protein by immunoblotting was observed (data not shown). These results indicate, therefore, that DG-blocking antibody does not prevent oligodendrocyte differentiation to the pre-myelinating stage. Due to the lack of any myelin markers specific for compacted sheaths and suitable for biochemical analysis, we quantified myelination using morphological criteria (Wang et al., 2007). First, we scored the percentage of myelinating oligodendrocytes within the MBP-positive oligodendrocyte population (Fig. 7F). A cell was termed a myelinating oligodendrocyte if it was MBP-positive and had two or more myelinating processes that appeared to surround neurofilament-positive DRG neurites. We observed a substantial decrease ($n=4$, $P=0.0041$) in the percentage of myelinating oligodendrocytes in DG-antibody-treated co-cultures ($2.7 \pm 3.2\%$) compared with control co-cultures ($22.1 \pm 10.3\%$). No change in oligodendrocyte survival was observed (data not shown). Second, in addition to scoring the percentage of myelinating oligodendrocytes per total oligodendrocyte population, we also scored the mean number of myelinating segments per field, as described in a recent study using the co-culture model (Zhang et al., 2006). We found that co-cultures treated with DG antibodies had significantly less

myelinating segments per field (7.3 ± 4.1 , $P=0.0122$) compared with co-cultures treated with control antibodies (39.8 ± 9.3). Finally, we evaluated the relative levels of myelinating oligodendrocytes as a function of neurite density. Neurite density can vary throughout the co-culture, and areas of higher neurite density have been correlated with increased percentages of myelinating oligodendrocytes within the MBP-positive oligodendrocyte population. To exploit this correlation, we recently employed a strategy to plot neurite density (as percent area covered by NF-positive neurites) against the myelinating:total oligodendrocyte ratio, after which the slope of the best-fit line under control conditions is compared to slope of the best-fit line obtained from test conditions (Wang et al., 2007). Using this strategy, we found that the average slope of best-fit lines from cultures treated with control antibodies was 0.0081 ± 0.0016 ($n=4$) compared with 0.0014 ± 0.0015 ($n=4$) for cultures treated with DG antibodies (Fig. 7I). A representative set of plots is shown in Fig. 7H. The overall conclusion of the various quantification methods is that blocking DG in oligodendrocyte-neuron co-cultures leads to a stalled oligodendrocyte differentiation phenotype.

DISCUSSION

The dysmyelination observed in laminin $\alpha 2$ -deficient mice and humans points to a role for laminins in myelination. The underlying mechanisms, however, remain unknown. Here, we have examined the potential role of DG, an important laminin receptor in skeletal muscle. We present the first evidence that (1) functional DG receptors are found on oligodendrocyte cell surfaces, and that, (2) by blocking DG interactions either in oligodendrocytes on laminin substrates or in oligodendrocyte-neuron co-cultures, the ability of oligodendrocytes to differentiate and to myelinate is perturbed. DG has also been shown to mediate interactions with several other extracellular molecules that are expressed in the brain, including agrin, perlecan and α -neurexin (Ford-Perriss et al., 2003; Gesemann et al., 1998; Halfter, 1993; Maresh et al., 1996; Sugita et al., 2001). Of these ligands, however, only laminins have been shown to play a role in regulating CNS myelination to date, and we suggest that the dysmyelination associated with laminin deficiency is likely to involve the disruption of at least two classes of receptor interactions: integrin and DG. Laminin $\alpha 2$ expression has been reported in the early stages of myelinating white matter tracts, yet it remains unclear how oligodendrocytes interact with this transient CNS laminin (Anderson et al., 2005; Colognato et al., 2002; Farwell and Dubord-Tomasetti, 1999; Georges-Labouesse et al., 1998; Hagg et al., 1997; Liesi et al., 2001; Morissette and Carbonetto, 1995; Powell et al., 1998; Tian et al., 1997). One possibility is that CNS $\alpha 2$ -laminins help to regulate axonal-glia interactions at early stages of axon ensheathment, when instructive cues for survival and/or differentiation are needed.

Several lines of evidence implicate signaling via the integrin receptor $\alpha 6 \beta 1$ 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 knockout of the $\alpha 6$ -subunit (Colognato et al., 2002) or a conditional knock-out in oligodendroglia of the $\beta 1$ -subunit (Benninger et al., 2006). Second, survival of oligodendrocytes grown in culture is reduced in the presence of antibodies that block oligodendrocyte $\alpha 6 \beta 1$ receptors (Colognato et al., 2002; Corley et al., 2001; Frost et al., 1999). Third, $\beta 1$ -integrin-blocking antibodies have been shown to reduce myelin membrane sheet formation in cultured oligodendrocytes (Buttery and French-Constant, 1999; Olsen and French-Constant, 2005; Relvas et al., 2001). Fourth, expression of dominant-negative $\beta 1$ -integrin in oligodendrocytes

perturbs remyelination in injured spinal cord (Relvas et al., 2001), and disrupts myelination in the developing spinal cord and optic nerve (Lee et al., 2006). Finally, several integrin-associated signaling molecules, including integrin linked kinase (ILK) and the Src family kinase Fyn proto-oncogene (FYN), have been shown to be activated downstream of laminins in oligodendrocytes (Chun et al., 2003; Colognato et al., 2004; Liang et al., 2004); at least one of these signaling effector molecules, FYN, is required for normal CNS myelination (Sperber et al., 2001; Sperber and McMorris, 2001; Umemori et al., 1999; Umemori et al., 1994).

Despite the evidence that laminins transmit signals to oligodendrocytes using integrin receptors, it remains unknown to what extent the dysmyelination associated with laminin deficiency is caused by the loss of integrin signaling. The $\alpha 6$ -integrin-null mouse dies at birth due to severe skin blistering and, thus, only the initial stages of myelination have been evaluated in the developing brain stem and spinal cord of such mice, up to E18.5 (Colognato et al., 2002; Georges-Labouesse et al., 1996). A more-comprehensive analysis of oligodendrocyte $\beta 1$ -integrin requirements has recently been performed, however, in which mice were engineered to lack the integrin $\beta 1$ subunit in oligodendrocytes and were found to form normal-appearing myelin in the brain and spinal cord (Benninger et al., 2006). Another recent study examined the development of CNS myelination in the presence of a dominant-negative $\beta 1$ -integrin receptor, and found that myelination was perturbed in the spinal cord and optic nerve, but not in the corpus callosum (Lee et al., 2006). In laminin-deficient mice, myelination defects have been reported to be present in the corpus callosum but absent in the spinal cord (Chun et al., 2003). Thus, laminin-deficient mice, $\beta 1$ -integrin-deficient mice and $\beta 1$ -integrin-compromised mice have different myelination phenotypes, pointing to additional oligodendrocyte laminin receptors that could contribute to myelination.

Our current study shows that DG probably represents one of these additional receptors. Although the persistence of binding of the laminin rE3 fragment to differentiated oligodendrocytes in the presence of anti-DG-blocking antibodies or following treatment with DG siRNA (J.G. and H.C., unpublished) points to the presence of other potential laminin receptors, we show here that DG has a specific role in promoting oligodendrocyte differentiation and myelination. We cannot exclude the possibility that inhibition of neuronal DG by the blocking antibodies might contribute to the inhibition of myelination we observe in the co-culture experiments. However, our current studies using purified oligodendrocytes grown on laminin substrates, in which oligodendrocyte myelin protein production is reduced, indicate that the perturbation of oligodendrocyte DG contributes to the lack of myelin formation in the co-culture system. We have also shown previously that $\beta 1$ -integrin-blocking antibodies can inhibit both process formation and myelin membrane formation in culture (Buttery and ffrench-Constant, 1999; Relvas et al., 2001; Olsen and ffrench-Constant, 2005). It appears, therefore, that integrins and DG may both contribute to oligodendrocyte differentiation, while integrins also have a more-specific role in survival and process outgrowth earlier in development. Two models can therefore be envisaged (Fig. 8). In one model (Fig. 8A), the receptors signal in a sequential manner, with myelin membrane formation in response to DG signaling requiring the prior activation of integrin signaling. In the other model (Fig. 8B), integrins promote survival and process outgrowth earlier in development, and both receptors are involved in the later stages of differentiation via parallel signaling pathways. Targeted knockout studies of DG in oligodendrocytes, in conjunction with $\beta 1$ -integrin deficiencies, will be required to test these models. The model of

parallel action (Fig. 8B), however, would facilitate compensation for the loss of integrins and would be consistent with the normal myelin seen in the conditional $\beta 1$ knockout in oligodendrocytes (Benninger et al., 2006).

Other cell types have also been shown to use both integrin and DG 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 the removal of the laminin ligand (reviewed in Feltri and Wrabetz, 2005; Jimenez-Mallebrera et al., 2005). In peripheral nerves, laminin removal causes a severe combination of amyelination and dysmyelination, whereas the removal of Schwann cell $\beta 1$ -integrin causes a less-severe failure in radial sorting, which, in many cases, halts normal myelination (Feltri et al., 2002). Following the removal of Schwann cell DG, however, radial sorting and myelination proceed, at least in most axon bundles, although the removal of DG results in disorganized myelin and improper nodal architecture that is susceptible to degeneration (Saito et al., 2003). In skeletal muscle, a different hierarchy emerges: DG removal has the more-severe consequence for muscle function whereas removal of the primary laminin-binding integrin, $\alpha 7\beta 1$, causes subtle deficits (Cote et al., 1999; Mayer et al., 1997). A further complication of these studies is the fact that, in the absence of one receptor type, other receptor types can be deregulated (Cohn et al., 1999; Cote et al., 2002; Cote et al., 1999; Moghadaszadeh et al., 2003). This occurs in skeletal muscle, in which integrin receptors are upregulated in the absence of DG. In the current study, we found that the interactions between laminin and oligodendrocytes resulted in an increase in the amount of DG protein that was expressed (Fig. 2). So far, it is not known how laminin alters DG protein expression. One potential scenario is that early laminin-integrin interactions in oligodendrocytes trigger an increase in DG, which is then poised to play a role in more-differentiated myelinating cells (as in the sequential model of Fig. 8A). In intestinal epithelia, however, it has recently been shown that laminin-DG interactions can regulate the activation state of integrins (Driss et al., 2006). An interesting aspect of this regulation was the finding that interactions between DG and different laminin types caused different outcomes for the integrin receptor in question: interactions between laminin-1 and DG caused an increase in $\beta 1$ -integrin activity, whereas laminin-2 interactions with DG caused a decrease in $\beta 1$ -integrin activity. Such receptor crosstalk could have significant effects on the balance of signaling activity between integrins and DG in the parallel-pathway model (Fig. 8B), providing a mechanism to alter the response to laminin at different developmental stages. Because laminin-2, but not laminin-1, expression is disrupted in laminin deficiencies that cause CNS myelin abnormalities, further studies that are focused on the interaction between oligodendrocytes and laminin-2 are needed to determine whether such oligodendrocyte DG interactions can alter integrin function, how different laminins could potentially regulate this response and what role such receptor crosstalk might play in the development of normal myelin.

Although a potential role for DG in oligodendrocytes had not previously been addressed, several roles have been proposed for DG in other CNS cell lineages (Gee et al., 1993; Gorecki et al., 1994; Guadagno and Moukhles, 2004; Henion et al., 2003; Moore et al., 2002; Moukhles and Carbonetto, 2001). DG is expressed in the radial glial endfeet that attach to the pial basal lamina and, like $\beta 1$ -integrins, is required for the stability of this pial basal lamina during the layering and organization of the cerebral cortex and cerebellum (Blank et al., 1997; De Arcangelis et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Koulen et al., 1998; Moore et

al., 2002; Noel et al., 2005; Schmitz and Drenckhahn, 1997; Tian et al., 1996; Ueda et al., 2000; Zaccaria et al., 2001). The loss of this anchorage between the pial basal lamina and the underlying glia results in ectopic clusters of neural cells termed cobblestone lissencephaly (Olson and Walsh, 2002). In addition, several human congenital muscular dystrophies are caused by mutations in glycosyltransferases that modify the unusual O-linked carbohydrate moieties that are found on DG (Cohn, 2005; Schachter et al., 2004). The loss of these carbohydrate modifications has been shown to disrupt DG binding to several ligands, including laminins (Kanagawa et al., 2005; Kim et al., 2004; Patnaik and Stanley, 2005; Saito et al., 2005). Like laminin deficiencies that cause MCD1A, these dystrophies also cause developmental brain abnormalities, including MDC1C (*myd* in mice), Fukuyama's muscular dystrophy (FCMD), muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (WWS). In MDC1C, mutations in the *LARGE* gene, a putative O-linked glycosyltransferase, have been found to cause aberrant white matter development, indicating a potential alteration in CNS myelination (Longman et al., 2003). Given that *LARGE* mutations cause both aberrant DG function and abnormal white matter, it may be that the dysregulation of oligodendrocyte DG contributes to the *LARGE* phenotype. Our finding that oligodendrocytes express DG 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. Further modification of DG may also take place during disease states: a recent report has shown that brain DG can be cleaved by matrix metalloproteinases (MMPs) during experimental autoimmune encephalomyelitis (EAE) and, importantly, that this cleavage contributes to the destabilization of the blood-brain barrier by disrupting DG linkage to the parenchymal basal lamina (Agrawal et al., 2006). EAE is generated by a severe immune reaction directed against myelin components, and is thought to provide a disease model for aspects of the inflammation and myelin loss that occur during multiple sclerosis (MS). It will therefore be interesting to learn whether oligodendrocyte DG is similarly processed by MMPs, which are known to be upregulated in active demyelinating lesions of MS (Anthony et al., 1997; Lindberg et al., 2001).

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