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Dystroglycan is a Novel Regulator of Stem Cell Niche Structure and Function in the Developing Postnatal Subventricular Zone

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Abstract of the Dissertation

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The extracellular matrix (ECM) has emerged as a potential regulator of neural stem cell quiescence and neurogenesis in the adult subventricular zone (SVZ). However, the nature and role of ECM in the developing SVZ has not been determined. During the first postnatal week, radial glia differentiate into ependymal cells and adult neural stem cells, which together organize into adult niche pinwheel structures at the ventricular surface. Using genetic and antibody blocking approaches *in vitro* and *in vivo*, we found that these events coincide with a unique developmental restructuring of ECM in the early postnatal SVZ and that this process is regulated by the ECM receptor dystroglycan. We found that dystroglycan is upregulated in maturing ependymal cells and required for their differentiation and assembly into niche pinwheel structures. Dystroglycan furthermore mediates the association of radial glia with ventricle surface-associated laminins, and genetic deletion of dystroglycan delayed their transition into intermediate gliogenic progenitors and led to abnormal progenitor distribution and proliferation.

Dystroglycan loss-of-function also had a dramatic impact on niche output; oligodendrogenesis was increased in dystroglycan-deficient mice and a single injection of dystroglycan blocking antibody into the ventricle of perinatal rats was sufficient to induce oligodendroglial fate in SVZ progenitors. However, the differentiation of dystroglycan-deficient oligodendrocytes was delayed, with the early postnatal corpus callosum containing more oligodendrocyte progenitor cells, and a higher proportion of progenitors with an immature phenotype, resulting in delayed myelination. These findings reveal, for the first time, dystroglycan's role as a master regulator, orchestrating both the assembly and function of the SVZ neural stem cell niche during postnatal gliogenesis.

For my parents, Eleanor and Sean,

who encouraged curiosity, creativity, wonder and perseverance
and, ultimately, raised two little scientists.

And for Fikers Birs,

my partner in crime, my colleague, my best friend;
who read countless pages, provided invaluable advice, made coffee every morning
and left me a stronger woman.

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List of Abbreviations

APC	adenomatous polyposis coli
AJ	adherens junction
BL	basal lamina
BLBP	brain lipid-binding protein
CD24	cluster of differentiation 24
CNS	central nervous system
CNP	2', 3'-cyclic nucleotide 3' phosphodiesterase
CSF	cerebrospinal fluid
CY3	cyanine 3
DAG cKO	neural cell-specific dystroglycan-deficient mouse
DAPI	4',6-diamidino-2-phenylindole
DGC	dystrophin-associated glycoprotein complex
DICD	dystroglycan intracellular domain
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EGF	epidermal growth factor
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FoxJ1	Forkhead box protein J1
FKTN	fukutin
FKRP	fukutin-related protein
FCMD	Fukuyama congenital muscular dystrophy

GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
ICD	intracellular domain
IHC	immunohistochemistry
INM	interkinetic nuclear migration
JAM	junctional adhesion molecule
LARGE	like-glycosyltransferase
LGMD	limb girdle muscular dystrophy
Lm	laminin
MAPK	mitogen activated kinase-like protein
MBP	myelin basic protein
MDC1C	congenital muscular dystrophy type 1C
MDC1D	congenital muscular dystrophy type 1D
MEB	muscle-eye-brain disease
NEC	neuroepithelial cell
NG2	nerve/glial antigen 2
NICD	notch intracellular domain
NS	not significant
NPC	neural progenitor cell
NSC	neural stem cell
oIPC	oligodendrogenic intermediate progenitor cell
Olig2	oligodendrocyte lineage transcription factor 2
OPC	oligodendrocyte progenitor cell
Pax6	paired box protein 6

PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor receptor alpha polypeptide
PDL	poly-D-lysine
PFA	paraformaldehyde
PI3K	phosphoinositide-3-kinase
POMT1	protein-O-mannosyltransferase 1
POMT2	protein-O-mannosyltransferase 2
POMGnT1	protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase
RGC	radial glial cell
RMS	rostral migratory stream
SEM	standard error of the mean
Shh	Sonic hedgehog
Sox2	sex determining region Y-box 2
SVZ	subventricular zone
TJ	tight junction
VS	ventricular surface
VZ	ventricular zone
WM	white matter
WT	wildtype
WWS	Walker-Warburg Syndrome

CHAPTER I: GENERAL INTRODUCTION

During late embryonic brain development, radial glia serve as the neural stem cells of the mammalian cerebral cortex, dividing asymmetrically to produce immature neurons (neuroblasts). Radial glial neural stem cells exhibit an extreme bipolar morphology, with processes that span the developing cortical plate, terminating apically at the surface of the lateral ventricle and basally at the pial basement membrane. Shortly after birth, radial glia detach from the pial surface and transform into adult neural stem cells (B cells) and multiciliated ependymal cells, which then arrange into "pinwheels", rings of ependymal cells that surround B cell apical processes[10]. Stem- and ependymal cell pinwheels, together with transit amplifying neural progenitors (C cells) and neuroblasts (A cells), comprise the adult ventricular/subventricular zone (VZ/SVZ), a specialized microenvironment that supports stem cell quiescence and the controlled production of neurons and glia. Intriguingly, neural stem and progenitor cells undergo the principal wave of dorsal gliogenesis during this postnatal transitional period. The process of VZ/SVZ postnatal niche construction and its correct coupling with early postnatal gliogenesis are likely critical to proper brain development, yet the factors regulating these processes remain poorly understood.

The extracellular matrix (ECM) has recently been identified as a potential regulator of cell proliferation in the adult SVZ niche. Actively dividing neural stem- and progenitor cells (NSCs/NPCs) remain associated with ECM through adhesion to the basal lamina surrounding

blood vessels and extra-vascular ECM structures unique to the SVZ, and these interactions have been implicated in the regulation of NSC/NPC quiescence and neurogenesis. Despite the recent attention the specialized ECM of the adult VZ/SVZ has garnered, it remains entirely unknown whether ECM acts during postnatal development to regulate either the assembly of the adult SVZ niche structure or the concurrent process of SVZ oligodendrogenesis. In the course of my dissertation research I investigated the role of the ECM receptor dystroglycan in the cellular maturation and structural development of the VZ/SVZ neural stem cell niche, and in the production and differentiation of oligodendrogenic progenitor cells.

The extracellular matrix

Extracellular matrices (ECMs) are networks of cell adhesion proteins that arose with the development of multicellularity, providing tissues with structural support, facilitating communication between cells and their local environment and integrating signals from other compartments. Layers of cell-associated ECM assemble into thin sheets to form basement membranes (BMs), or *basal lamina* (BL), which line the basal surfaces of epithelial and endothelial cells and surround muscle, fat and Schwann cells. Although the exact composition varies by tissue, the basic protein constituents of BMs are laminins, type IV collagen, nidogen and the heparan sulfate proteoglycans (HSPG) perlecan and agrin [11,1,12]. Of these ECM ligands, only laminins are indispensable for initial BM assembly.

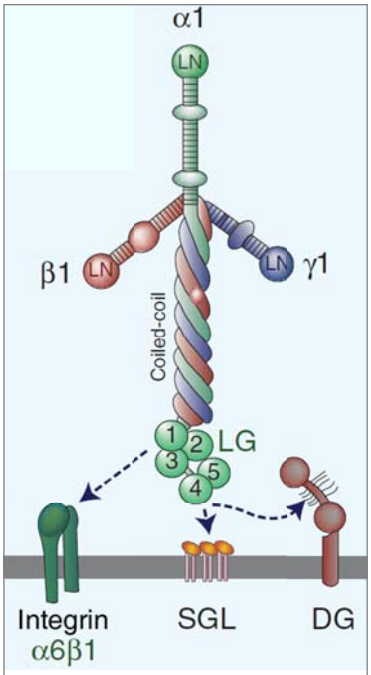


Figure I-1. Laminin heterotrimer structure and functional domains
Adapted from [1]

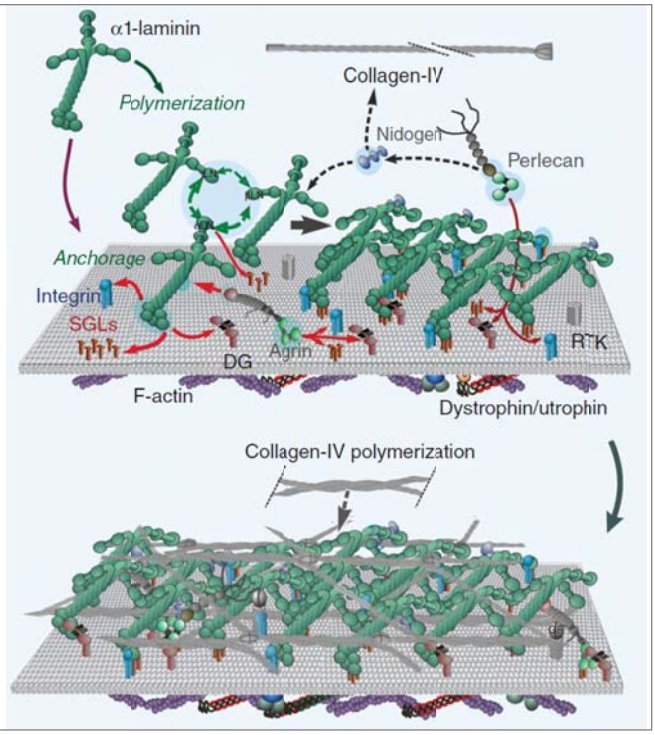
Laminins and basement membrane assembly

Laminins are heterotrimeric extracellular matrix proteins consisting of one α, one β and one γ chain, each produced as a different gene product. To date, five α, three β and three γ chains have been identified in vertebrates. The laminin trimer naming convention currently in use refers to their subunit composition, for example, laminin-111 is composed of the α1, β1 and γ1 chains (Figure I-1.) [13]. Genetic deletion of either the laminin β1or γ1 chain prevents laminin heterotrimerization and basement membrane (BM) assembly, resulting in early embryonic lethality (E5.5 in mouse) [14] [15]. In contrast, deletion of other major ECM ligands produces comparatively minor developmental defects, does not impact BM assembly in most tissues and does not preclude the viability of embryos [16-20].

To initiate BM assembly, laminins bind to cell surfaces through interactions between laminin LG domains and integrins, dystroglycan, sulfated glycolipids and haparan sulfates. This

Figure I-2. Basement membrane assembly: Laminins first

Laminin LG domains bind to sulfated glycolipids (SGL), integrins and α-dystroglycan (DG) on the cell surface. Laminins then polymerize through their LN domains, which also bind to cell surface SGLs and integrins to lock laminin heterotrimers in a sheet configuration. This is followed by the binding of nidogen to laminin coiled-coil domains and collagen-IV to form a stabilizing network, with further stability provided by collagen-IV polymerization. Agrin and perlecan bind to both the laminin-nidogen network and cell surface receptors, and recruit heparan-binding growth factors (GF) that initiate intracellular signaling through their receptor tyrosine kinases (RTK). Adapted from [1]



initial step establishes a scaffold, enabling the subsequent recruitment and self-assembly of nidogen, type IV collagen, perlecan and agrin (Figure I-2.) [21-25]. In mature basement membranes the transduction of extracellular signals occurs mainly through the integrin family of receptors, dystroglycan and growth factor (GF) receptor tyrosine kinases. Not only are laminins crucial for the formation of basement membranes, they have emerged as arguably the most permissive substrate in supporting the proliferation and differentiation of both embryonic stem cell lines and primary cortical neural progenitors [26,27]. Furthermore, recent studies have implicated laminins, both in vascular basal lamina and in unique non-BM ECM structures, in the regulation of SVZ neural stem- (NSC) and progenitor cell (NPC) proliferation and neurogenesis (more below).

Dystroglycan

Dystroglycan is a transmembrane ECM receptor, known to mediate cell-matrix adhesions through interactions with a variety of ligands, including laminins, neurexins and the heparan sulfate proteoglycans perlecan and agrin. Dystroglycan, originally termed cranin, was first described as a laminin-binding protein isolated from the brain [28]. Shortly thereafter, the same protein was identified as a member of the

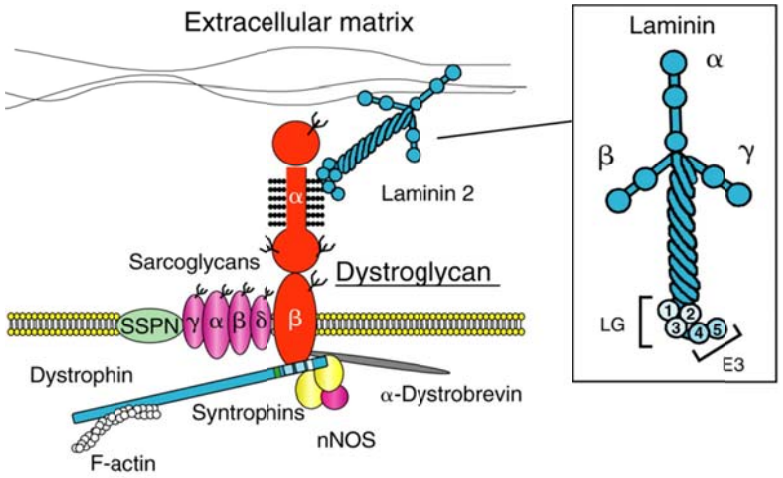


Figure I-3. Dystroglycan is a member of the DGC
Dystroglycan links the ECM to the actin cytoskeleton through interactions with extracellular ligands, such as laminins, and intracellular association with other DGC proteins. Inset: Dystroglycan binds to laminin globular (LG) domains at the C-terminus of the a1 and a2 subunits. The E3 fragment is composed of LG4 and LG5. Adapted from [4]

dystrophin-glycoprotein complex (DGC) in skeletal muscle and given its current name [29-31]. Dystroglycan is still best known as a transmembrane component of the DGC (Figure I-3.), connecting the ECM to the actin cytoskeleton either directly [32] or through interaction with other actin-binding DGC members, such as dystrophin [33], utrophin [34] and ezrin [35].

Dystroglycan is produced as a single gene product that is post-translationally cleaved into an α and β subunit [36], which then reassociate non-covalently (Figure I-4.). The extracellular α subunit undergoes substantial post-translational *N*- and *O*-linked glycosylation. *O*-linked glycosylation of the central mucin domain of α-dystroglycan is of particular importance as it is a requisite for the ability to bind laminin G (LG) domain-containing extracellular ligands [37], with variations in the degree of glycosylation reflected in ligand binding affinity [38,39]. Dystroglycan has been shown to be differentially glycosylated in different tissues, different cell types and even within a single cell line ([40], with reported molecular weights ranging from 180 kDa in cerebellar Purkinje neurons [41] to 156 kDa in skeletal muscle [29] to 120 kDa in the rest of the brain and PNS [28,41].

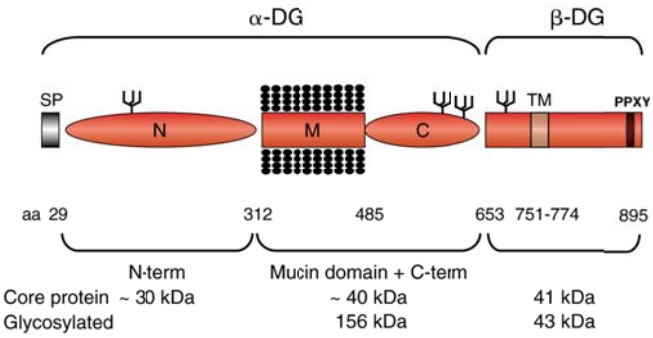


Figure I-4. Dystroglycan domain organization
Circles indicate O-linked sugar chains; branches indicate N-linked sugar chains. SP, signal peptide; TM, transmembrane domain; PPXY, dystrophin-binding site. [4]

β-dystroglycan is the transmembrane component of the receptor, linking α-dystroglycan to the cytoskeleton through the direct binding of actin or via other DGC proteins. In addition to this well-described structural function, β-dystroglycan is capable of a diverse set of intracellular interactions (reviewed in [42]). The cytoplasmic domain of β-dystroglycan contains 19 different functional motifs and over 40 predicted interaction sites ([42], ELM prediction). These include

binding motifs for extracellular signal-related kinase (ERK), ezrin-radixin-moesin (ERM), and receptor-associated protein of the synapse (rapsyn) and consensus sequences for Src homology 2 (SH2) and Src homology 3 (SH3). Along with ERK, MEK2 [43] and rapsyn [44], known binding partners of β-dystroglycan include growth factor receptor 2 (Grb2) [45], caveolin-3 [46] and dynamin-1[47]. Furthermore, it has recently been shown that the juxtamembrane domain of β-dystroglycan also contains a functional nuclear localization sequence. A cleaved form of the β-dystroglycan intracellular domain is capable of nuclear translocation and acts as a nuclear scaffold protein [48,49].

To date, there are only two reports of mutations in the *dag1* gene in human patients, both resulting in amino acid substitutions; a homozygous missense mutation affecting the extracellular portion of β-dystroglycan, predicted to interfere with its association with the α subunit [50] and a distinct homozygous mutation corresponding to the N-terminus of α–dystroglycan, preventing its proper glycosylation and therefore greatly reducing its ability to bind ECM ligands [51]. The scarcity of human *dag1* mutations likely reflects the necessity of dystroglycan function in development. Constitutive deletion of *dag1* in mice is embryonic lethal [52], resulting from the malformation of Reichert's membrane, one of the first BMs to form during rodent development, which acts as a barrier between the embryo and the maternal circulation and uterine environment, and may play a role in materno- embryonic exchange. Rather, the known conditions associated with dystroglycan loss-of-function manifest as "secondary dystroglycanopathies", congenital muscular dystrophies in which mutations in genes encoding glycosyltransferases lead to hypoglycosylation.of α–dystroglycan. As mentioned above, the proper glycosylation of α-dystroglycan is required for ECM ligand binding. Dystroglycan-

associated glycosyltransferases known to be affected, and their associated conditions, are detailed in Table I-1.

Protein (*encoding gene*)	Associated conditions	Reference
Protein-O-mannosyltransferase 1 (*POMT1*)	Walker–Warburg syndrome (WWS)	53
	Limb girdle muscular dystrophy (LGMD)	54
Protein-O-mannosyltransferase 2 (*POMT2*)	Walker–Warburg syndrome (WWS)	55
Protein-*O*-linked mannose beta 1,2-*N*-acetylglucosaminyltransferase (*POMGnT1*)	Muscle-eye-brain disease (MEB)	56
Like-glycosyltransferase (*LARGE*)	MDC1D	57
Fukutin (*FKTN*)	Fukuyama congenital muscular dystrophy (FCMD)	58
	WWS	59
	LGMD	60
Fukutin-related protein (*FKRP*)	MDC1C	61
	LGMD	62
	WWS-like	63
	MEB-like	64

Table I-1. Glycosyltransferases implicated in the secondary dystroglycanopathies

With the exception of certain subtypes of limb girdle muscular dystrophy, all dystroglycanopathies have some degree of developmental brain defects. Brain abnormalities commonly observed in secondary dystroglycanopathies include disordered cortical lamination, thickening of the cortical plate, hydrocephaly, delayed myelination, complete or partial absence of the corpus callosum and abnormal white matter volume (Figure I-5.).

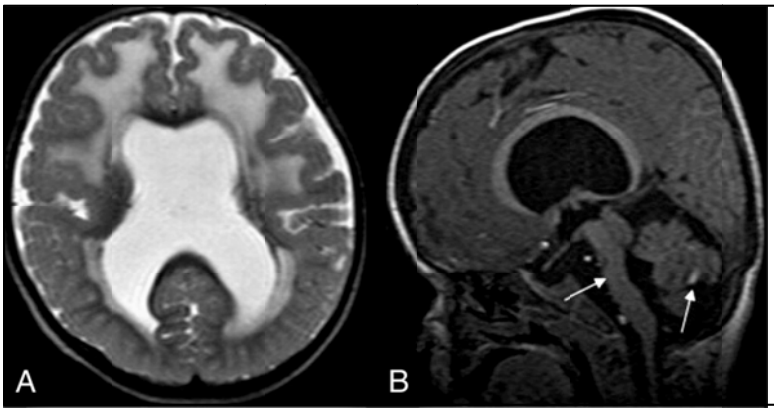


Figure I-5. Dystroglycanopathy-related brain abnormalities
Brain MRI of a child with muscle-eye-brain disease. **(A)** T2-weighted transverse and **(B)** T1-weighted sagittal images demonstrate features characteristic of the dystroglycanopathies, such as polymicrogyria, white matter defects and hydrocephaly. Adapted from [2]

The cortical lamination defects observed in these conditions are thought to be due to both the malformation of the pial basal lamina and the loss of dystroglycan-mediated radial glial cell (RGC) endfoot attachment. This disrupts the positioning of RGC basal processes and, therefore, their ability to function as scaffolds for migrating neuroblasts. As a result, neuroblasts become mislocalized throughout the cortical plate, with some migrating past the glia limitans and into the subarachnoid space (Figure I-6.).

While the investigation of these phenotypes has provided valuable insight into dystroglycan's role in the developing brain, such studies have failed to detect mutations in known glycosyltransferases in approximately 50% of dystroglycanopathy patients [65-67,64], indicating there may be a significant number of dystroglycan-modifying or –interacting proteins yet to be found.

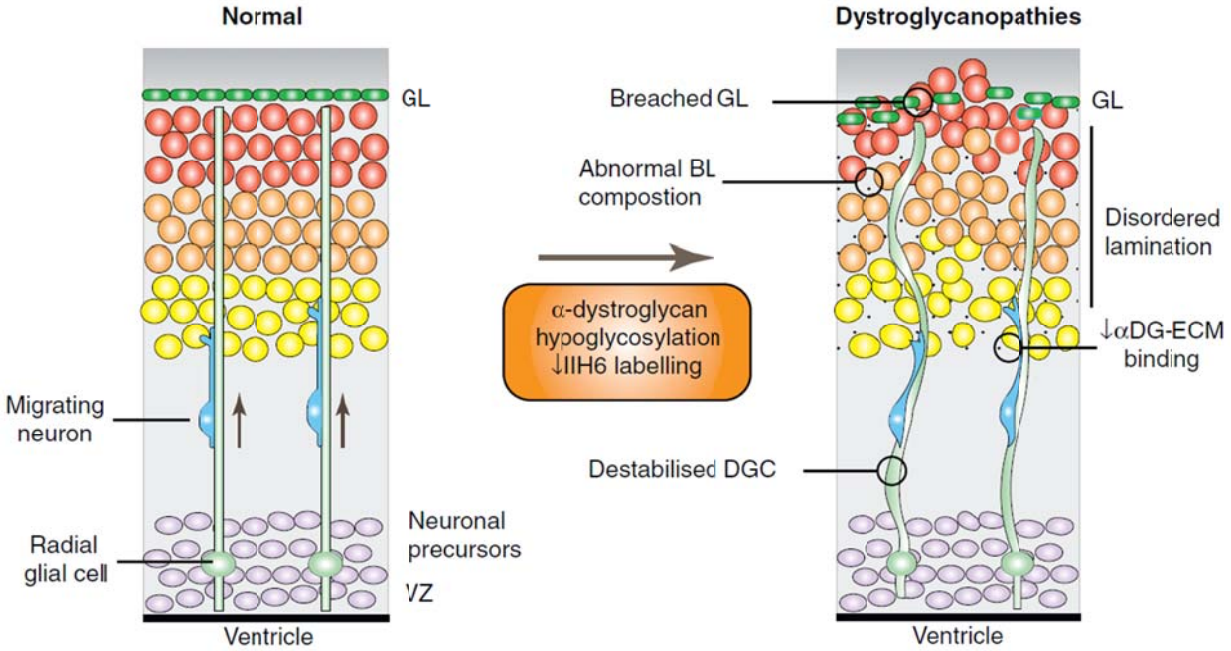


Figure I-6. Hypoglycosylation of a-dystroglycan results in neuronal migration defects
GL: glia limitans; BL: basal lamina; αDG: α-dystroglycan; ECM: extracellular matrix; DGC: dystrophin-glycoprotein complex; VZ: ventricular zone. [5]

The human DG gene has been mapped to chromosome 3p21, a locus that has been associated with tumor suppression [68]. Indeed, dystroglycan hypoglycosylation is also a characteristic of many epithelial-derived tumors, including breast, colon, cervix and prostate cancers [69], as well as gliomas [70], and is associated with poor prognosis.

Dystroglycan-mediated laminin assembly

The interaction between dystroglycan and its ligands extends beyond a passive adhesive function. Dystroglycan is involved in the binding and aggregation of laminins and other ECM proteins, most notably agrin and perlecan, in a variety of cell types, including embryonic stem cells and embryoid bodies [71,72]{Li, 2002 #316}, myotubes [73-77] and Schwann cells [78], with genetic deletion of dystroglycan leading to reduced cell surface ligand binding and the inability of ligands to organize into aggregates and more complex ECM structures. The ability of

dystroglycan to bind and cluster its ligands is dependent on the proper glycosylation of its α subunit, as cell surface laminin binding and BM formation were similarly impaired in neural stem cells isolated from protein O-mannose N-acetylglucosaminyltransferase 1 (POMGnT1) KO mice [79]. Defective laminin binding and aggregation have knock-on effects; it has been shown that laminin assembly is required for the binding and BM incorporation of other integral ECM proteins, including collagen IV, nidogen-1 and perlecan [80] [79]. Furthermore, dystroglycan-mediated laminin aggregation is required for the establishment of cell polarity, and therefore proper tissue morphogenesis and function, in a number of epithelia, including those of the kidney, mammary gland, lung, salivary gland, and Drosophila ovary follicle and disc [81-86]. These findings raise the possibility that dystroglycan may have a similar function in neuroepithelial cells and their descendants, radial glial- and ependymal cells, which could have important implications for cortical development.

Epithelial cell polarization

The establishment of apical-basal polarity is a necessary prerequisite to epithelial morphogenesis and, in turn, to the generation of distinct tissues and organs. Intracellular asymmetry is achieved by the recruitment of lipids and cell-surface proteins, such as transporters, ion channels and pumps, to discrete membrane domains. The polarization of epithelial cells is initiated in response to spatial cues provided by the formation of adhesive contacts, both with other cells and between cells and the extracellular matrix. Upon cell contact, nascent adhesions are formed through interaction between the extracellular domains of nectins in adjacent epithelial cells. The nectin intracellular domain contains a binding site for activators of Rap1 or cdc42 (cell division control protein 42) GTPases, and recruits aPKC, PAR (partitioning defective) complex and Afadin adaptor proteins to the apical cell surface[87-90]. Together, this

complex forms a scaffold for the activation of Rap1, which results in the further recruitment of either cadherins or JAMs (junctional adhesion molecules) to form adherens- (AJs) or tight junctions (TJs), respectively [91-95].

Rap1 activation also results in the recruitment of cdc42 GEF to the apical membrane, resulting in cdc42 GTPase activation [96,97]. In this manner, the aPKC-cdc42-PAR-3-PAR-6 complex specifies and stabilizes the apical domain and triggers actin cytoskeleton-dependent domain maturation [98-103]). Downstream of PAR-3 and PAR-6, the threonine kinase PAR-1 (EMK1/MARK2) is excluded from the apical membrane by aPKC phosphorylation and regulates basolateral membrane domain maturation [104 105,106]. In a canine kidney epithelial cell line this process was shown to be mediated by the PAR-1b-dependent assembly and targeting of the dystroglycan complex, resulting in laminin binding at the basal cell surface and initial formation of the apical domain [81,107]. Indeed, many studies have found that dystroglycan mediates laminin binding at the basal surface of epithelial cells and contributes significantly to basement membrane assembly and, therefore, to epithelial polarization[108-111,71,72]. In contrast, β1-integrin is required for the expression of the laminin α1 subunit [112,113,22,114] but is less essential for basement membrane assembly and initial cell-surface laminin binding. Evidence from several cell lines suggests that epithelial cells further contribute to tissue morphogenesis through the dynamic regulation of extracellular laminin following initial dystroglycan-mediated basal surface ECM assembly [115,85,82]. The subsequent reorganization of laminin into more complex structures is generally integrin-dependent and is thought to be achieved by Rac1 acting downstream of PAR-1b to regulate cytoplasmic F-actin networks [107,116]. As the proper epithelial polarization is crucial for a number of processes relevant to cortical development and adult neurogenesis, so, too are functional interactions between neuroepithelial cells and the extracellular matrix.

Embryonic ventricular zone neurogenesis

Prior to the onset of cortical neurogenesis, the neural plate and neural tube are comprised of a single layer of neuroepithelial cells (NECs). NECs divide symmetrically to produce two daughter NECs, thereby expanding the progenitor pool in preparation for neurogenesis (McConnell 1995; Rakic 1995). Though NECs exhibit a columnar morphology, with processes extending from the luminal (apical) surface of the neuroepithelium to the basal lamina, their nuclei are found at various locations along the apico-basal axis, giving the impression of multiple cell layers [117-120]. For this reason, the neuroepithelium is referred to as "pseudostratified". Pseudostratification is the result of interkinetic nuclear migration (INM), an interesting property of NE cell division. While mitosis of NECs takes place at the apical surface of the neuroepithelium, NECs undergo S phase near the basal lamina. Therefore, NEC nuclei must shuttle back and forth, migrating basally during G1 and apically during G2 (Figure I-7) [9].

In basal-to-apical INM, the nucleus is shuttled along microtubules, mediated by minus-end-directed dynein [121-123]. Lis1, an adaptor protein that regulates microtubule dynamics in a variety of neural contexts[124-127], binds to dynein and dynactin, which link microtubules to the nucleus via nuclear envelope-associated proteins Syne2a and

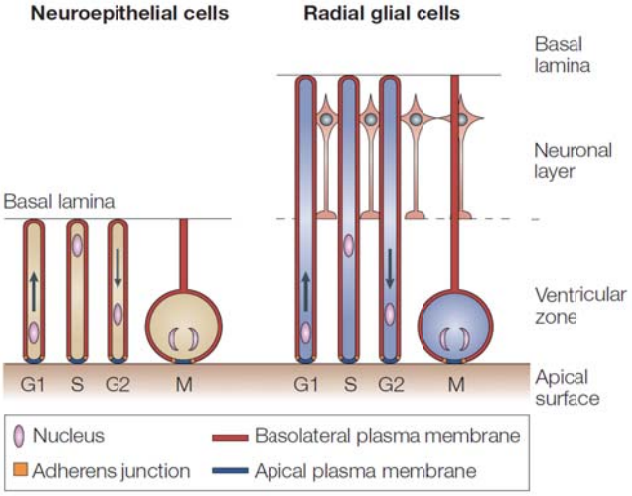


Figure I-7. Interkinetic nuclear migration in NECs and RGCs

NECs and RGCs are highly polarized, spanning the developing cortical plate. S phase nuclei reside in more basal locations and migrate apically during G2 to undergo mitosis at the apical surface. G1 nuclei then migrate basally to begin the next cycle. NEC INM traverses the entire neuroepithelium, whereas basal migration of RGC nuclei is restricted to the dorsal boundary of the ventricular/subventricular zone. Adapted from [9].

SUN1/2[122,128,129]. During mitosis, the apical process is retracted but the basal process remains attached to the pial BL and is split evenly between daughter cells [130,131]; [132]; [133]. In contrast, apical-to-basal INM requires actomyosin contractility, with nuclear transport mediated by directional myosin-II-dependent constriction [134].

The exact function that INM serves is unclear; INM is not required for cell cycle progression in NE cells, as interrupting INM through treatment with cytochalasin (to inhibit F-actin polymerization)[135-137] or blebbistatin (myosin II inhibitor) [134] leads to mislocalized mitosis but not cell cycle arrest. On the other hand, cell cycle progression *is* required for nuclear migration; pharmacological agents that cause S phase arrest block basal-to-apical INM, while those that block G2/M transition prevent apical-to-basal INM [117] [138]. There is some evidence to suggest that INM may serve a more practical purpose in preventing the overcrowding of NE cell bodies at the ventricular surface [139].

At approximately E13 in the mouse, NECs transform into radial glial cells (RGCs), signaling the onset of embryonic cortical neurogenesis. During this transition RGCs acquire characteristics generally associated with the astrocytic lineage, such as the expression of Nestin [140], BLBP (brain lipid-binding protein)[141], GLAST (glutamatergic astrocyte-specific transporter) [142]and Vimentin [143]. A series of genetic fate mapping experiments have led to the widespread acceptance that RGCs act as neural stem cells in the embryonic and early postnatal telencephalon, capable of producing all neuronal and glial subtypes [144,141,145-147,132,148]. Like their neuroepithelial predecessors, RGCs undergo INM, though the basal migration of RGC nuclei is restricted to the dorsal boundary of the subventricular zone (Figure I-3). RGCs display two modes of division: symmetric (proliferative) divisions produce two RGCs and serve to maintain the neural stem cell pool, while asymmetric (neurogenic) divisions produce one RGCs and one

intermediate progenitor cell. As the developing cortical plate expands, newly produced neuroblasts remain associated with the mother RGC, using the basal process as a scaffold to assist in their dorsal migration.

The precise manner in which the balance between symmetric and asymmetric divisions is maintained is not fully understood, but several important factors have been identified. Generally speaking, in polarized cells the plane of cell division dictates the inheritance of fate determinants by daughter cells; division planes perpendicular (vertical) to the apical (ventricular) surface result in the equal segregation of fate determinants (symmetric division) while oblique or parallel planes (horizontal) result in the inheritance of fate determinants by only one daughter cell (asymmetric division) [149-151]. Although there is a strong correlation between absolute division angle and daughter cell fate in *Drosophila*, this is not always the case in the developing mammalian cortex, where fate is also influenced by progenitor cell type and developmental stage. For example, RGCs typically exhibit a vertical cleavage plane, yet undergo both symmetric and asymmetric divisions [152]. A more reliable metric appears to be the differential partitioning of adherens junction domains. NECs are separated by tight junctions that prevent interactions between the apical domains of neighboring cells. As NECs transition into RGCs, apical tight junctions are replaced by adherens junctions, which anchor RGC apical endfeet to the ventricular surface and each other [153,154]. The formation of adherens junctions then triggers the recruitment of aPKC and Par-complex proteins to the junctional domain (as described above). In addition to their polarizing functions, aPKC-Par-3-Par-6 and adherens junctional complexes may also have a role in cell fate determination. They have been shown to consistently partition with the apical membrane during RGC division such that symmetric RGC divisions partition AJ and Par complexes equally, while asymmetric divisions result in their acquisition by

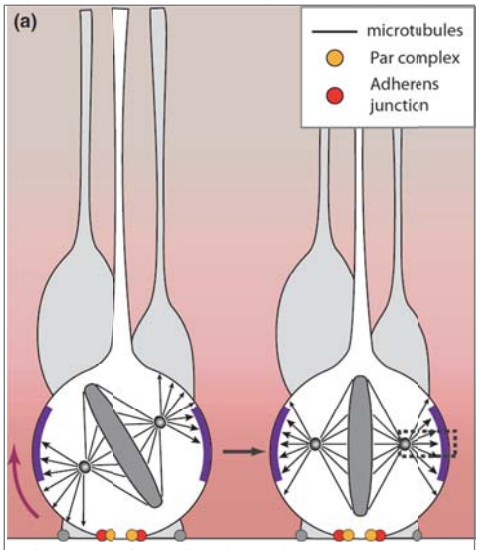


Figure I-8. Mitotic spindle orientation dictates the inheritance of fate determinants

The mitotic spindle is oriented randomly at the start of M-phase and is then rotated by the LGN complex acting on astral microtubules. Spindle angles that lead to the inheritance of adherens junctional and Par complexes by only one cell result in asymmetric divisions (left), with the inheriting cell retaining RGC fate. Angles that divide these junctional complexes equally result in symmetric divisions (right). Adapted from [3]

only one daughter cell, leading to the retention of RGC fate by that cell (Figure I-8)[155]. Loss- and gain- of function experiments also suggest a more active role in the regulation of RGC division mode, finding that overexpression of Par-3 and Par-6 promoted self-renewing divisions while Par-3 knockdown led to premature cell cycle exit and neuronal differentiation[156].

The plane of cell division is determined by the orientation of the mitotic spindle, which requires the correct positioning and stability of centrosomes and microtubules (reviewed in [157]). The mitotic spindle consists of kinetochore microtubules, which connect the chromosomes to the centrosomes, and astral microtubules, which connect the centrosomes to the cell cortex. The scaffold proteins Lis1 and Nde1, which have been implicated in microtubule dynamics in a variety of neurogenic contexts, have been shown to regulate mitotic spindle assembly and orientation in mouse RGCs [127,125,158,159]. Through interactions between Nde1 and dystroglycan, the Lis1-Nde1 complex stabilizes the dystrophin-glycoprotein complex (DGC) at the lateral membrane [124]. Together, the Lis1-Nde1-DGC complex anchors the astral microtubules to the cell cortex and links microtubule-associated structures to the actin cytoskeleton and, consequently, to the ECM. Lis1-Nde1 loss-of-function led to the dispersal of dystroglycan from the lateral membrane and the loss of basolateral adhesions and membrane integrity, as well as profound disturbances in

RGC morphology. Mitotic spindle orientation in Lis1-Nde1-deficient RGCs was randomized, leading to an increase in asymmetric divisions. Both Lis1-Nde1 and dystroglycan-deficient mouse models recapitulate many aspects of human cortical lissencephaly (discussed in greater detail below), suggesting that the defects in cortical development observed in dystroglycan-deficient mouse models and patients with dystroglycanopathies are not restricted to those resulting from the loss of RGC attachment to the pial basement membrane.

Dystroglycan expression during cortical development

From E10, dystroglycan is found at the basal endfeet of embryonic radial glia, where it mediates their attachment to the pial basement membrane [160-162]. The loss of radial glial attachments is thought to underlie neuronal migration defects often observed in the "dystroglycanopathies", a subset of congenital muscular dystrophies arising from defective dystroglycan glycosylation (more below) [160,41]. Mouse models in which dystroglycan is conditionally deleted from neural cells recapitulate many aspects of the developmental brain defects observed in the dystroglycanopathies, including severe neuronal migration defects [160,41]. This phenotype has been shown to be a result of the loss of dystroglycan in glial cells, as cortical lamination defects were not observed in mice with neuron-specific deletion of dystroglycan [41].

During early corticogenesis, when the majority of cell divisions are symmetric (from approximately E10.5 to E13.5), dystroglycan is expressed along the entire basolateral surface of both NECs and RGCs, as well as in their apical processes and the apically retracted cell bodies of metaphase progenitors [162,161,124,41]. Reports differ regarding the expression of dystroglycan in RGCs as neurogenesis peaks (approximately E13.5- late embryonic period). Lathia et al. (2007) observed continued high expression of α-dystroglycan (clone VIA4-1) in the VZ throughout late embryonic development. Another group found that α-dystroglycan (clone IIH6C4) levels

dropped in the VZ at the onset of neurogenesis, becoming largely restricted to RGC basal endfeet by E15.5 [124]. Myshrall et al. (2012) also found β-dystroglycan protein expression limited to pial endfeet at E15.5, despite high levels of Dag1 mRNA expression in the VZ at the same timepoint. These conflicting results are likely due to differences in immunohistochemical protocol but could reflect real phenomena, such as the differential glycosylation of α-dystroglycan in different cortical regions. Immunohistochemical evidence suggests that dystroglycan may also play a role in embryonic neurogenesis; at E12, dystroglycan is upregulated in the ventricular zone (VZ), particularly concentrated in the apical processes of NSCs, where it remains highly expressed throughout late embryonic development. At E15, dystroglycan is also found at high levels in the neuron-containing region of the cortical plate [160,162,161].

In the adult cortex, dystroglycan continues to be expressed in neurons, particularly pyramidal cells in layers II-VI [163]. However, the best understood function of dystroglycan in the adult brain is in the maintenance of the blood-brain barrier. Dystroglycan is expressed in vascular endothelial cells and the perivascular endfeet of cortical astrocytes, where it mediates their adhesion to blood vessel basal lamina and regulates the polarized expression of Kir 4.1 and aquaporin-4 [41,164-167]. Preliminary evidence suggests that, through similar mechanisms, dystroglycan also contributes to the establishment of the blood-brain-barrier during postnatal development (Michael Menezes, unpublished data). In the adult SVZ, β-dystroglycan 'globules' have been observed at the basal surface of ependymal cells, where it was suggested that these structures may serve to tether fractones to the ependymal layer [168]. These and other studies have established dystroglycan as a critical regulator of cortical histogenesis. However, despite its observed expression and temporal regulation within the VZ/SVZ, it remains unknown whether dystroglycan has further roles in cortical development.

The adult VZ/SVZ neural stem cell niche

As neurogenesis comes to an end in the perinatal period, RGCs retract their basal processes and begin to mature. Most RGCs detach from the niche and undergo terminal differentiation to become protoplasmic astrocytes [169]. A subset of transitional RGCs remain associated with the adult SVZ, either transforming into slowly dividing adult neural stem cells (B cells) [170,171] or differentiating to form ependymal cells [172]. B cells retain the polarized morphology of their RGC predecessors, with an apical process that contacts the ventricle and a basal process that adheres to the basement membrane surrounding SVZ blood vessels [173,10 174,175], and exhibit INM-like nuclear movement during mitosis. In the first postnatal week, B cells and immature ependymal cells organize into "pinwheel" formations at the ventricular surface (Figure I-9).

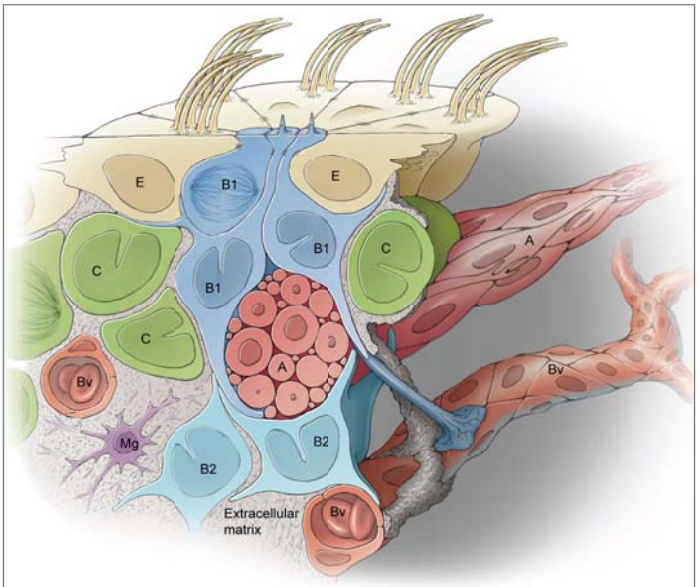


Figure I-9. Structural organization of the adult VZ/SVZ stem cell niche

At the ventricular surface (top), multiciliated ependymal cells (E) encircle the apical processes of B1 neural stem cells in a pinwheel formation. B1 cell basal processes and intermediate progenitor (C) cells make contact with the SVZ vasculature (Bv) and surround chains of migratory neuroblasts (A cells). Extracellular matrix structures (grey) emanate from the blood vessel basal lamina to contact all SVZ cell types [8].

Pinwheels are comprised of a ring of ependymal cells surrounding the apical processes of one or more B cells. B cells, like RGCs, interact with each other via adherens junctions and form adherens and tight junctions with neighboring ependymal cells [10,176]. Pinwheel structures are associated with neurogenic regions of the SVZ [10], suggesting that contact with the ventricle and/or adjacent ependymal cells plays some role in the regulation of B cell

neurogenic potential. One possibility is the reciprocal modulation of BMP (bone morphogenic protein) signaling amongst ependymal niche cells. B- and C cells express BMP2 and BMP4, as well as their receptors [177]; Peretto 2004). The addition of exogenous BMP4 to brain tumor stem cells resulted in increased differentiation at the expense of self-renewal and neurosphere production (Piccirello 2006). Ependymal cells participate in the local regulation of BMP signaling by secreting the BMP inhibitor Noggin, which has been shown to reduce neurogenesis and promote oligogliogenesis in the adult SVZ [178]. As B cells also express Noggin (Peretto 2004), it appears that the fine regulation of BMP signaling in the ependymal niche may support the balance between the maintenance of stem cell characteristics and the continued neurogenic potential of adult VZ progenitors. Additional support for this theory is provided by the discovery that upregulation of the adapter protein Ankyrin-3 by developing ependymal cells, downstream of the ependymal cell-specific transcription factor Foxj1, is required for the assembly of a structurally intact ependymal niche through NSC/ependymal lateral adhesion [179]. Deletion of this pathway in the established niche resulted in greatly reduced the neurogenic capacity of SVZ progenitors. As Ank3 is required for the appropriate localization of dystroglycan in skeletal muscle [180] and the mislocalization of dystroglycan led to disrupted RGC lateral contacts [124], it is possible that dystroglycan may play a similar role in the structural development of the ependymal niche.

Although ependymal cell fate is specified in RGCs between E14 and E16, their maturation is delayed until early in the first postnatal week [172]. In earlier studies of SVZ cell lineage relationships, the lack of cell type-specific markers led to the belief that ependymal cells retained neurogenic potential after differentiation [181-184]. However, modern lineage-tracing experiments and improved imaging techniques have established that mature ependymal cells are

post-mitotic under normal circumstances [185-188,172,170,189]. Ependymal cell motile cilia exhibit planar cell polarity (PCP), a quality that is established in the primary cilium of their RGC precursors. Ependymal cell cilia also express PDGFRα and EGFR [176], though the source of their endogenous ligands is not clear.

The extracellular matrix (ECM) has recently been identified as a potential regulator of cell proliferation in the adult SVZ niche. The SVZ is highly vascularized relative to other cortical regions and contains extra-vascular extracellular matrix structures unique to the SVZ. In particular, spindle-like highly branched "fractones" emanate from blood vessels to contact all SVZ cell types, although actively dividing NSCs/NPCs exhibit a particularly close association with these structures [175,174]. Fractones contain the heparan sulfate proteoglycan perlecan, which can trap and activate FGF-2, enhancing FGF-2-mediated stimulation of SVZ cell proliferation [190-192]. The laminin receptor α6β1 integrin has been found to mediate the adhesion of mitotically active B and C cells to the vasculature, an interaction that has been implicated in maintaining the balance between proliferation and quiescence. It was demonstrated that transiently disrupting the laminin binding capability of α6β1 integrin caused B/C cells to detach from blood vessels, leading to aberrant proliferation and, ultimately, precocious neuronal differentiation [175]. A recent study from the same group [193] clarified that the expression of α6β1 integrin *precedes* the mitotic activation of SVZ progenitors downstream of the SDF-1 (stromal-derived factor 1)/CXCR-4 (CXC chemokine receptor 4) signaling pathway. B and C cells home to blood vessels in response to SDF-1 secreted by vascular endothelial cells. SDF-1 upregulates EGFR and α6β1 integrin in SVZ progenitors, resulting in heightened mitotic activation and increased laminin-binding capability. Laminin-containing ECM aggregates observed near the ventricle surface, on the other hand, remain of unknown function [175]. Despite the recent attention the specialized ECM of the

adult VZ/SVZ has garnered, it remains entirely unknown whether ECM acts during postnatal development to regulate either the assembly of the adult SVZ niche structure or the concurrent process of SVZ gliogenesis. It is also not clear if extracellular matrix ligand/receptor interactions have any direct role on the regulation of SVZ niche cell behavior or if their function is purely adhesive, holding niche cells in place to optimize their exposure to other regulatory factors.

Postnatal subventricular zone oligogliogenesis

In the embryonic forebrain, the lateral and medial ganglionic eminences produce two waves of oligodendrocyte progenitor cells (OPCs), commencing at approximately E11.5 in the mouse [194]. These early OPCs migrate into all areas of the forebrain, including the developing cortex. However, most of these cells die prior to the first postnatal week and thus do not contribute substantially to the adult glial pool or to cortical myelination. During the perinatal period, the neurogenic capacity of SVZ progenitors declines and they begin to produce intermediate progenitors that are largely restricted to the astrocyte and oligodendrocyte (OL) lineages.

Two families of transcription factors are particularly important for oligodendroglial development; bHLH proteins Olig1 and Olig2 [195-200] and SoxE proteins (Sox8-10) [201-207]. Olig2 is a master regulator of all stages of oligodendrocyte lineage progression and is necessary and sufficient for oIPC specification. Olig1 is dispensable for fate induction but has a minor role in oligodendrocyte (OL) maturation and myelination [208]. Newly produced oIPCs downregulate Sox1-3 and begin to express Sox8-10. All SoxE proteins promote OL specification and differentiation [201-204,206] but Sox10 has an additional role, in conjunction with Olig1, in promoting MBP expression in mature OLs [205]. Sox 10 expression inhibits the transcription of Sufu (Suppressor of Fused) [206], which regulates several morphogenic signaling pathways [209-211]

and may also activate PDGFRα [212]. Other transcription factors like Mash1/Ascl1 and Dlx1 and 2 contribute to, respectively, the enhancement and inhibition of oligogliogenesis [200,213]. Newly produced oligodendrogenic progenitor cells (oIPCs) can be identified by the expression of OL lineage-specific markers such as Olig2, Sox10 and PDGFRα in combination with the residual expression of the stem/progenitor cell marker Sox2 (Figure I-10). Shortly after exiting the SVZ, oligodendrocyte progenitor cells (OPCs) begin to express the chondroitin sulfate proteoglycan NG2.

The Wnt signaling pathway is a major extrinsic regulator of the neurogenic-gliogenic switch in the postnatal SVZ. Wnts are secreted glycoproteins with known roles in the regulation of progenitor cell proliferation and differentiation [214 215] and, more specifically, in the repression of oligodendroglial fate [216-218] and differentiation [219,220]. Wnt signaling is very high in SVZ progenitors during late embryonic neurogenesis but decreases during the perinatal and early postnatal period, coinciding with the onset of gliogenesis. Inhibition of Wnt signaling in the embryonic SVZ results in the premature production of OPCs, suggesting that the Wnt pathway regulates the timing of early postnatal oligodendrogenesis [218], perhaps through transcriptional regulation of Olig2 [217]. Shh also contributes to the initial stages of oligogliogenesis by increasing the expression of Olig2 and PDGFRα [221]. Notch signaling has been shown to promote oligodendroglial fate during gliogenic periods [222,223], with constitutive activation leading to the overproduction of OPCs [224]. Importantly, though Notch signaling promotes gliogenesis, it simultaneously acts to inhibit OL terminal differentiation and myelination [225]. PDGF [226,227], FGF [228,229] and EGF [230,231] signaling pathways all have potent morphogenic effects on oligodendroglial specification and proliferation. However, like Notch, FGF and PDGF have

antagonistic effects on subsequent OL differentiation and myelination, both separately and by FGF-dependent maintenance of PDGFRα expression in OPCs [232-234,229].

Newly born OPCs begin to proliferate and leave the niche, migrating radially and tangentially to colonize the cortical plate and subcortical white matter tracts like the corpus callosum. Collosal OPCs then begin to differentiate, signaled by the expression of mature OL markers like CNPase and APC (clone CC1), and extend elaborate processes to make contact with axons. Post-mitotic OLs that have successfully made axon contact begin to synthesize myelin components, including MBP, and initiate the wrapping process.

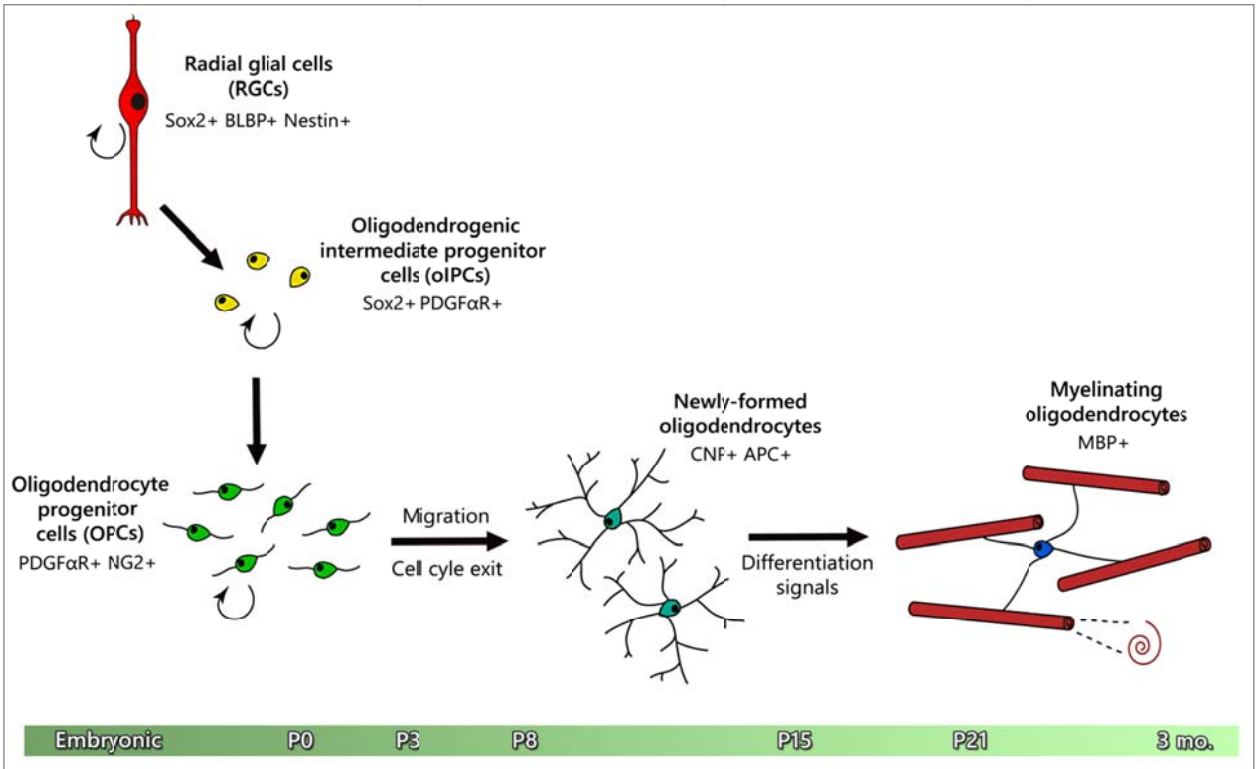


Figure I-10. Oligodendrocyte lineage progression
Oligodendrocyte precursor cells are derived from early postnatal radial glia and adult B cells, via C (transit amplifying) cells, in the subventricular zone. Green cells: intermediate progenitors; blue cells: neural stem cells. Protein markers commonly used to identify each glial cell type are also shown. [6]

Dystroglycan in oligodendrocyte development

Unlike Schwann cells, the myelinating glia of the peripheral nervous system, oligodendrocytes lack a basal lamina[235]. Nevertheless, OLs express the laminin receptors α6β1 integrin and dystroglycan[236-238], and interactions with the ECM have been found to influence various aspects of oligodendrocyte maturation. In some white matter tracts in the CNS, axon-associated laminin a2 expression increases just before the start of myelination[239] and the use of laminin as a substrate *in vitro* has been shown to support OL survival and myelin membrane production[239,237,240]. While integrin supports OL survival[241,238], its loss does not impact the myelination of CNS axons[242]. Dystroglycan, however, has been shown to promote laminin-mediated OL differentiation and the production of myelin components by potentiating IGF-1 signaling [238,243]. Dystroglycan also supports OL maturation by localizing to focal adhesions in OL filopodia, where it modulates cytoskeletal remodeling to promote filopodial outgrowth and the extension of elaborate processes [244], elements that are critical for successful axon contact and myelination. It remains unknown, however, if dystroglycan functions to promote earlier events in the oligodendrocyte lineage, a question that I seek to address in this dissertation.

Potential roles for dystroglycan in the early postnatal gliogenic SVZ

A common thread running through various aspects of the regulation of neural stem cell proliferation and the fate of resulting progenitors is the establishment and maintenance of apicobasal polarity. For example, if interkinetic nuclear migration, and therefore neural stem cell division, is to proceed correctly there are two main (and overlapping) requirements: radial morphology and a polarized cytoskeleton. The concept of polarity is relative; it requires a landmark. That cue is provided by the extracellular matrix. Dystroglycan is known to be responsible for the proper aggregation and membrane localization of ECM proteins in a variety

of epithelial cell types and is involved in orchestrating the cellular response to changes in the ECM, including apical domain remodeling and the re-establishment of apico-basal polarity. Intracellularly, dystroglycan loss has been shown to result in mislocalization of key polarity proteins and the subsequent loss of adherens junctions. As the segregation of the apical domain during asymmetric cell divisions dictates the fate of daughter cells, improper localization of apical proteins could result in alterations to the cellular output of the niche. Therefore, it seems reasonable to hypothesize that in the postnatal SVZ, where ECM is both ubiquitous and undergoing remodeling to produce complex adult SVZ ECM structures, dystroglycan may play an important role in ECM assembly, establishing niche cell-ECM interactions, repolarizing cells relative to ECM contacts and regulating the production of oligogliogenic progenitors.

In its role as a regulator of actin cytoskeleton and microtubule dynamics, dystroglycan is necessary for the cytoskeletal modifications required for formation of filipodia and microvilli, which could impact intercellular communication between transitional postnatal radial glia and other niche cell types. Furthermore, defective filopodia formation as a result of dystroglycan loss could also impact the ability of postnatal radial glial cells and their progeny to reorganize into the adult SVZ niche configuration. As the cytoarchitecture of the adult SVZ is thought to be important for regulating the proliferation and neuro-/gliogenic capacity of niche cells, structural disturbances could have further implications for the functional integrity of the niche and for the subsequent development of oligodendroglial cells produced during the early postnatal period.

In conclusion, over the course of my dissertation research I have sought to address the hypothesis that dystroglycan acts in the developing postnatal SVZ to regulate 1. the structural and functional development of the neural stem cell niche and 2. the production of appropriate

numbers of oligodendrogenic progenitor cells and their maturation into mature, myelinating oligodendrocytes.

CHAPTER II: DYSTROGLYCAN REGULATES LAMININ REMODELING AND PROMOTES EPENDYMAL NICHE MATURATION IN THE EARLY POSTNATAL VZ/SVZ

INTRODUCTION

Radial glial neural stem cells exhibit an extreme bipolar morphology, with processes that span the developing cortical plate, terminating apically at the surface of the lateral ventricle and basally at the pial basement membrane. Shortly after birth, radial glia detach from the pial surface and transform into adult neural stem cells (B cells) and multiciliated ependymal cells, which then arrange into "pinwheels", rings of ependymal cells that surround B cell apical processes [10]. Stem- and ependymal cell pinwheels, together with transit amplifying neural progenitors (C cells) and neuroblasts (A cells), comprise the adult ventricular/subventricular zone (VZ/SVZ), a specialized microenvironment that supports stem cell quiescence and the controlled production of neurons and glia. Intriguingly, neural stem and progenitor cells undergo the principal wave of dorsal gliogenesis during this postnatal transitional period. The process of VZ/SVZ postnatal niche construction and its correct coupling with early postnatal gliogenesis are likely critical to proper brain development, yet the factors regulating these processes remain poorly understood.

The extracellular matrix (ECM) has recently been identified as a regulator of cell proliferation in the adult SVZ niche. Like their radial glial predecessors, adult B cell basal processes remain associated with ECM through adhesion to the laminin-rich basal lamina of the SVZ vascular network [10,175,174]. Activated B cells and transit-amplifying neural progenitors

express higher levels of the laminin receptor α6β1 integrin than more lineage-restricted cells, and blocking this integrin impairs the adhesion of B/C cells to the vasculature, resulting in aberrant proliferation [245,175]. In addition to the vascular basal lamina, the adult SVZ has a unique extra-vascular ECM organization, featuring ECM aggregates at or near the ventricular surface as well as "fractones"; thin, highly-branched ECM structures that appear to emanate from the vascular basal lamina. Actively proliferating stem cells have been observed in contact with fractone ECM, where the heparan sulfate proteoglycan perlecan can trap and activate FGF-2, enhancing FGF-2-mediated stimulation of SVZ cell proliferation [190,191]. Laminin-containing ECM aggregates near the ventricle surface, on the other hand, remain of unknown function. Despite the recent attention the specialized ECM of the adult VZ/SVZ has garnered, it remains entirely unknown whether ECM acts during postnatal development to regulate either the assembly of the adult SVZ niche structure.

Dystroglycan is a transmembrane ECM receptor known to mediate cell interactions with a variety of ECM ligands, including laminins. Dystroglycan is best known as a member of the dystrophin-glycoprotein complex (DGC), linking ECM with the actin cytoskeleton of skeletal myocytes. However, dystroglycan also participates in intracellular signal transduction through its interactions with a variety of signaling effectors [43]. In the developing brain, dystroglycan is found on the basal endfeet of embryonic radial glia, and is required for their attachment to the pial basement membrane [246]. Thus the loss of radial glial attachments is thought to underlie neuronal migration defects observed in the "dystroglycanopathies"; a subset of congenital muscular dystrophies arising from defective dystroglycan glycosylation [160]. In the adult brain, dystroglycan found on the perivascular endfeet of astrocytes, where it mediates their adhesion to the vascular basal lamina at the blood-brain barrier and regulates the polarized expression of Kir

4.1 and aquaporin-4 [164-167]. However, whether dystroglycan regulates postnatal brain development or participates in the development or function of the postnatal VZ/SVZ neural stem cell niche remains unknown.

Here, we identified the ECM receptor dystroglycan as a novel and critical regulator of adult SVZ niche development. Using genetic and antibody blocking approaches *in vitro* and *in vivo*, we demonstrate that dystroglycan regulates neural stem and progenitor cell proliferation, promotes radial glial and ependymal cell maturation, and is required for ependymal niche pinwheel formation in the early postnatal VZ/SVZ.

RESULTS

Laminin organizes into niche hubs and tethers during early postnatal VZ/SVZ niche assembly

Three-dimensional imaging of VZ/SVZ architecture, facilitated by confocal microscopy of lateral wall whole mount preparations, has illuminated the complex spatial arrangement of extracellular matrix proteins in the adult neural stem cell niche, both in the basal lamina ensheathing the dense SVZ vascular network and in extra-vascular ECM structures unique to the SVZ [175,174]. However, the spatiotemporal expression and role of ECM in the developing postnatal VZ/SVZ is unknown. To similarly visualize ECM structures in the early postnatal VZ/SVZ, I performed IHC to detect laminin in whole mounts from wild type mice. At P0, a complex SVZ vascular plexus was already in place, denser but otherwise largely resembling that seen in adult mice (Figure II-1A). Fractones, previously described in the adult SVZ as spindle-like ECM structures that project from the vascular basal lamina [190], were also clearly visible from birth (Figure II-1B). Between P3 and P8, laminin-rich aggregates began to appear at the ventricular surface (z-plane inset, bottom panels Figure II-1B).

To more carefully assess the spatiotemporal organization of laminin in the VZ, I performed IHC against laminin and β-catenin (to visualize adherens junctions) in whole mounts from wild type mice. At P3, en face views revealed ventricular surface laminin concentrated around putative immature ependymal cells, which have a ventricle surface area that is several-fold larger than radial glia or type B stem cells (Figure II-1C). At P8, large laminin aggregates, or "hubs" appeared to coalesce on the ventricular surface of these laminin-positive cells, concurrent with an overall decrease in more generalized ependymal cell-associated laminin (Figure II-1C, arrowheads). By P21, general cell-associated laminin expression was greatly diminished, leaving ventricular surface laminin largely restricted to hubs. Most laminin hubs remained associated with ependymal cells (Figure II-1C, arrowheads) and some were found at the center of pinwheels at the interface between ependymal cells and type B stem cells (Figure II-1C, arrows). Three-dimensional reconstructions of confocal stacks helped to clarify the spatial relationships amongst extra-vascular ECM structures and between those structures and the underlying vascular basal lamina (Figure II-1D). At P3, I found that ventricular surface cell-associated laminin was contiguous with the vascular basal lamina, either directly (Figure II-1D, arrowheads) or via "tethers" (Figure II-1D, arrows). 3D reconstruction of VZ/SVZ laminin structures at P8 allowed me to discern two classes of laminin aggregates; laminin hubs visible at the ventricular surface, generally localized toward the center of laminin-positive cells (Figure II-1E, arrowheads) and "bulbs" found beneath the developing ependymal cell layer, often associated with fractone termini (Figure II-1E, arrow).

To confirm that the laminin-positive cells we observed were, indeed, ependymal cells, I performed IHC to detect laminin and GFP in whole mounts from FoxJ1-GFP mice. FoxJ1 is a transcription factor involved in ciliogenesis that is expressed early in ependymal cell

development. En face views and 3D reconstructions of GFP and laminin at postnatal days 3 and 8 (Figure II-1F) confirmed that (i) cell-associated laminin was almost exclusively restricted to immature ependymal cells, and that (ii) laminin tethers served as a bridge, connecting immature ependymal cells to the vascular basal lamina during niche cellular reorganization. At postnatal day 21, when pinwheel arrangement was nearly complete, most laminin hubs remained central to ependymal cells (Figure II-1G, arrow), whereas some had relocated to the center of pinwheels (Figure II-1G, closed arrowhead) and others appeared to be in transit, protruding into the center of a pinwheel while retaining attachment to an ependymal cell (Figure II-1G, open arrowhead). To determine the final positioning of laminin hubs relative to adult B cells in pinwheels, I performed IHC against laminin, GFAP and GFP in whole mounts from adult FoxJ1-GFP mice. I found that virtually all pinwheels contained one or more laminin hubs, which were localized to the interface between ependymal cells and B cell apical processes (Figure II-1H). Taken together, these observations suggest that extra-vascular ECM is developmentally regulated during SVZ niche construction and displays a high level of structural diversity. I found that in the developing VZ/SVZ, laminin was concentrated around immature ependymal cells and became more restricted with niche maturation to achieve a final configuration of discrete laminin hubs at the interfaces between type B stem cells and ependymal cells.

Dystroglycan regulates laminin restructuring in the developing ependymal niche

The association between laminin and immature ependymal cells during niche development (Figure II-1C, F), as well as the association among laminin hubs, ependymal cells, and type B stem cells in mature niche pinwheels (Figure II-1H), suggests that laminin-receptor interactions may regulate the transformation of RGCs into adult NSCs and ependymal cells or other aspects of niche construction. While α6β1 integrin has been found to mediate laminin

interactions in the embryonic cortex and adult SVZ niche [245,175,247], it is dystroglycan, another laminin receptor expressed in the brain, that is required for initial laminin clustering in response to changes in the extracellular environment in a variety of cell types [71,72,75,116,248,82]. To investigate the role of dystroglycan in ependymal cell development and niche construction, I deleted dystroglycan from neural cells using *nestin-cre;DAG$^{Flox/Flox}$* (DAG cKO) mice (Figure II-2A) and compared them to *nestin-cre$^{-/-}$;DAG$^{Flox/Flox}$* (WT) littermates. Previous characterization of *nestin-cre;DAG$^{Flox/Flox}$* mice determined that *DAG1* recombination occurs between E9.5 and E18.5, with β-dystroglycan immunoreactivity absent in the brain from E13.5 onward[249]. I confirmed appropriate removal of dystroglycan protein in DAG cKOs by evaluating western blots of protein lysates generated using either neurospheres or cerebral cortex, as well as dystroglycan IHC (Figure II-2B, C). The DAG cKO cortex recapitulates many aspects of the cortical abnormalities in human disorders related to dystroglycan hypoglycosylation (Figure II-2D,E), including disordered cortical layering, neuronal overmigration and ectopias (arrowhead in D) and hydrocephaly (E).

First, to determine if loss of dystroglycan impacted laminin levels in the early postnatal SVZ, I performed IHC to detect laminin and nestin in coronal sections from P0 mice (Figure II-3A). In WT mice, the apical processes of nestin+ radial glia (RGCs) made contact with laminin-positive puncta at the ventricular surface. However, it appeared that there were fewer laminin puncta in the VZ of DAG cKO mice, with those present appearing smaller than in WT littermates. As a result, the association of RGC apical processes with laminin puncta was largely diminished in the dystroglycan-deficient SVZ. IHC analysis of BLBP+ RGCs in coronal sections from WT and DAG cKO mice revealed no change in RGC density or signs of apical detachment ($45.1 \pm 4.21 \times 10^5$ cells/mm^3 vs. $44.6 \pm 2.28 \times 10^5$ in WT) (Figure II-3B,C) at birth, suggesting

that apical interaction with ventricular surface laminin aggregates is not required for the retention of RGCs in the perinatal niche. In contrast, laminin expression in the vascular basal lamina appeared normal and contacts between RGC basal processes and blood vessels did not appear to be impacted by dystroglycan loss.

To more closely examine the relationship between laminin and dystroglycan in the VZ, I next assessed laminin and dystroglycan expression in en face views of the ventricular surface from SVZ whole mounts at various stages of early postnatal development. In the newborn (P0) wildtype VZ, laminin and dystroglycan were localized mainly to radial glial adherens junctions (Figure II-4A). By postnatal day 3, emerging ependymal cells, identified by their large apical surface areas, had increased levels of cell surface-associated laminin and dystroglycan. By postnatal day 8, cell-associated laminin was restricted to a subset of cells and dystroglycan immunoreactivity was even more limited, with laminin- and dystroglycan-positive hubs now found at the ventricular surface.

The conditional removal of dystroglycan expression from neural cells led to the delayed recruitment of VZ cell-associated laminin (Figure II-4B). From an en face perspective, at postnatal day 0 the morphology and density of radial glial cell apical surfaces appeared normal in DAG cKO mice, though laminin immunoreactivity appeared diminished from that in wild type. By P3, developing ependymal cells were now readily apparent in en face views from SVZ whole mounts from both WT and DAG cKO mice, but here the dystroglycan-deficient VZ had a marked decrease in pericellular laminin associated with developing ependymal cells. However, by P8, cells at the ventricular surface of DAG cKO mice exhibited a massive *increase* in cell-associated laminin, surpassing levels seen in the VZ of wildtype littermates. These data indicate

that laminin recruitment and/or retention at ependymal cell surfaces is abnormal in the absence of dystroglycan.

I next turned to an ependymal cell culture approach to more carefully examine dystroglycan and laminin during ependymal cell maturation. I isolated SVZ cells from newborn FoxJ1-GFP mice (wild type for dystroglycan) and plated them on PDL, ensuring that the only ECM in the system would be that produced by the cells in culture (Figure II-5A). Following 7 days of ependymal cell differentiation, dystroglycan IHC was performed in conjunction with CD24 (to visualize ependymal cells) and acetylated α-tubulin IHC (to visualize cilia in mature ependymal cells) (Figure II-5B). Recapitulating my *in vivo* observations, I found that dystroglycan expression was tightly correlated to ependymal cell maturation and was highest in mature, multiciliated ependymal cells relative to non-ependymal cells or immature ependymal cells that were not yet multiciliated (Figure II-5C). In a separate set of cultures, radial glial cells were allowed to proliferate normally for 5 days, with dystroglycan blocking or IgM control antibodies added during the differentiation stage (7 days). The neonatal SVZ cultures produced thin, fibrillary laminin- and perlecan-containing structures, which appeared less abundant and disorganized in the presence of dystroglycan-blocking antibodies (Figure II-5D,E).

Dystroglycan is required for ependymal cell maturation and niche assembly

An early step in SVZ stem cell niche construction is the transformation of radial glia into adult NSCs and ependymal cells. As extracellular matrix interactions have been implicated in the regulation of NPC proliferation and differentiation in the adult niche [174,175,250,192], I wondered if extracellular matrix interactions might also regulate the controlled maturation of RGCs into ependymal cells. As mentioned above, DAG cKO mice had normal numbers of radial glia at birth (Figure II-3C). However, at P3, DAG cKO mice had fewer CD24+ ependymal cells than

their wildtype littermates (3.01 ± 0.23 x10^3 cells/mm^2 vs. 4.82 ± 0.34 x10^3 in WT) (Figures II-6A,B). At the same time, DAG cKO mice had more BLBP+ radial glia than did WT littermates (44.2 ± 2.16 x10^5 cells/mm^3 vs. 38.2 ± 2.05 x10^5 in WT) (Figure II-6C,D) and, indeed, the levels of BLBP+ cells in DAG cKO mice had not changed from birth, whereas they had decreased in WT mice (Figures II-3C and 6D). Together, my findings of decreased ependymal cell densities concurrent with elevated radial glial cell densities are indicative of a delayed transformation of radial glia into ependymal cells. I also observed delayed ependymal cell maturation through the third postnatal week, with significantly lower densities of multiciliated ependymal cells observed both at P8 (39.8% ± 2.3% of total area vs. 62.0% ± 3.1% in WT) (Figure II-7B) and P21 (41.5% ± 3.0% of total area vs. 61.8% ± 5.0% in WT) (Figure II-7E). Dystroglycan loss further impacted the ability of ependymal cells to arrange into pinwheels. At P8 (Figure II-7C) and P21 (Figure II-7F), dystroglycan-null ependymal-NSC clusters were smaller and had a disorganized appearance (P8: 6.2 ± 0.6 x10^2 μm^2 vs. 10.8 ± 1.2 x10^2 in WT; P21: 8.7 ± 1.5 x10^2 μm^2 vs. 12.2 ± 1.7 x10^2 in WT). Furthermore, at P21, the polarity of ependymal cell cilia appeared abnormal, with lateral clustering frequently observed (Figure II-7D).

To explore the possibility that the observed ependymal phenotype stemmed from an existing defect in radial glial cells, I again turned to a cell culture approach where I applied dystroglycan-blocking or control antibodies to SVZ cultures prepared from FoxJ1-GFP (WT for dystroglycan) newborn mice (as in Figure II-5A). For this purpose I used the antibody IIH6, which recognizes a unique O-linked glycoepitope on α-dystroglycan, competitively inhibiting the binding of LG domain-containing ligands [251]. In agreement with the ependymal phenotype seen in DAG cKO mice, the addition of dystroglycan blocking antibodies resulted in a significant reduction in GFP+ ependymal cells (49.4% ± 5.5% of total cells vs. 71.5% ± 5.1% in controls)

(Figures II-8A,B), as well as a significant reduction in the proportion of GFP+ cells that achieved a multiciliated phenotype (17.5% ± 2.2% vs. 31.8% ± 3.1% in controls) (Figures II-8C,D). Additionally, dystroglycan block reduced the ability of GFP+ cells to arrange themselves into pinwheel-like clusters, with cluster area reduced by over 50% (2.48 ± 0.08 $x10^3$ μm^2 vs. 5.62 ± 0.75 $x10^3$ in controls) (Figure II-8E). Together these results indicate that laminin-dystroglycan interactions play a crucial role in ependymal cell maturation and niche construction. Furthermore, my *in vitro* findings suggest that dystroglycan regulates ependymal cell development independent of any potential undetected defect in RGCs, as wild type RGC cultures exposed to dystroglycan blocking antibodies were unable to produce appropriate numbers of mature ependymal cells or to assemble them into pinwheel-like polarized clusters encircling neural stem cells.

DISCUSSION

I found that the ECM receptor dystroglycan is necessary for niche development, i.e. ependymal cell maturation and organization into niche pinwheels, during this critical gliogenic period. Moreover, I describe for the first time the extensive dystroglycan-dependent reorganization of ECM that occurs concurrent with niche building, and suggest that this unique and dynamic postnatal ECM is central to the establishment of proper supracellular organization of niche cells and provides important cues for ependymal cell development.

The neonatal SVZ vascular plexus, with its associated vascular basal lamina, is denser but morphologically similar to that observed in adult mice. Additionally, I observed that extra-vascular ECM structures thought to have regulatory roles in the adult niche, such as fractones and fractone bulbs [190,191,175,250,192], are present in the neonatal SVZ during niche construction and gliogenesis. Focusing on the developing ependymal niche, I probed niche ECM dynamics during

this developmental window and found that laminin and its receptor, dystroglycan, were upregulated on the cell surface of differentiating ependymal cells. I furthermore identified novel extra-vascular ECM structures; transient laminin-rich "tethers" that link ependymal cell-associated laminin to the vascular basal lamina during SVZ niche construction. As immature ependymal cells emerge, they lose their long radial glial basal process and, therefore, their connection to basement membrane ECM. Laminin tethers may serve as an ECM contact point that promotes ependymal cell maturation and pinwheel formation, perhaps by reinforcing intra- or supracellular polarity during this transitional period,. The observed delay in ependymal cell maturation and pinwheel organization in the absence of dystroglycan supports this interpretation. Finally, analysis of SVZ whole mounts from FoxJ1-GFP mice shed new light on the origins and spatial plasticity of laminin/dystroglycan-positive "hubs", a class of extra-vascular ECM structures found at the ventricular surface from approximately P8 onward. These ECM aggregates have previously been observed in association with GFAP+ apical processes in the adult niche and referred to as "specks" [175]. I propose that the term "hub" better describes their location at the interface between ependymal cells and type B cells within mature pinwheels. However, the cellular association of ECM hubs appears to be more varied during early development, first appearing toward the center of ependymal cell apical surfaces of and later transposed to the center of pinwheels. As they were previously found to be nidogen-positive, it was proposed that ECM pinwheel hubs originated from the endothelial basal lamina [175]. However, unlike fractones and fractone bulbs, I did not observe any contact between the vascular basal lamina and ECM pinwheel hubs during development or in the adult SVZ. In light of the abundant laminin deposition I witnessed in ependymal niche cultures and the localization of ECM pinwheel hubs relative to developing ependymal cells *in vivo*, it seems likely that hubs are

produced, or at least assembled, by ependymal cells. These results suggest that ependymal niche cells may therefore play a key role in adult VZ niche construction, participating in the establishment of their own regulatory environment via the expression of both ECM ligands and receptors. Taken together, my observations indicate that the ECM organization of the early postnatal VZ/SVZ is considerably more complex than that in the adult niche. And, while the early postnatal niche is "under construction", it houses distinct ependymal and vascular niche ECM elements, with the immature ependymal niche particularly enriched in laminin and dystroglycan.

As cortical neurogenesis subsides perinatally, most radial glial cells (RGCs) undergo terminal division, giving rise to transit amplifying progenitors. Others transform directly into relatively quiescent adult neural stem cells or post-mitotic ependymal cells [172]. Intermediate RGC progenitors simultaneously withdraw their basal processes, exchanging endfoot attachment to the pial basement membrane for contact with the basal lamina surrounding SVZ blood vessels. Laminin-dystroglycan interactions are known to mediate RGC contact with the pial basal lamina, as the loss of brain dystroglycan or its laminin binding domain lead to basal endfoot detachment and aberrant neuronal migration [160,41,246]. Given that laminin-dystroglycan signaling is required for basement membrane integrity and RGC attachment at the cortical surface, I hypothesized that it might also mediate cellular interactions with laminin-rich ECM in the neonatal SVZ. I found that WT RGCs associate with laminin-positive aggregates at the ventricular surface at birth. Although deletion of brain dystroglycan virtually eliminated these laminin aggregates I did not observe any evidence of RGC apical detachment, suggesting that the functional significance of perinatal RGC contact with VZ laminin extends beyond that of simple adhesion. This is not surprising, as the apical endfeet of RGCs are attached to each other through adherens junctions,

which have been demonstrated to mediate the retention of RGCs in the niche [252,253]. As such, it appears that while dystroglycan may facilitate laminin aggregation at the ventricular surface, a) these aggregates are not necessary for RGC apical attachment and b) dystroglycan does not contribute substantially to the formation or maintenance of adherens junctions between perinatal RGCs.

When I analyzed the numbers and proliferation of BLBP+ cells in the perinatal DAG cKO SVZ, I found that the proliferative capacity of P0 RGCs was greatly reduced. However, I found no correlation between reduced RGC proliferation and RGC density, which may indicate that dystroglycan supports the production of progenitors through asymmetric cell division and that these divisions were specifically reduced in DAG cKO mice. The decreased numbers of oIPCs in the DAG cKO SVZ at P0 (see Chapter III) would support this interpretation. The maturation of dystroglycan-deficient RGCs also appeared to be delayed. While BLBP expression and proliferation decreased in WT RGCs between P0 and P3, RGC transition to ependymal cells was stalled in the DAG cKO SVZ. Although dystroglycan loss is known to cause RGC detachment from the pial basement membrane, studies of other mouse models in which RGC attachment is impaired (α6 integrin$^{-/-}$, perlecan$^{-/-}$, laminin γ1 nidogen-binding site mutation) found no disruption in embryonic RGC polarity, proliferation or fate [254].

Although the cellular architecture of the adult VZ/SVZ has been extensively characterized, much less is known about cellular reorganization during niche construction. Furthermore, to my knowledge, no information exists regarding the role of ECM interactions in driving this process. Here I reveal that maturing ependymal cells, but not FoxJ1-negative postnatal RGC progenitors, upregulate laminin and dystroglycan at their apical surfaces. Laminin assembly was delayed in the absence of dystroglycan but later rebounded, with cell surface

laminin binding surpassing that seen in WT mice, suggesting a profound dysregulation of ECM dynamics. I furthermore demonstrated that upregulation of dystroglycan in developing ependymal cells is required for both their timely differentiation and the cellular reorganization necessary to produce adult niche pinwheel structures. Analysis of WT ependymal cell development *in vitro*, with dystroglycan ligand binding blocked only during RGC differentiation, revealed that this effect was niche-independent and was not predicated on an existing RGC phenotype.

These results reveal a novel and distinct function of dystroglycan *within* the developing SVZ, regulating both radial glial cell division and their ability to mature into adult SVZ niche cells. The systematic study of dystroglycan interactions in the developing SVZ will be an important next step, but the current investigation indicates that the pial detachment of RGCs is only one of many defects underlying the widespread dysfunction seen in the cortices of both mice and human patients as a result of dystroglycan loss-of-function.

Figure II-1. Laminin-rich extracellular matrix structures in the early postnatal SVZ

(A) IHC staining of an SVZ whole mount from a WT P0 mouse, showing laminin immunoreactivity in the vascular basal lamina. The distinctive SVZ vascular plexus is clearly visible at birth. Box denotes area of further whole mount analysis. **(B)** Projections of confocal stacks from P0, P3 and P8 WT whole mounts highlighting blood vessel-associated- and extra-vascular laminin. Extensive blood vessel-fractone networks were observed throughout the early postnatal period, with laminin anchors appearing around P8. Bottom panels: XZ projections of above panels. **(C)** IHC staining against laminin and b-catenin on whole mounts taken from WT mice at P3, P8 and P21. Ventricular surface cell-associated laminin was localized to developing ependymal cells at P3. At P8, generalized cell surface laminin decreased as laminin aggregated into "anchors" on the apical surfaces of ependymal cells. **(D)** 3D reconstruction of a confocal stack showing laminin expression in the VZ/SVZ of a P3 WT mouse. Ventricular surface cell-associated laminin was contiguous with the blood vessel basal lamina either directly (arrowheads) or via "tethers" (arrows). **(E)** 3D reconstruction of laminin expression in a P8 WT mouse. At P8, two types of globular laminin structures were observed; ventricular surface "anchors", typically associated with laminin-positive cells (arrowheads) and "bulbs", found at fractone termini deeper in the SVZ (arrows). **(F)** IHC staining against laminin in whole mounts from P3 and P8 FoxJ1-GFP mice. Left panels: en face view of cell-associated laminin in developing ependymal cells. Top right: 3D reconstructions of confocal stacks, showing laminin-containing ECM tethering young ependymal cells to the underlying vasculature. Bottom right: 2D orthogonal view of top panel. **(G)** IHC staining in a wholemount from a P21 FoxJ1-GFP mouse. Left panel: en face views of the ventricular surface. Right panel: 3D reconstructions of confocal stacks taken from the same field. At P21, VZ laminin was largely restricted to pinwheel anchors. Most anchors remained associated with ependymal cell surfaces (arrow), but many had relocated to the center of pinwheels (closed arrowhead) or were in the process of relocating (open arrowhead). **(H)** IHC against laminin and GFAP in a whole mount from an adult FoxJ1-GFP mouse. Left panel: En face view. Right panel: 3D reconstruction. In adult mice, one or more laminin anchors were found at the center of pinwheels, in contact with GFAP+ B cell apical processes. Scale bars: 50 μm (B), 25 μm (C), 20 μm (F, G, H).

Figure II-1. Laminin-rich extracellular matrix structures in the early postnatal SVZ

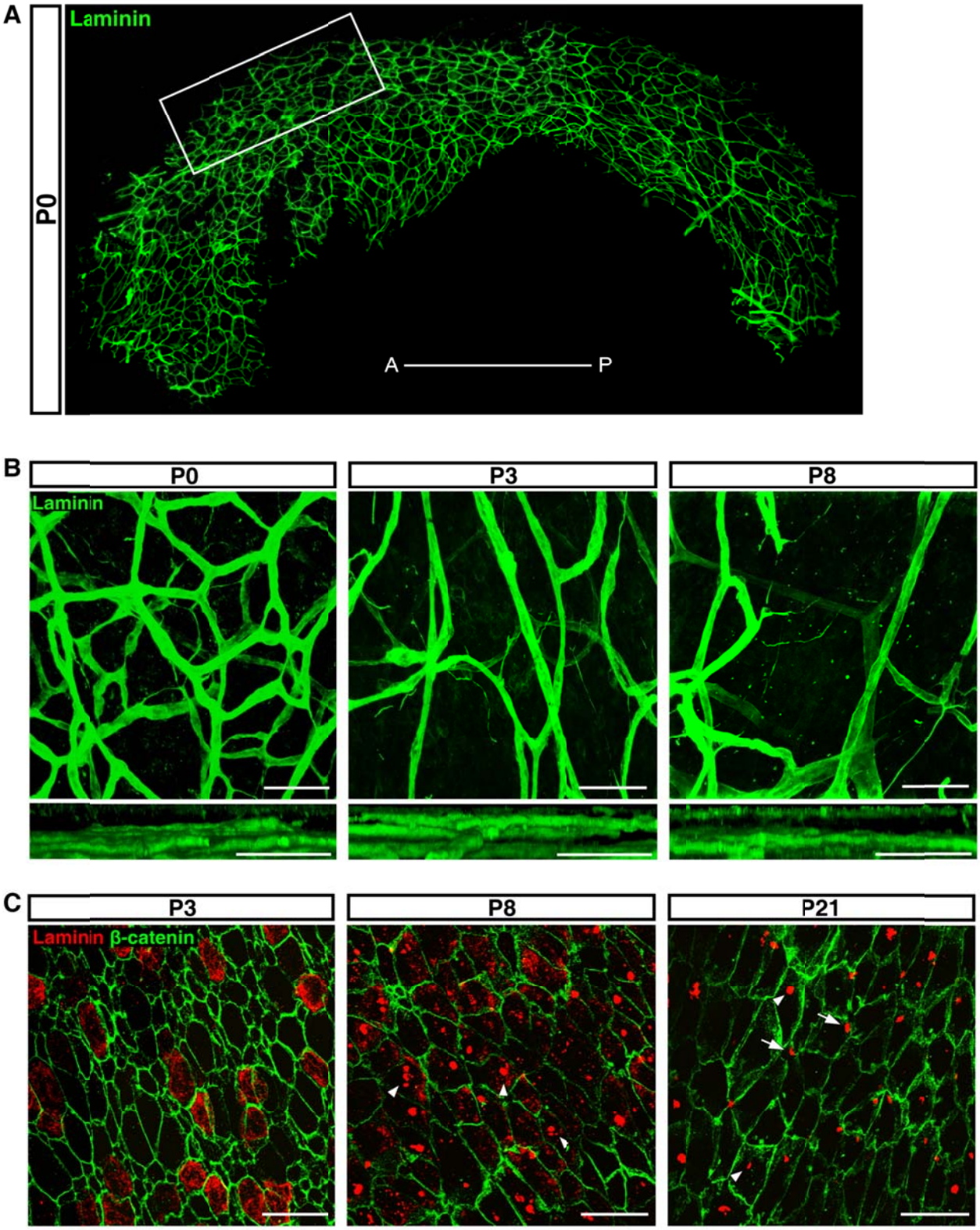
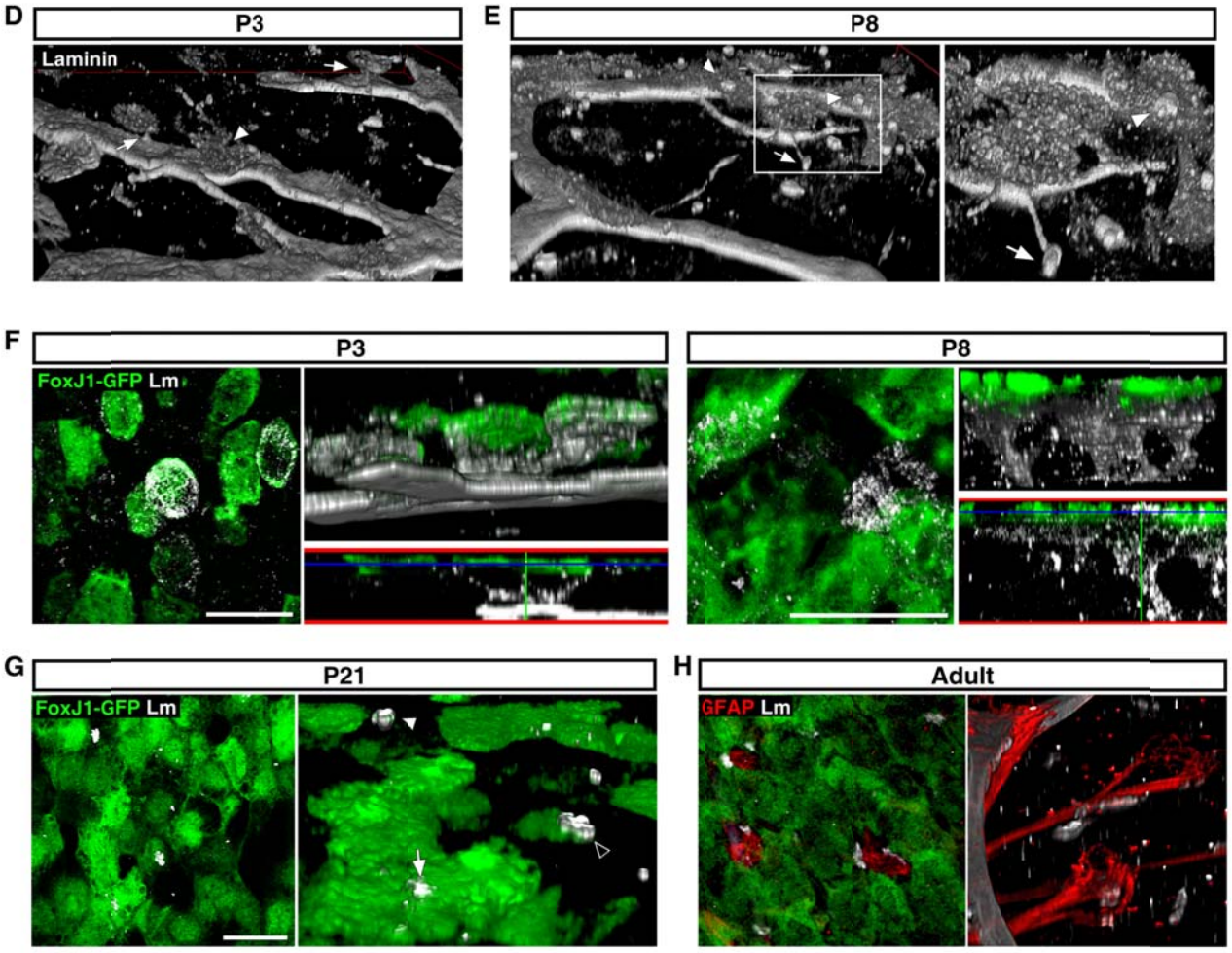


Figure II-1. Laminin-rich extracellular matrix structures in the early postnatal SVZ



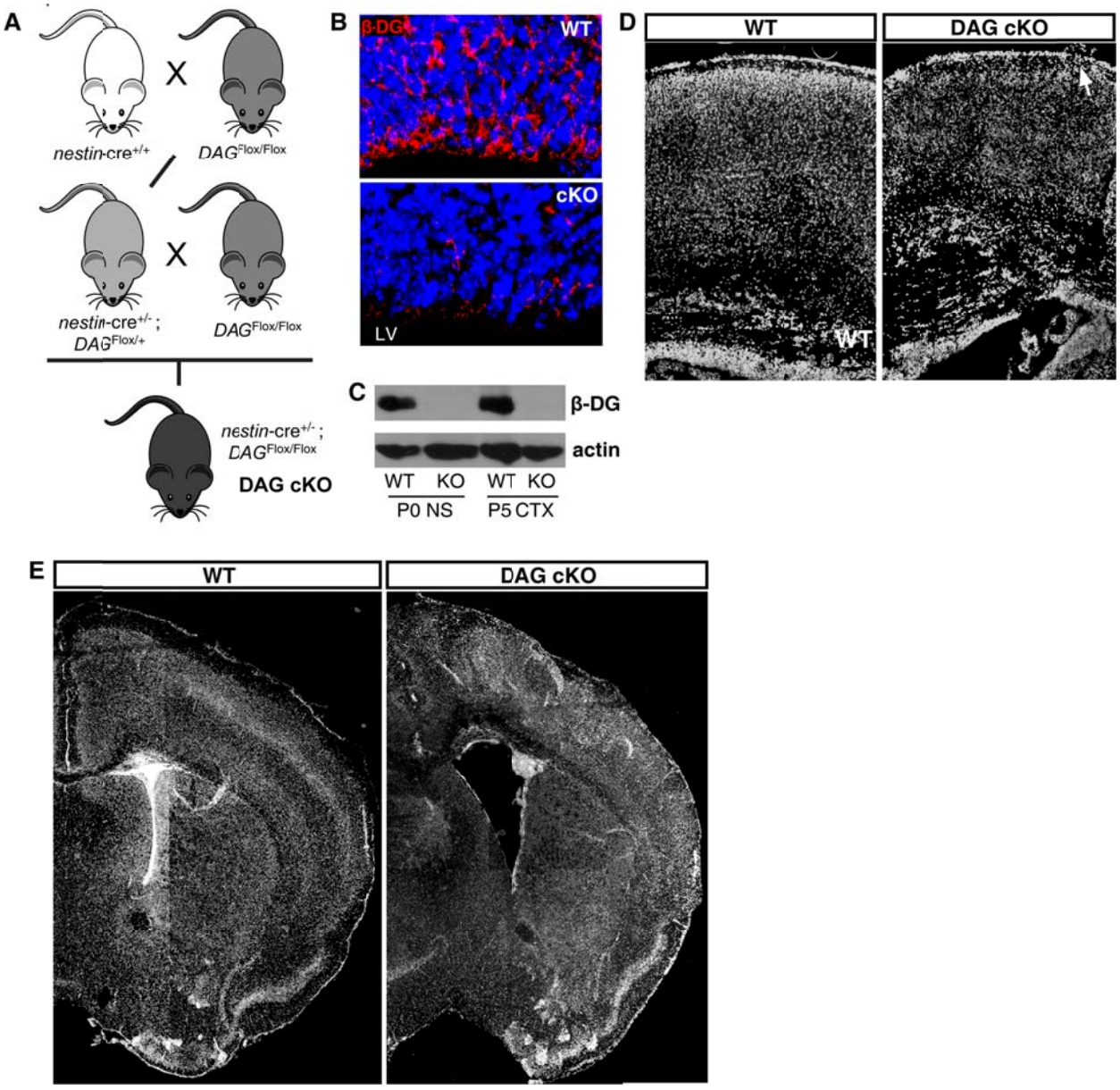


Figure II-2. Generation of *nestin*-cre$^{+/-}$; *DAG*$^{Flox/Flox}$ (DAG cKO) mice

(A) Schematic of breeding scheme used to obtain *nestin*-cre$^{+/-}$; *DAG*$^{Flox/Flox}$ (DAG cKO) mice. **(B)** IHC against β-dystroglycan in coronal sections taken from P0 WT and DAG cKO mice. Scale bar: 25 μm. LV: lateral ventricle. **(C)** Western blotting against β-dystroglycan on lysates taken from i) second passage neurospheres isolated from P0 WT and DAG cKO mice and ii) cortices from P5 WT and DAG cKO mice. **(D-E)** DAPI staining in coronal sections from **(D)** P3 and **(E)** P8 WT and DAG cKO mice.

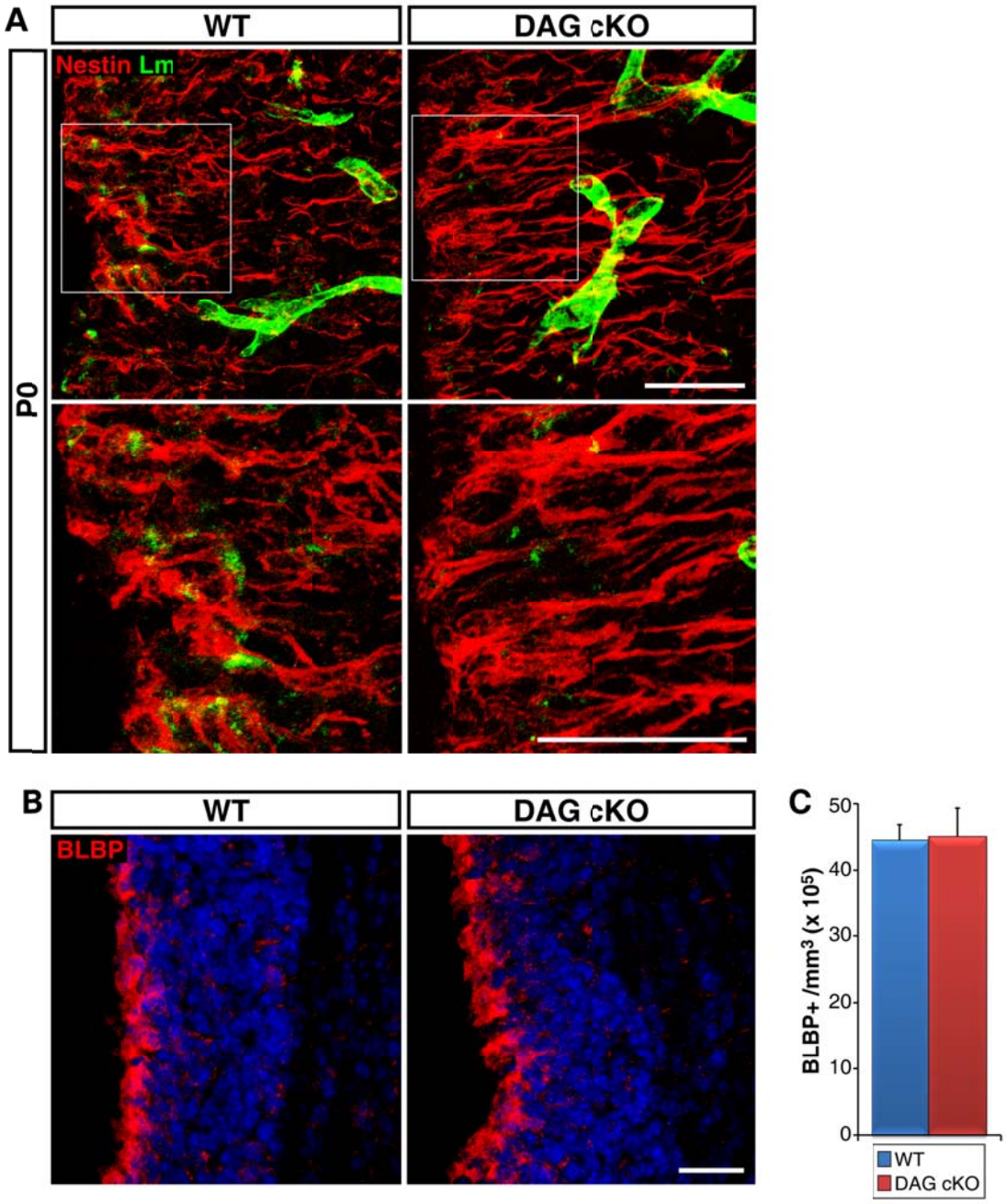


Figure II-3. Ventricular surface laminin aggregates are lost in the perinatal DAG cKO SVZ

IHC staining of coronal sections from P0 WT and DAG cKO mice. **(A)** In WT mice, the apical processes of nestin+ radial glia make contact with laminin puncta at the ventricular surface (dashed lines, bottom panels). In DAG cKO mice there were fewer laminin-positive puncta and radial glial association with the remaining puncta was reduced. The association of radial glial basal processes with SVZ blood vessels did not appear to be altered in DAG cKO mice. **(B)** Perinatal WT and DAG cKO mice had similar BLBP+ radial glial cell densities. **(C)** Quantification of BLBP+ cell density at P0 in WT and DAG cKO mice. Error bars, SEM; n=3. Scale bars: 25 μm.

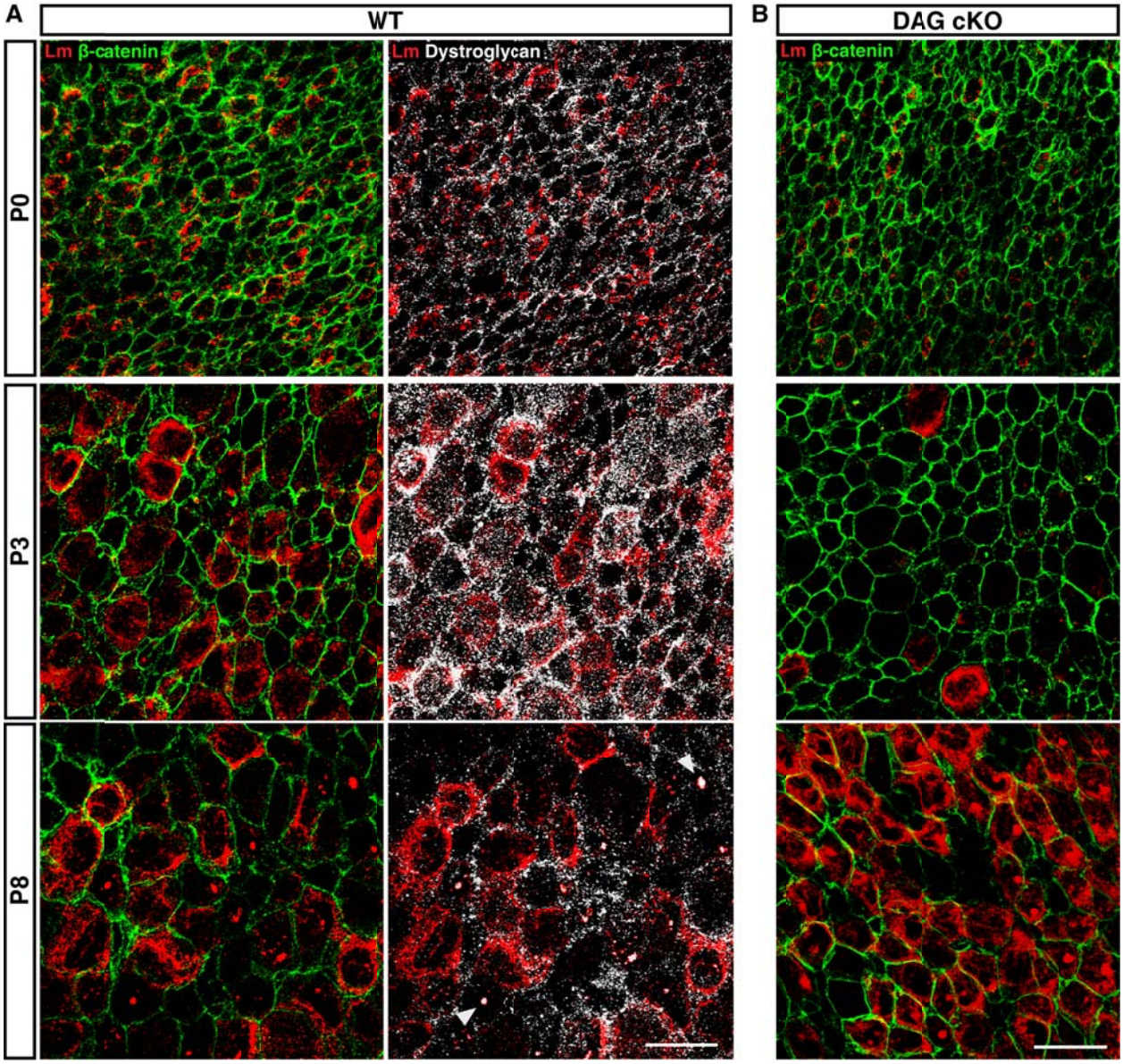


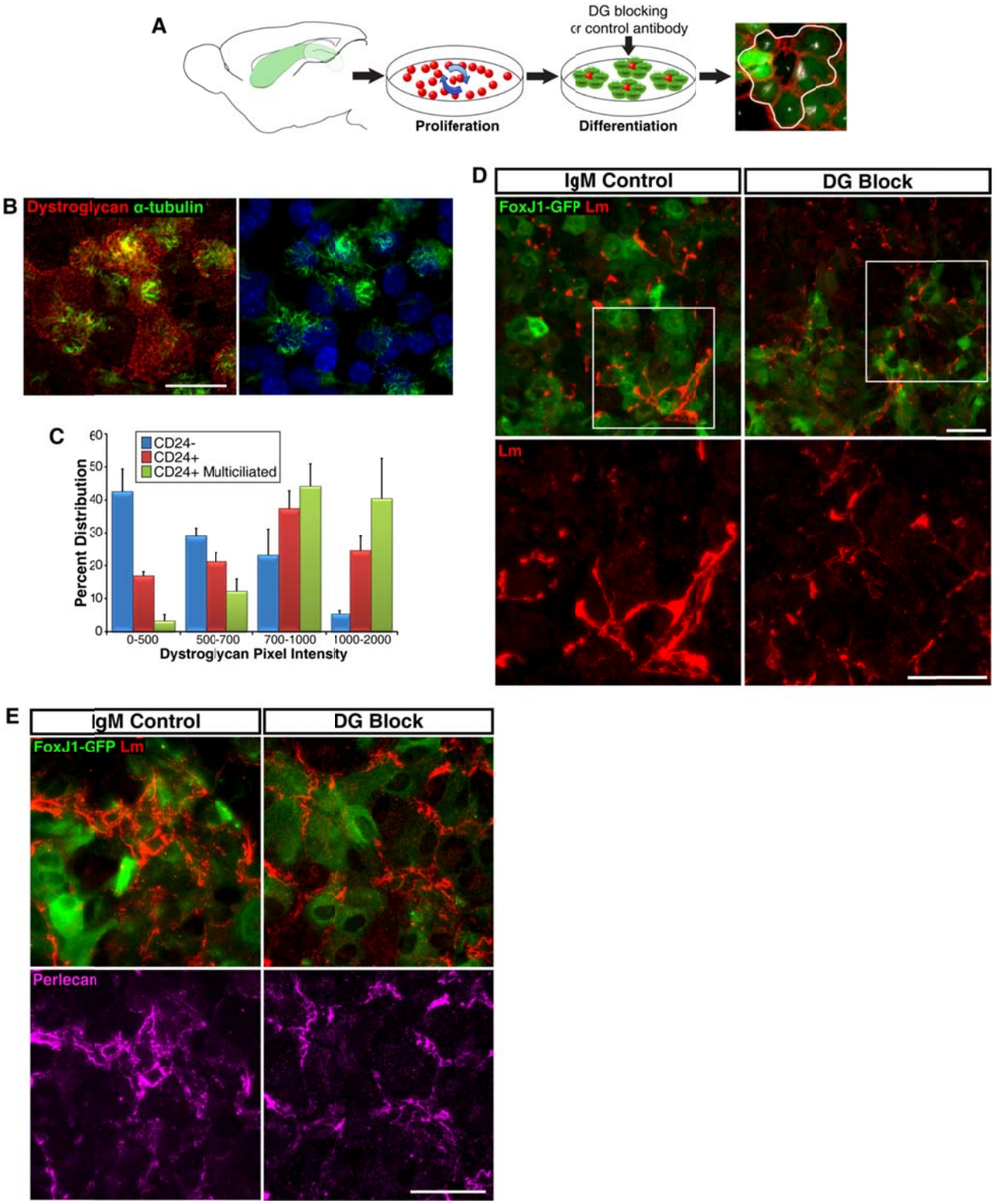
Figure II-4. Dystroglycan regulates laminin restructuring in the developing ependymal niche

(A) IHC staining of lateral ventricular wall whole mounts from P0, P3 and P8 WT mice. Maturing ependymal cells upregulate laminin and dystroglycan at the cell surface. By P8, laminin- and dystroglycan-positive anchors appear at the ventricular surface (arrowheads). (B) IHC staining of DAG cKO whole mounts at the same timepoints. Ependymal cells lacking dystroglycan show an initial delay in acquisition of cell-associated laminin followed by rapid upregulation, exceeding that of WT ependymal cells by P8. Scale bars: 25 μm (A,B).

Figure II-5. Blocking dystroglycan ligand binding disrupts ECM structure in ependymal niche cell cultures

(A) Schematic of ependymal cell culture protocol. SVZ cells were isolated from P0 FoxJ1-GFP mice, plated on PDL in high serum media and allowed to proliferate for 3-4 days. Cells were then switched to low serum media with control or DG-blocking antibodies and allowed to differentiate for 7 days. For measurement purposes, clusters were defined as 3 or more directly adjacent GFP+ cells also in contact with at least one primary-ciliated GFP- cell. **(B)** IHC staining of differentiated ependymal cell cultures. Dystroglycan expression increases as radial glia transition into ependymal cells, with the highest expression levels in fully mature, multiciliated ependymal cells. **(C)** Quantification of DG IHC pixel intensity in CD24-, CD24+ and CD24+ multi-ciliated cells. Error bars, SEM; n=3. **(D)** IHC staining of cultures with control or DG-blocking antibodies added during the differentiation phase. Ependymal cell cultures produce thin, fractone-like laminin-containing ECM structures, which appear fractured and disorganized following DG block. **(E)** Laminin-positive ECM structures also contain perlecan. Scale bars: 25 μm (B), 50 μm (D,E).

Figure II-5. Blocking dystroglycan ligand binding disrupts ECM structure in ependymal niche cell cultures



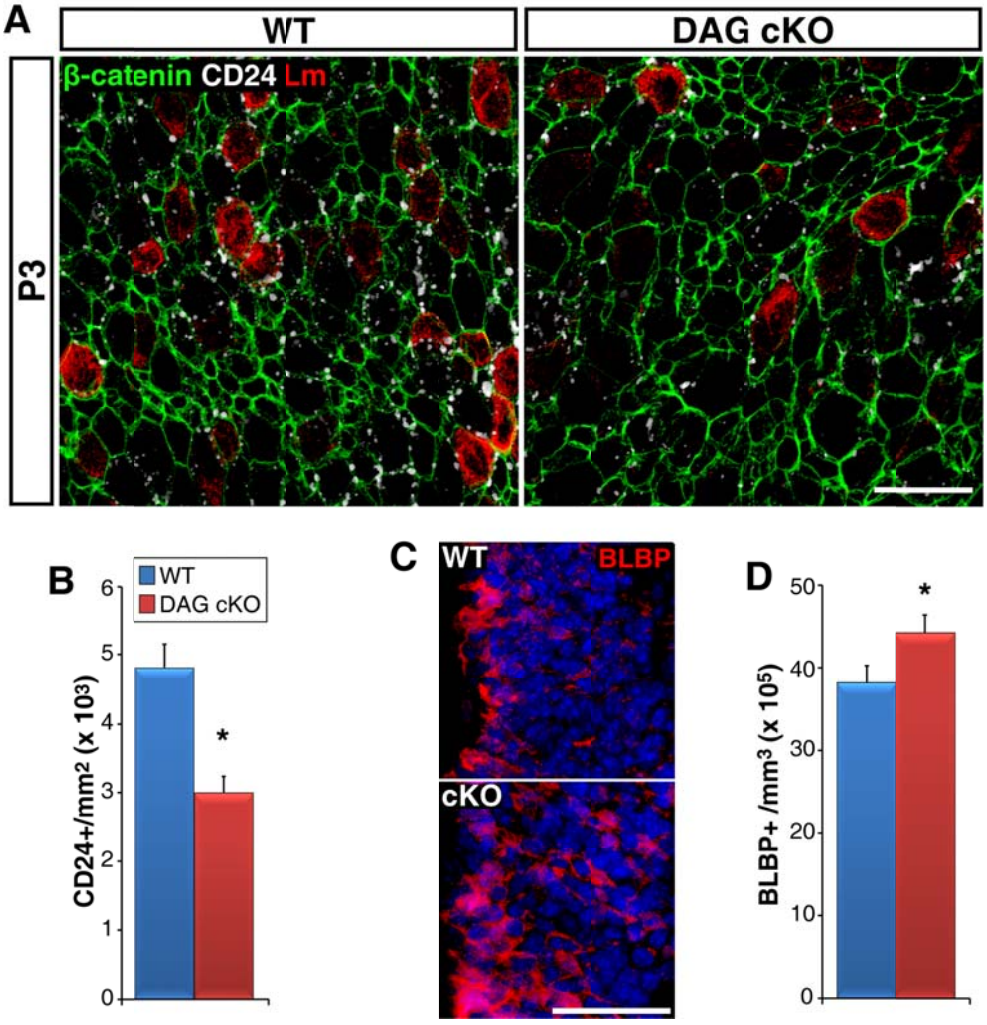


Figure II-6. The transformation of radial glia into ependymal cells is delayed in the DAG cKO SVZ

(A) IHC staining of lateral ventricular wall whole mounts from P3 WT and DAG cKO mice. The VZ of DAG cKO mice contains fewer ependymal cells. (B) Quantification of CD24+ cells at the ventricular surface in P3 WT and DAG cKO mice. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. (C) IHC staining of the SVZ in coronal sections taken from P3 WT and DAG cKO mice. The SVZ of DAG cKO mice contains more BLBP+ radial glial cells. (D) Quantification of BLBP+ cells in the dorsal SVZ in P3 WT and DAG cKO mice. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. Scale bars: 25 μm (A), 50 μm (C).

Figure II-7. Dystroglycan is required for ependymal cell maturation and niche assembly

(A) IHC staining of whole mounts from P8 WT and DAG cKO mice. Fewer DAG cKO ependymal cells have attained a multi-ciliated phenotype and clusters are smaller than in WT littermates, with a disorganized appearance. Right panels show examples of measured clusters. (B) Quantification of relative surface area coverage of multi-ciliated cells in P8 WT and DAG cKO whole mounts. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. (C) Quantification of average cluster area in P8 WT and DAG cKO whole mounts. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. (D) IHC staining of whole mounts from P21 WT and DAG cKO mice. Pinwheel formation in the DAG cKO VZ has begun to normalize, though polarization of cilia appears abnormal. (E) Quantification of relative surface area coverage of multi-ciliated cells in P21 WT and DAG cKO whole mounts. **$p < 0.01$, Student's t-test; error bars, SEM; n=5. (F) Quantification of average cluster area in P21 WT and DAG cKO whole mounts. *$p < 0.05$, Student's t-test; error bars, SEM; n=5. Scale bars: 25µm.

Figure II-7. Dystroglycan is required for ependymal cell maturation and niche assembly

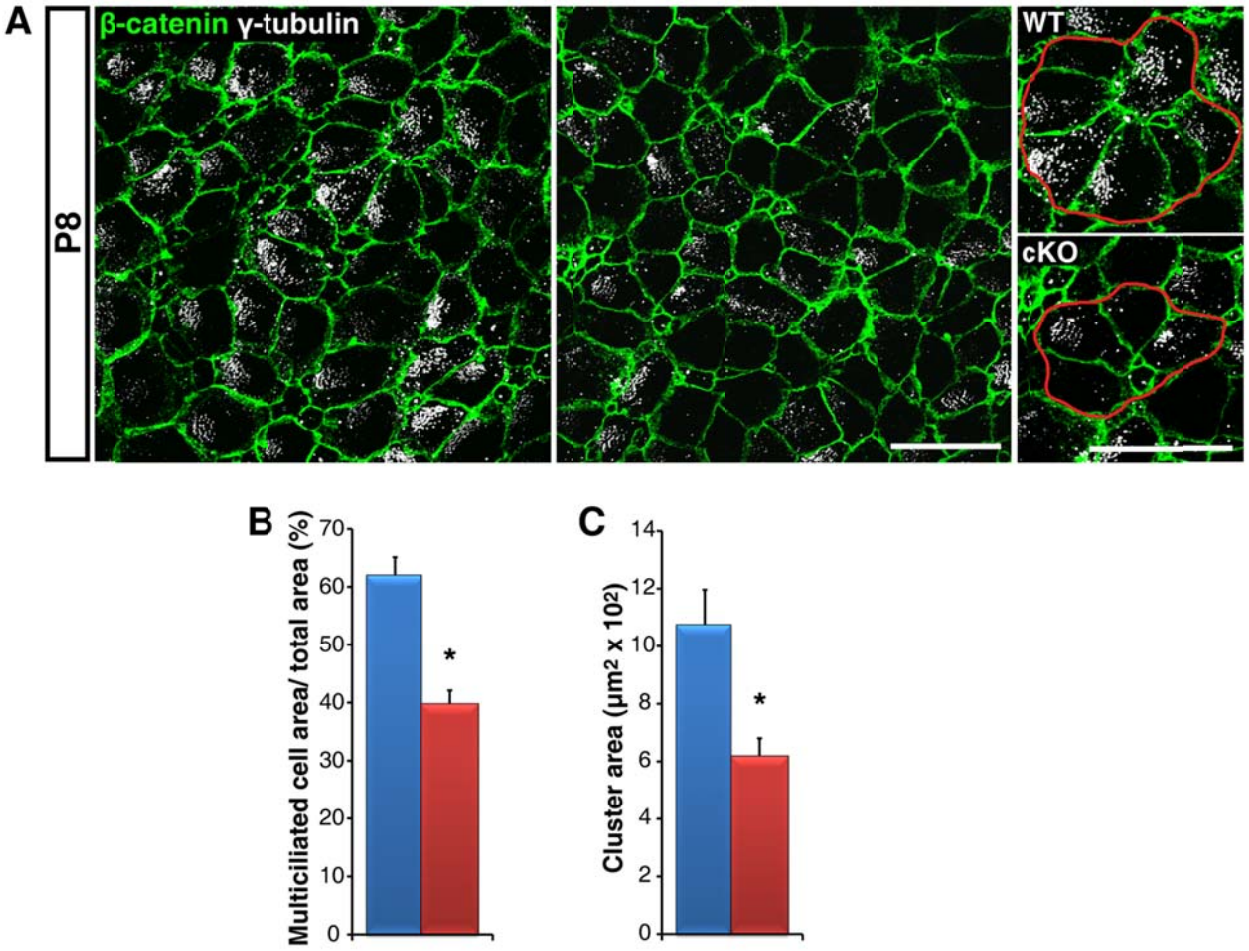
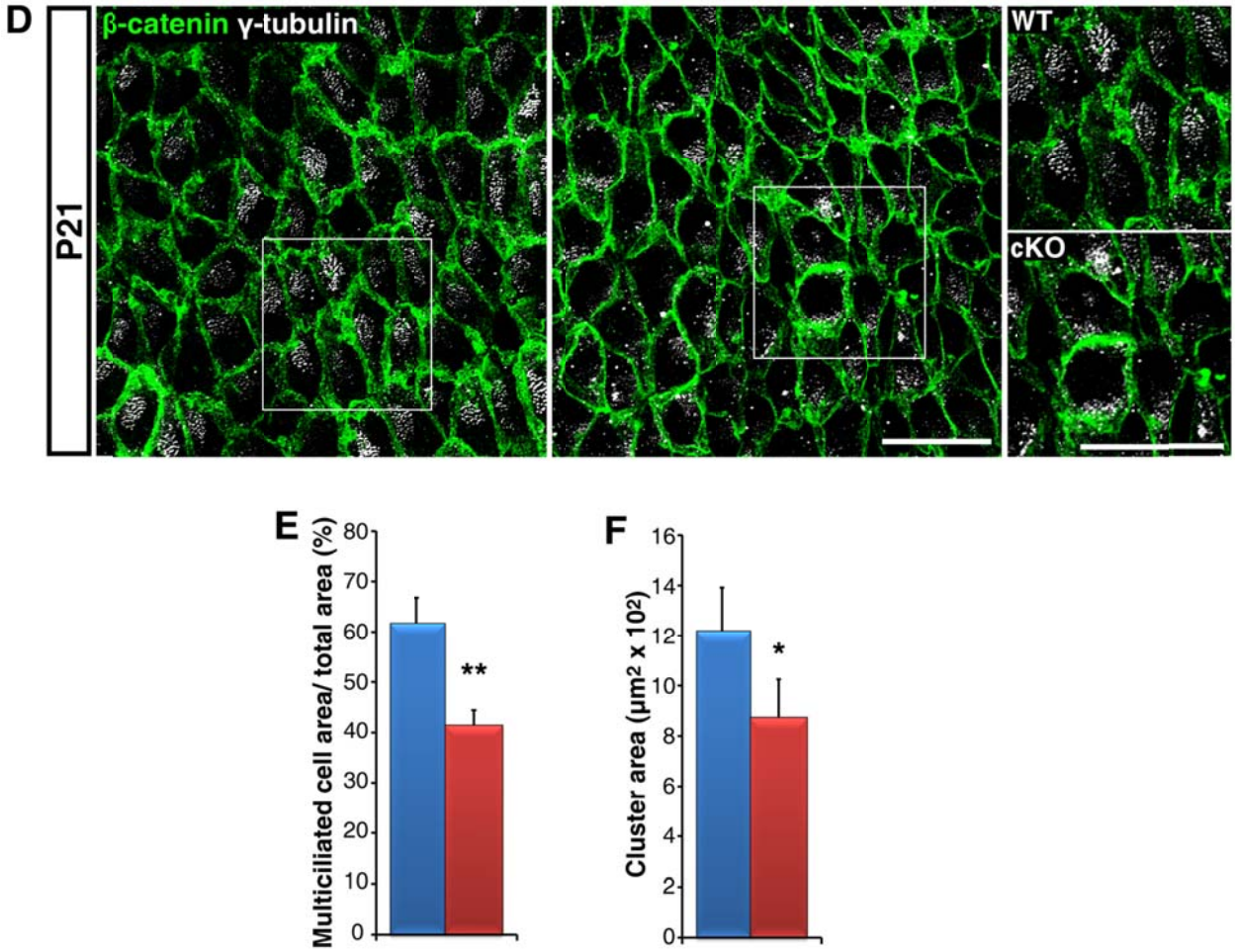


Figure II-7. Dystroglycan is required for ependymal cell maturation and niche assembly



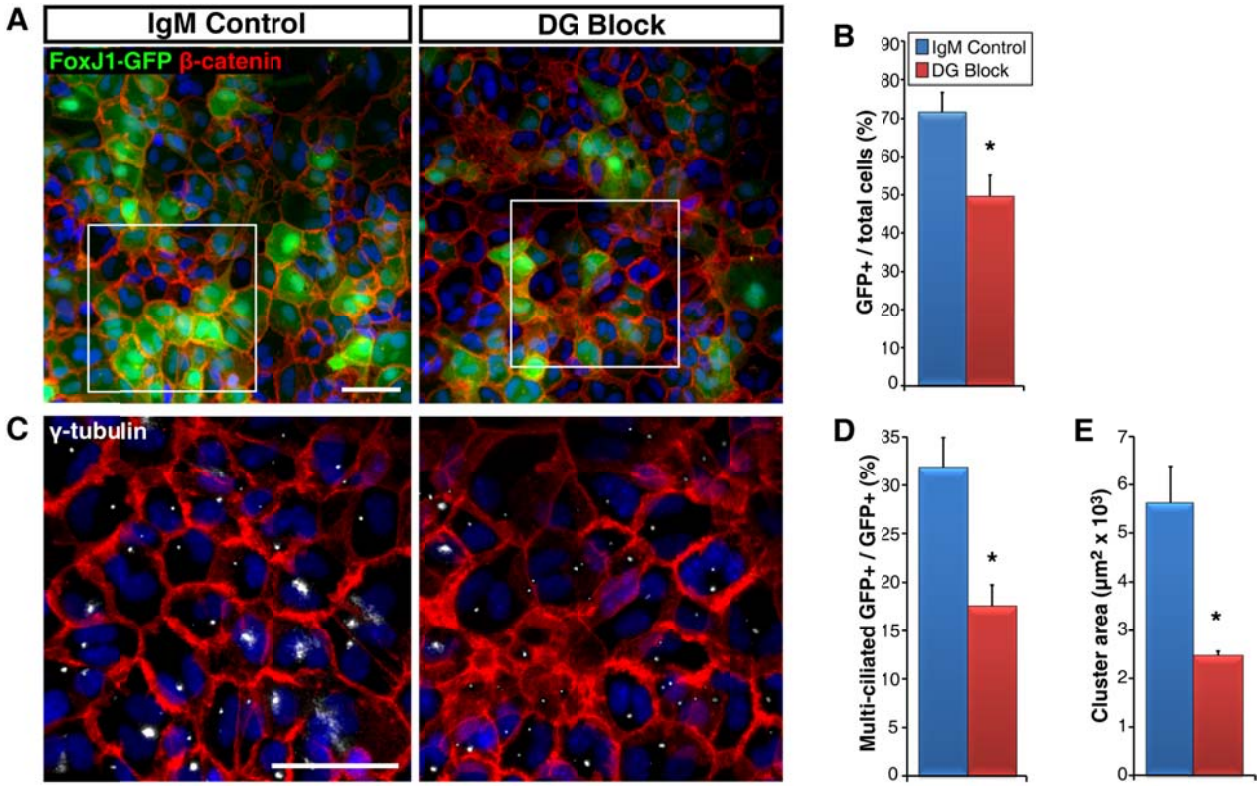


Figure II-8. Dystroglycan block impacts ependymal cell maturation and clustering *in vitro*

(A) IHC staining of differentiated cultures after adding control or DG-blocking antibodies. Applying DG-blocking antibodies during RGC differentiation inhibited their transformation into ependymal cells. **(B)** Quantification of GFP+ ependymal cell numbers following differentiation with control or DG-blocking antibodies. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. **(C)** Inset denoted in K, with y-tubulin staining to visualize cilia. Blocking DG led to decreased numbers of FoxJ1+ ependymal cells in a mature, multiciliated state. DG block also reduced the ability of ependymal cells to form pinwheel-like clusters *in vitro*. **(D)** Quantification of subset of FoxJ1+ cells with multiple cilia after differentiation with control or DG-blocking antibodies. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. **(E)** Quantification of ependymal cell/RGC cluster area in control or DG-blocked cultures. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. Scale bars: 50μm.

CHAPTER III: DYSTROGLYCAN REGULATES POSTNATAL OLIGOGLIOGENESIS AND OLIGODENDROCYTE MATURATION

INTRODUCTION

In the embryonic VZ and adult SVZ, extracellular matrix interactions have been proposed to regulate the timing and placement of neural stem cell division and, in so doing, help to maintain the balance between self-renewal and the controlled production of neural progenitors. Furthermore, though laminin and dystroglycan are known promoters of oligodendroglial survival, differentiation and myelination [255,238,256,243,257,244], the role of ECM in oligodendroglial fate determination remains unknown. In this chapter I examine the role of ECM in the postnatal SVZ, as the developing niche shifts from neurogenesis to the production of large numbers of oligodendrogenic progenitors that will go on to populate the adult cortical grey matter and myelinate forebrain axon tracts.

RESULTS

Gliogenesis is dysregulated in the dystroglycan-deficient SVZ stem cell niche

The principal wave of dorsal oligodendrogenesis begins perinatally and extends into the second postnatal week. During this time, oligodendrogenic intermediate progenitor cells (oIPCs) are produced by asymmetric divisions of neural stem- and transit amplifying cells. oIPCs, as they transition into oligodendrocyte progenitor cells (OPCs), proliferate and migrate out of the SVZ into the overlying white matter. As I found that perinatal DAG cKO radial glial cells (RGCs) were defective in their ability to transform into ependymal cells and establish a normal SVZ stem cell niche, I wanted to determine if these disturbances impacted the niche's ability to produce

lineage-specific progenitors. First, to investigate both the production of oIPCs and subsequent oIPC/OPC proliferation, I performed IHC to detect the oIPC/OPC marker PDGFαR and PCNA on sections obtained from DAG cKO mice and their wild type littermates at P0 and P3 (Figure III-1A). At P0, oligodendrogenesis had begun in DAG cKO mice, albeit to a lesser extent than their WT littermates, with slightly fewer PDGFαR+ oIPC/OPCs found in the dystroglycan-null SVZ ($10.6 \pm 1.76 \times 10^5$ cells/mm^3 vs. $15.1 \pm 3.58 \times 10^5$ in WT) (Figure III-1B). By P3, however, DAG cKO animals had caught up and far surpassed WT in terms of oligodendrogenesis, with an SVZ oIPC/OPC density of more than double that of their WT littermates ($30.6 \pm 3.07 \times 10^5$ cells/mm^3 vs. $14.0 \pm 1 \times 10^5$ in WT). oIPC/OPC production by the DAG cKO SVZ remained elevated over WT levels until the beginning of the second postnatal week.

Given that I observed increased numbers of oIPC/OPCs in the DAG cKO SVZ I next sought to determine if inappropriate proliferation contributed to this phenotype. IHC was used to detect the proliferation marker PCNA on coronal sections from newborn mice, revealing that DAG cKO mice exhibited significantly decreased SVZ cell proliferation relative to their WT littermates ($38.8 \pm 3.61 \times 10^5$ cells/mm^3 vs. $63.1 \pm 2.06 \times 10^5$ in WT) (Figure III-2B). And, while proliferation sharply declined in the WT SVZ between P0 and P3, proliferation in the DG-null SVZ remained relatively constant between P0 and P3, resulting in a slight elevation over WT levels at P3 ($44.2 \pm 8.38 \times 10^5$ cells/mm^3 vs. $28.7 \pm 4.19 \times 10^5$ in WT) (Figure III-2B). Additionally, while WT NSC/NPC proliferation was concentrated mainly in the VZ, proliferative Sox2+ cells were mislocalized throughout the DAG cKO SVZ (Figure III-2C).

To determine which cell types contributed to altered proliferation, I next performed IHC to detect the radial glial marker BLBP in conjunction with PCNA on coronal sections from P0 and P3 mice (Figure III-1C). At P0 I found that the percentage of BLBP+ RGCs that were

PCNA+ was significantly lower in DAG cKO mice, mirroring the phenotype observed in the general PCNA+ population (36.1% ± 3.5% vs. 64.8% ± 1.9% in WT) (Figure III-2B). However, while WT animals underwent the typical marked decrease in RGC proliferation between P0 and P3, RGC proliferation levels remained constant in DAG cKO mice between P0 and P3, again leaving RGC proliferation levels slightly higher than those in WT littermates at P3 (34.4% ± 1.9% vs. 27.8% ± 1.8% in WT) (Figure III-1C). Dystroglycan loss had additional effects on the newborn oIPC/OPC population, significantly increasing oIPC/OPC proliferation at P3 (57.7% ± 2.8% vs. 39.3% ± 5.3% in WT), although not at P0 (65.6% ± 8.6% vs. 67.6% ± 5.7% in WT) (Figure III-1D). I further analyzed the proliferation data at P3 to assess the relative contribution of each cell type and determined that while radial glia contribute to the overall increase in SVZ proliferation seen in P3 DAG cKO mice, OPCs are the primary drivers of hyperproliferation in the dystroglycan-deficient SVZ (Figure III-1E).

These results led me to question whether the observed increase in oIPC/OPC density was due to increased generation of these cells from RGCs or solely due to increased oIPC/OPC proliferation. To address this issue, I performed single injections into the lateral ventricles of P2 rats of either control antibodies or antibodies that blocked dystroglycan-ligand interactions (Figure III-3A). The rats were sacrificed either 6 or 24 hours post-injection to assess acute changes in SVZ cell phenotypes. I first performed IHC to detect laminin and β-catenin at the ventricular surface of whole mounts from control- and dystroglycan blocking antibody-injected rats. 6 hours post-injection, ventricular surface laminin in control rats was found mainly in aggregates at the apical surface of immature ependymal cells (Figure III-3B). However, the injection of dystroglycan-blocking antibodies led to the loss of laminin aggregates, with laminin immunoreactivity appearing more diffuse with only minor puncta apparent. At 24 hours after the

injection of dystroglycan-blocking antibodies I observed multiple, larger laminin puncta on the apical surfaces of VZ cells, suggesting that laminin aggregation was recovering.

I next performed IHC on coronal sections to detect PDGFαR (to label oIPC/OPCs), PCNA (to label proliferating cells) and Sox2 (to label NSC/IPCs) within the SVZ. I found that dystroglycan-blocking antibodies produced a robust increase in PDGFαR+ cells that was apparent at just 6 hours post-injection (Figure III-3C) and resulted in a twofold increase in oIPC/OPC density relative to control antibody-injected littermates (14.3 ± 2.33 x10^5 cells/mm^3 vs. 6.96 ± 1.94 x 10^5 in controls) (Figure III-3D). However, this increase in OPC numbers was not explained by increased proliferation within the oIPC/OPC population, as PCNA immunoreactivity within PDGFαR+ cells was similar in control and dystroglycan-blocking conditions at the same timepoint (25.5% ± 11.7% vs. 36.3% ± 9.7% in controls) (Figure III-3E). To further confirm that these OPCs were indeed the result of *de novo* gliogenesis, I also assessed PDGFαR expression within the Sox2+ NSC/IPC population. Six hours after the injection of dystroglycan-blocking antibodies, I found that the proportion of Sox2+ cells co-expressing PDGFαR was nearly fourfold that of controls (38.2% ± 8.3% vs. 9.6% ± 4.4% in controls), a result consistent with an acute induction of gliogenesis by neural stem- and/or uncommitted progenitor cells (Figure III-3F). Increased PDGFαR expression within the SVZ subsided by 24 hours post-injection, returning to levels in line with control antibody-injected littermates and suggesting that the population of newly-formed oligodendrogenic progenitor cells had exited the SVZ. To further confirm the oligodendrocyte lineage identity of the PDGFαR+ cells, I performed IHC against the oligodendroglial transcription factor Olig2 and found greater numbers of Olig2+ cells 6 hours following the injection of dystroglycan-blocking antibodies (6.15 ± 2.15 x10^5 cells/mm^3 vs. 4.33 ± 3.03 x10^5 in controls) (Figure III-3G). In contrast, dystroglycan block

had no effect on the number of cells expressing Pax6 (26.3 ± 2.87 x 10^5 cells/mm^3 vs. 25.2 ± 2.44 x 10^5 in controls) (Figure III-3H,I), a transcription factor that represses *olig2* to promote neuronal fate in postnatal SVZ progenitors [258,259]. In addition to increased gliogenesis, the dystroglycan block also led to a twofold increase in Sox2+ cell proliferation, relative to controls (32.6% ± 2.0% vs. 15.7% ± 1.7% in controls) (Figure III-3J,K). Intriguingly, this effect was not observed until 24 hours post-injection, well *after* the induced NSC/IPC gliogenesis had concluded. These findings suggest that detachment from dystroglycan ligands deregulates the proliferation and maturation of RGCs and drives oligodendroglial fate specification in SVZ progenitors without an intermediate transit amplifying step. Overall, these results indicate that dystroglycan has multiple roles within the neonatal gliogenic SVZ, regulating both the development and structural integrity of the niche and the functional production of progenitors from the niche.

Dystroglycan loss delays the maturation of oligodendrocyte lineage cells

Newly produced OPCs migrate dorsally and tangentially out of the SVZ into the overlying corpus callosum, where they differentiate into oligodendrocytes (OLs), make contact with axons and begin to myelinate. To determine if the niche defects observed in the DAG cKO mice affected the development of oligodendroglial cells after exiting the SVZ, I performed IHC to detect PDGFαR, Sox2 and CC1 (a marker for mature oligodendrocyte cell bodies) on sections from DAG cKO and WT mice at P3 and P8 (Figures III-4A,D). At P3, although WT and DAG cKO mice had similar numbers of PDGFαR+ OPCs in the corpus callosum (Figure III-4B), OPCs derived from the DAG cKO SVZ displayed a less mature phenotype, with a significantly higher proportion of DG-deficient OPCs co-expressing the NSC/IPC marker Sox2 relative to

those in WT littermates (36.0% ± 3.9% vs. 22.1% ± 5.4% in WT) (Figure III-4C). These data suggested that OPCs in the DAG cKO corpus callosum at P3 were less mature. By P8, DAG cKO callosa contained more OPCs and OLs than those of WT littermates (39.6 ± 1.21 x 10^4 PDGFαR+ and 30.5 ± 1.24 x 10^4 CC1+ cells/mm^2 vs. 22.1 ± 4.20 x10^4 PDGFαR+ and 15.2 ± 0.75 x 10^4 CC1+ in WT) (Figure III-4E), though the OPC:OL ratio did not differ significantly (not shown). Furthermore, WT and DAG cKO OPC proliferation in the corpus callosum was similar (29.9% ± 5.7% vs. 34.7% ± 3.4% in WT) (Figure III-4F), indicating that the progenitor surplus in DAG cKO white matter is the product of SVZ glial overproduction. These data furthermore suggest that OPCs produced in the DAG cKO SVZ are not defective in their ability to migrate to the overlying corpus callosum, as OPC densities in the callosum were actually higher than those in WT littermates, i.e. OPCs were able to reach their target.

To assess the myelination capacity of OLs in DAG cKO white matter, I performed IHC to detect MBP in coronal sections taken from WT and DAG cKO mice at P8 and P21 (Figure III-4G). At P8, MBP expression was greatly reduced in the corpus callosa of DAG cKO mice relative to that in WT, despite the presence of higher numbers of mature OLs (Figure III-4E). Similarly, although the DAG cKO corpus callosum contained normal numbers of mature OLs at P21 (not shown), MBP immunofluorescence remained at lower levels than in WT littermates (Figure III-4G). Western blotting analysis of lysates taken from WT and DAG cKO cerebral cortices at P21 and 3 months reveal lower levels of MBP protein in the DAG cKO cortex at P21 (Figure III-4H). By 3 months, however, MBP expression in DAG cKO animals had normalized, indicating that dystroglycan loss delays, but does not prevent, the production of myelin components by DAG cKO OLs.

To further monitor the effects of dystroglycan loss on oligodendrogenesis and oligodendrocyte lineage progression, I isolated SVZ cells from WT and DAG cKO mice at P0 and cultured them as neurospheres. Second passage neurospheres were dissociated into single cells, plated on PDL and allowed to differentiate for 3 or 7 days. I then performed IHC to detect Sox2 (NSC/IPCs), PDGFαR (oIPC/OPCs), NG2 (OPCs), CNPase (mature OLs)and MBP (myelin) (Figures III-5A,D). In line with my *in vivo* observations, 3 days after growth factor removal, PDGFαR+ oIPC/OPCs originating from DAG cKO neurospheres retained Sox2 expression at a higher level than those from WT neurospheres (59.6% ± 6.1% vs. 25.0% ± 3.5% in WT) (Figure III-5B), indicative of the presence of more oIPCs relative to OPCs. Dystroglycan-deficient cultures also contained higher percentages of NG2+ OPCs (56.1% ± 4.5% vs. 39.7% ± 2.7% in WT) and, although a small percentage of CNP+ OLs had begun to differentiate from WT OPCs, almost no mature OLs were observed in DAG cKO cultures (1.1% ± 0.3% vs. 4.5%±1.4% in WT) (Figure III-5C). After 7 days of differentiation, a significantly higher percentage of DAG cKO cells persisted in the OPC stage (43.2% ± 5.1% vs. 25.1% ± 2.6% in WT), while of the percentage of mature CNP+ OLs was significantly lower than in WT cultures (5.2% ± 1.8% vs. 12.3% ± 3.2% in WT) (Figure III-5E). These results suggest that dystroglycan can promote the timely differentiation of oligodendrocyte lineage cells in a niche-independent manner. I did not, however, observe any significant proliferative changes in OPCs derived from DG-deficient neurospheres (not shown), indicating that increased OPC proliferation in the DAG cKO SVZ may be a direct result of the structural disruption of the niche.

DISCUSSION

Recent reports have demonstrated that the temporal and spatial regulation of integrin receptors is essential to SVZ niche maintenance and controlled neurogenesis. In the adult SVZ, mitotically active NSC/NPCs upregulate α6β1 integrin, which mediates adhesion to the vascular basal lamina. Blocking integrins causes cell detachment from SVZ blood vessels, leading to increased proliferation and precocious neuronal differentiation [175,193,245]. In contrast, the regulation of ECM signaling and its significance in postnatal gliogenesis are not understood. Thus, while laminin and dystroglycan are known promoters of oligodendroglial survival, differentiation and myelination [255,238,256,243,257,244], the role of ECM in oligodendroglial fate determination remains unknown.

I analyzed oligodendrocyte progenitor types (oIPC and OPCs) in the early postnatal SVZ and corpus callosum of WT and DAG cKO mice, finding that an initial delay in gliogenesis was followed by overproduction of oIPC/OPCs in the P3 dystroglycan-deficient SVZ. Enhanced oligodendrogenesis was accompanied by a significant increase in oIPC/OPC proliferation within the SVZ of DAG cKO mice, leading to higher numbers of OPCs in the P8 corpus callosum. Although dystroglycan-deficient OPCs were able to differentiate, the resulting oligodendrocytes did not myelinate collosal axons in the correct developmental window.

As was the case in ependymal cell development, I found that the ability of dystroglycan to promote the production and differentiation of oligodendrocyte lineage cells is niche-independent, as neurospheres isolated from P0 DAG cKO mice and differentiated on the non-physiological substrate PDL also gave rise to higher numbers of OPCs that appeared to be stalled at the point of differentiation. Thus, while DAG cKO oligodendrocyte densities were higher than normal in vivo, they were lower than normal using the neurosphere assay. The observed

disconnect in dystroglycan-dependent oligodendrocyte differentiation *in vitro* and *in vivo* may result from the absence of proliferative abnormalities in cultured DAG cKO OPCs (not shown) or the contribution of other factors present *in vivo*.

In antibody injection experiments, I found that even the transient block of dystroglycan ligand binding was sufficient to induce rapid oligodendroglial fate specification in early postnatal SVZ progenitors. The short timeframe in which this event occurred, coupled with the lack of concomitant SVZ proliferation, suggests that dystroglycan loss drove the direct acquisition of OPC identity by SVZ niche cells. Together, these results demonstrate that dystroglycan is a critical regulator of fate specification in postnatal neural progenitors, both within the developing SVZ and in the absence of niche ECM structures or other extrinsic cues. The increased production of OPCs by the dystroglycan-deficient SVZ may result from the increased responsiveness of postnatal RGCs to gliogenic mitogens such as PDGF [226,227], FGF [228], EGF [230,231] or IGF [260,243,261] . The PDGF and FGF signaling pathways are particularly attractive candidates, as both have also been shown to inhibit oligodendrocyte differentiation and myelination separately and by FGF-dependent maintenance of PDGFαR expression in OPCs [232-234]. Alternatively, the results of several studies suggest that increased Notch signaling could contribute to the phenotypes reported here following dystroglycan loss-of-function. Firstly, constitutive Notch signaling was shown to increase BLBP+ expression while inhibiting RGC proliferation in the late embryonic VZ [262], a result similar to our finding of delayed maturation and decreased proliferation in P0 DAG cKO radial glia. Notch has also been shown to promote oligodendroglial fate during gliogenic periods [222,223], with constitutive activation leading to the overproduction of OPCs [224]. Importantly, though Notch signaling promotes gliogenesis, it simultaneously acts to inhibit oligodendrocyte terminal differentiation and myelination [225]. This

could help to explain why dystroglycan loss results in delayed oligodendrocyte differentiation *in vitro* but not *in vivo*, as cultured OPCs remain in closer proximity to potential sources of Notch ligands.

Together, these results suggest that dystroglycan continues to regulate oligodendrocyte lineage progression outside of a structured, ECM-rich niche. From gliogenesis to the myelination of axons, dystroglycan function is critical to the production of a competent oligodendrocyte pool.

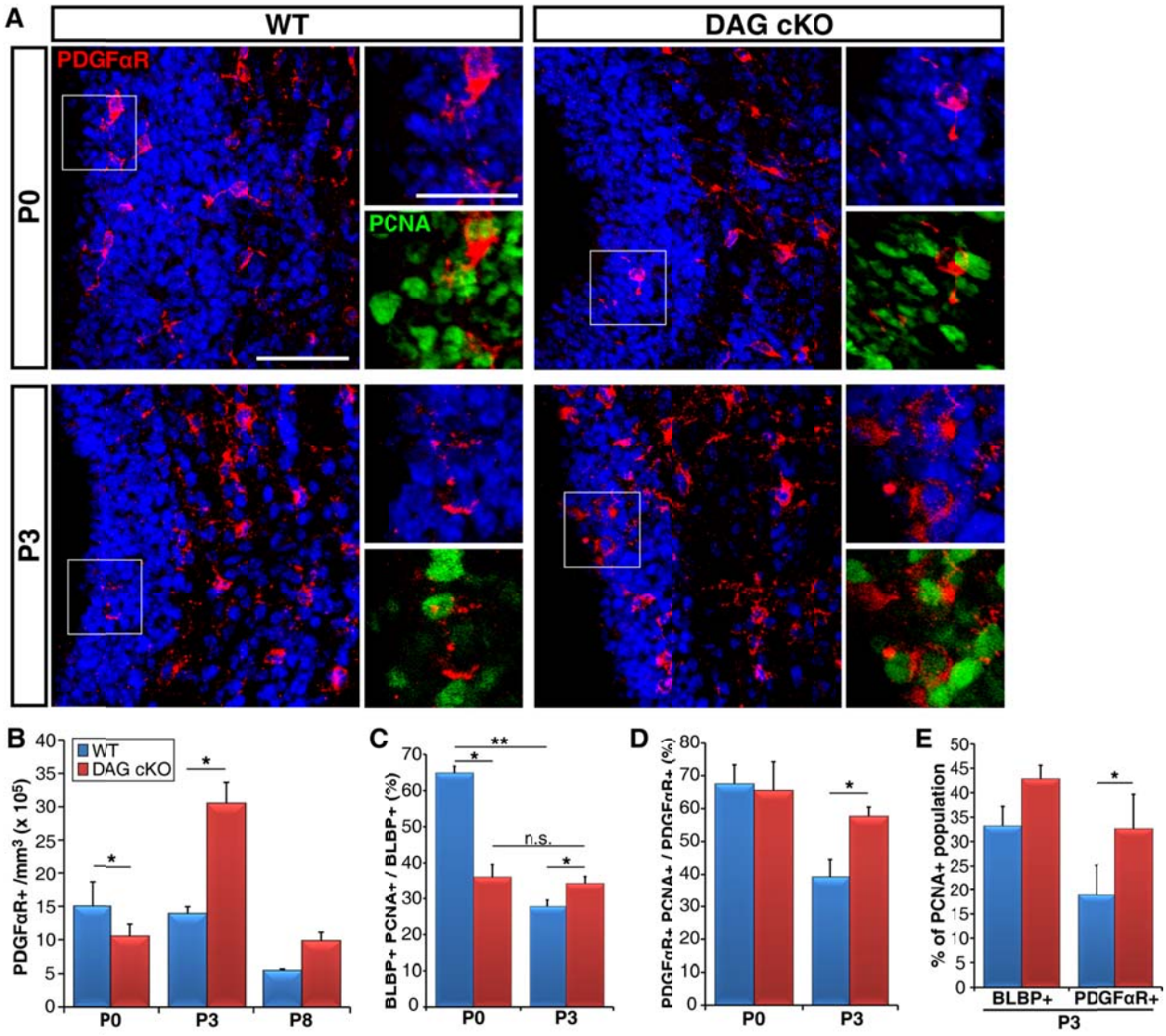


Figure III-1. Dysregulated Gliogenesis in the Dystroglycan cKO SVZ

(A) IHC staining of the dorsal SVZ in coronal sections from P0 and P3 WT and DAG cKO mice. Gliogenesis is delayed in the DAG cKO SVZ, with fewer OPCs present at P0 (top panels) but no decrease in OPC proliferation (insets). At P3 (bottom panels), OPC numbers in DAG cKO mice far exceed those of WT littermates and newborn DAG cKO OPCs are more proliferative. (B) Quantification of PDGFαR+ cell density in the SVZ of P0, P3 and P8 WT and DAG cKO mice. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. (C) Quantification of BLBP+ cell proliferation at P0 and P3 in WT and DAG cKO mice. *$p < 0.05$, **$p < 0.01$, Student's t-test; error bars, SEM; n=3. (D) Quantification of PDGFαR+ cell proliferation in P0 and P3 WT and DAG cKO mice. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. (E) Quantification of BLBP+ and PDGFαR+ cells within the PCNA+ population proliferation in P3 WT and DAG cKO mice. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. Scale bars: 50µm, insets 25µm.

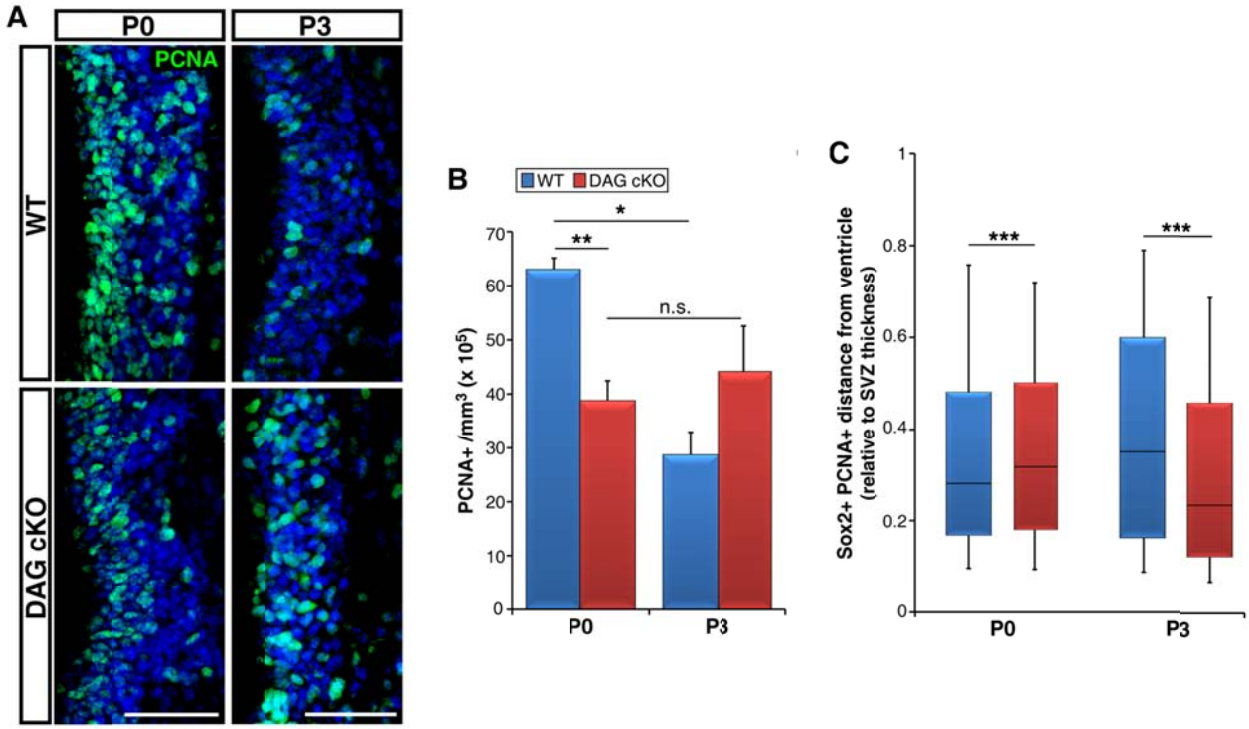


Figure III-2. Neural stem and progenitor cell proliferation is dysregulated in the DAG cKO SVZ

(A) IHC staining of coronal sections from P0 and P3 WT and DAG cKO mice. While stem and progenitor cell proliferation in WT mice is high at P0 and decreases sharply by P3, proliferation in the DAG cKO SVZ is greatly reduced at P0 and does not change significantly from P0 to P3. **(B)** Quantification of PCNA+ cell density in the SVZ of WT and DAG cKO mice at P0 and P3. *$p < 0.05$, **$p < 0.01$, Student's t-test; error bars, SEM; n=3. **(C)** Quantification of sox2+ PCNA+ nuclear distance from the lateral ventricle, relative to total SVZ thickness, in P0 WT and DAG cKO mice. ***$p < 0.001$, Wilcoxon rank sum test; error bars, 90^{th} and 10^{th} percentiles; n=3.

Figure III-3. Gliogenesis is increased following ventricular injection of dystroglycan blocking antibodies

(A) Schematic of antibody injection protocol. P2 rat pups were given single injections of control or DG-blocking antibody into the lateral ventricle and sacrificed 6 or 24 hours later. **(B)** IHC staining of whole mounts taken from rats 6 and 24 hours after the injection of control or DG-blocking antibodies. DG block led to the dispersal of ventricular surface laminin anchors 6 hours post-injection. At 24 hours following DG block, small laminin clusters reappeared on the apical surfaces of ependymal cells. **(C)** IHC staining of the dorsal SVZ in coronal sections taken from control or blocking antibody-injected rats 6 hours post-injection. DG block led to a rapid increase in gliogenesis, with higher numbers of OPCs observed in the SVZ and increased expression of OPC markers by Sox2+ NSCs/NPCs. This increase in OPC numbers was not linked to altered OPC proliferation and had subsided by 24 hours post-injection. **(D)** Quantification of OPC density in the SVZ 6 hours after the injection of control or DG-blocking antibodies. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. **(E)** Quantification of PDGFαR+ OPC proliferation in control and DG-blocking antibody-injected rats 6 hours post-injection. Error bars, SEM; n=3. **(F)** Quantification of PDGFαR expression by Sox2+ NSC/NPCs 6 and 24 hours after control or DG-blocking antibody injection. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. **(G)** Quantification of Olig2+ cell density in the SVZ 6 hours after the injection of control or DG-blocking antibodies. **$p < 0.01$, Student's t-test; error bars, SEM; n=3. **(H)** IHC staining of the dorsal SVZ in coronal sections taken from control or blocking antibody-injected rats 6 hours post-injection. DG block had no effect on the number of Pax6+ neuronal progenitor cells. **(I)** Quantification of Pax6+ cell density 6 hours after the injection of control or DG-blocking antibodies. Error bars, SEM; n=3. **(J)** IHC staining of coronal sections from rats injected with control or DG-blocking antibodies at P2 and sacrificed 24 hours later. DG block led to increased NSC/NPC proliferation 24 hours post-injection, after NSC/NPC gliogenesis had ceased. **(K)** Quantification of Sox2+ cell proliferation 24 hours after injection of control or DG-blocking antibodies. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. Scale bars: 25μm (B), 50μm (C,G,I).

Figure III-3. Gliogenesis is increased following ventricular injection of dystroglycan blocking antibodies

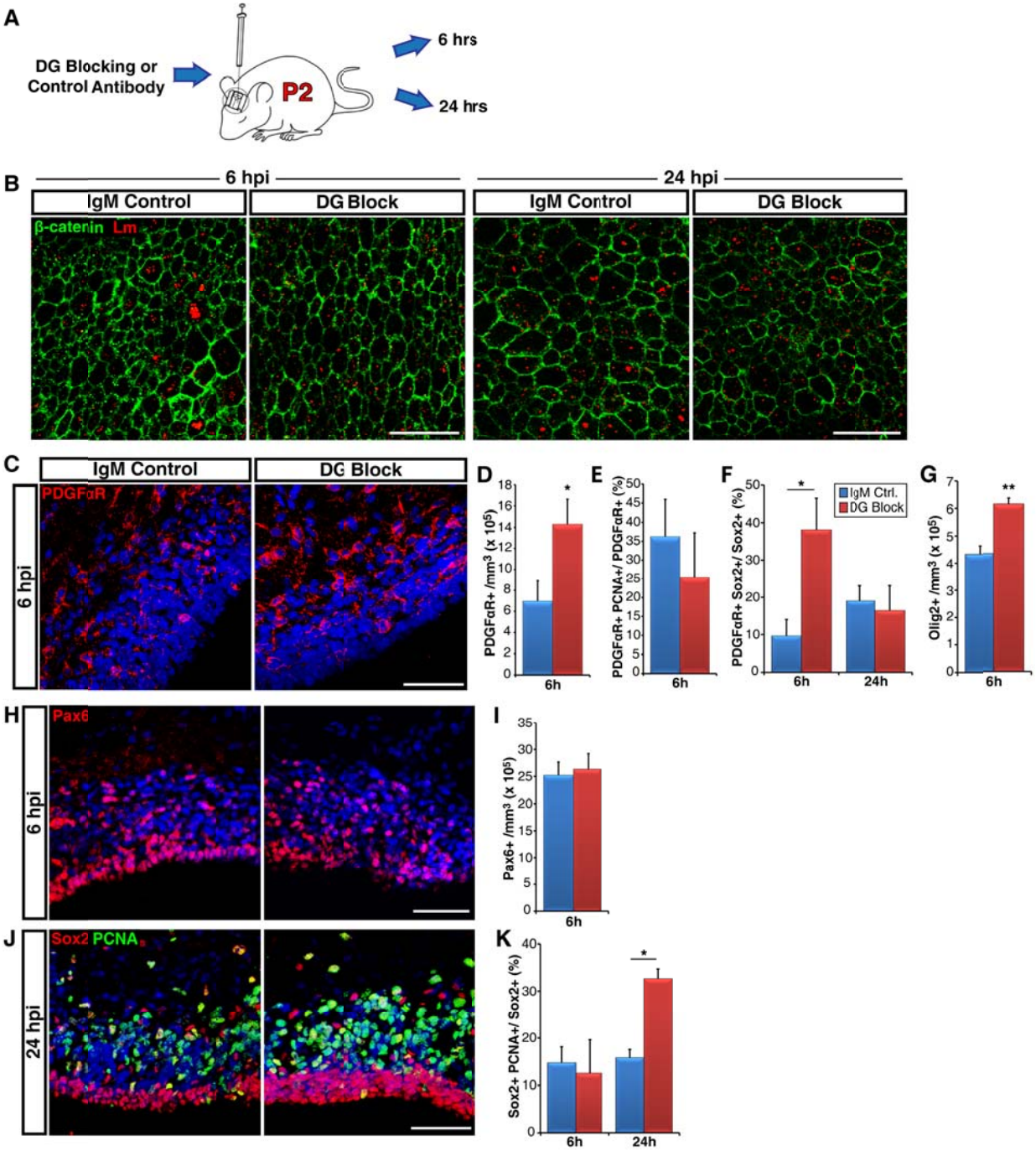
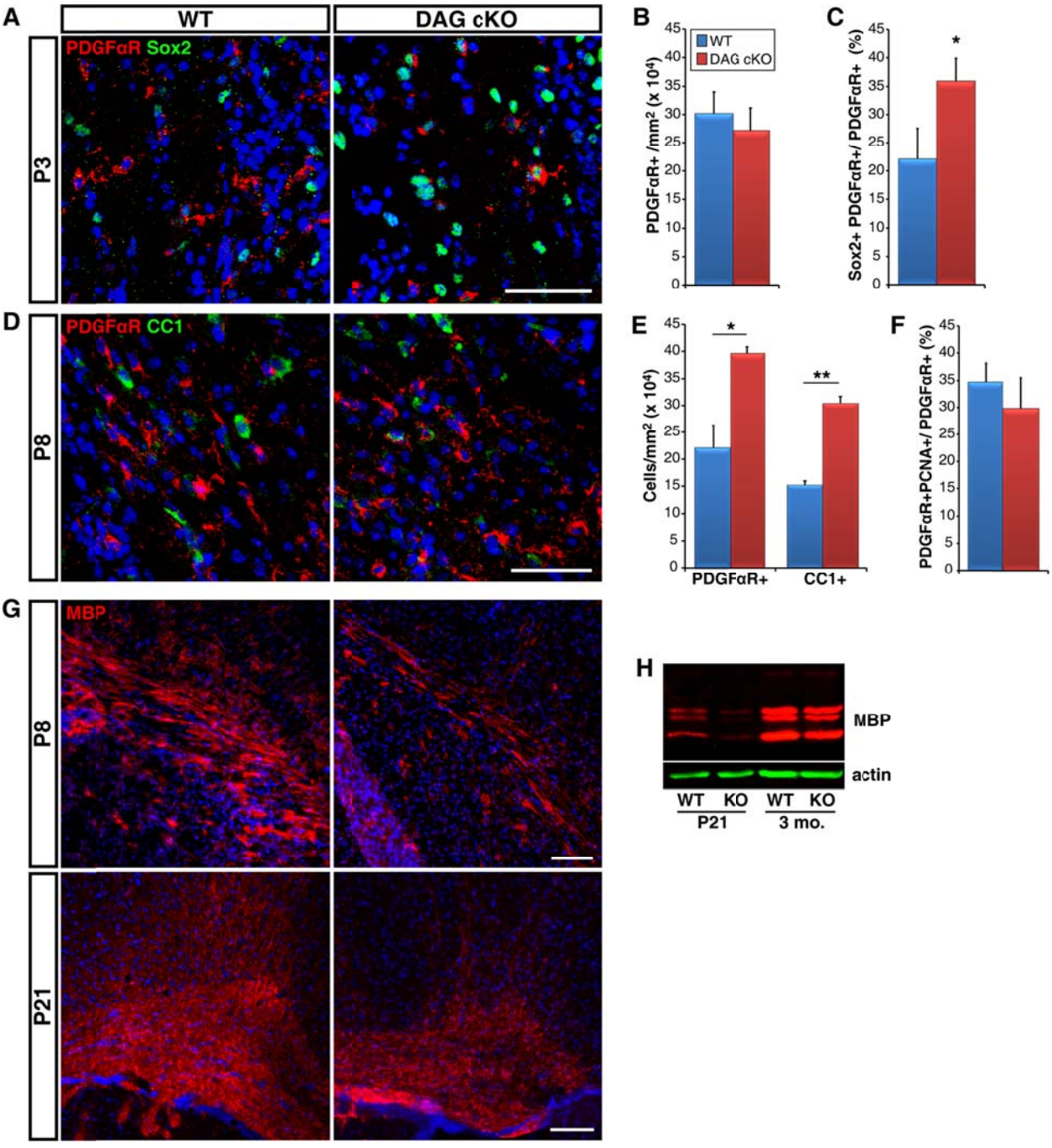


Figure III-4. Dystroglycan promotes timely oligodendrocyte lineage progression

(A) IHC staining of the corpus callosum in coronal sections from P3 WT and DAG cKO mice. Although similar numbers of OPCs were present in the corpus callosa of DAG cKO mice, more OPCs retained Sox2 expression, indicative of a less mature phenotype. **(B)** Quantification of PDGFαR+ OPC density in the corpus callosum of P3 WT and DAG cKO mice. Error bars, SEM; n=3. **(C)** Quantification of Sox2 expression in PDGFαR+ OPCs in the corpus callosum of P3 WT and DAG cKO mice. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. **(D)** IHC staining of the corpus callosum in coronal sections from P8 WT and DAG cKO mice. At P8, the corpus callosa of DAG cKO mice contained more OPCs and mature oligodendrocytes, without an associated decrease in OPC:OL ratio (not shown). There was no significant difference in OPC proliferation between WT and DAG cKO mice, indicating that the increased OPC numbers in the DAG cKO corpus callosum are the result of earlier OPC overproduction. **(E)** Quantification of PDGFαR+ and CC1+ cell density in the corpus callosum of P8 WT and DAG cKO mice. *$p < 0.05$, **$p < 0.01$, Student's t-test; error bars, SEM; n=3. **(F)** Quantification of PDGFαR+ OPC proliferation in the corpus callosa of P8 WT and DAG cKO mice. Error bars, SEM; n=3. **(G)** IHC staining of the corpus callosum in coronal sections from WT and DAG cKO mice at P8 and P21. Myelination is delayed in DAG cKO mice, with reduced MBP immunofluorescence apparent at both P8 and P21. **(H)** Western blot analyses of MBP protein levels in cortical lysates from WT and DAG cKO mice at P21 and 3 months. Scale bars: 50 μm (A,D), 100 μm (G).

Figure III-4. Dystroglycan promotes timely oligodendrocyte lineage progression



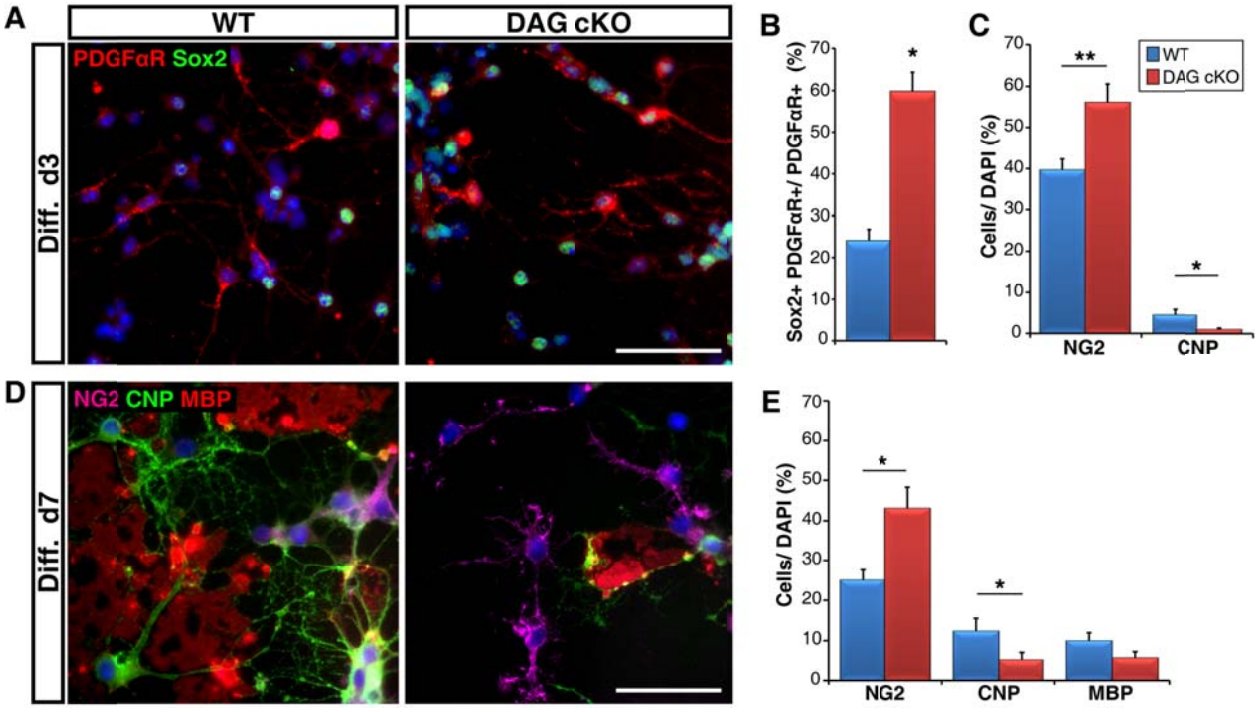


Figure III-5. Delayed maturation of dystroglycan cKO oligodendrocytes *in vitro*

(A) IHC staining of WT and DAG cKO cells dissociated from second passage neurospheres and differentiated on PDL for 3 days. OPCs derived from DAG cKO neurospheres exhibited persistent Sox2 expression, delaying their differentiation into mature OLs. **(B)** Quantification of Sox2+ cells within the PDGFαR+ OPC population in WT and DAG cKO cultures, 3 days after growth factor removal. *$p < 0.05$, Student's t-test; error bars, SEM; n=3. **(C)** Quantification of NG2+ OPCs and CNP+ OLs in cultures from WT and DAG cKO neurospheres, 3 days after growth factor removal. *$p < 0.05$, **$p < 0.01$, Student's t-test; error bars, SEM; n=7. **(D)** IHC staining of cells dissociated from WT and DAG cKO neurospheres and differentiated on PDL for 7 days. A week after growth factor removal, a greater proportion of DG-deficient oligodendrocyte lineage cells remain stalled at the progenitor stage, leading to a subsequent reduction in the number of post-mitotic OLs. **(E)** Quantification of NG2+ OPCs, CNP+ OLs and MBP+ myelin-competent OLs in cultures derived from WT and DAG cKO neurospheres, 7 days after growth factor removal. *$p < 0.05$, Student's t-test; error bars, SEM; n=4. Scale bars: 50 µm.

CHAPTER IV: CONCLUSIONS AND FUTURE CONSIDERATIONS

In this work, the developmental regulation of laminin expression and localization in the early postnatal SVZ was examined, as was the contribution of the ECM receptor dystroglycan to ependymal niche maturation and postnatal SVZ oligogliogenesis. I found that laminin is highly expressed in the neonatal SVZ and is closely associated with the apicolateral surfaces of developing ependymal cells, as well as in complex extra-vascular ECM structures unique to the early postnatal niche. Using transgenic and antibody-blocking approaches, I have shown that ECM-dystroglycan interactions are critical for the timely differentiation of ependymal cells from postnatal RGCs and to the proper organization of neural stem- and ependymal cells into niche pinwheel structures. Furthermore, I have demonstrated that dystroglycan function is necessary for the controlled production of oligodendrocyte lineage cells from SVZ progenitors and to the timely maturation of OPCs into myelin-competent mature oligodendrocytes.

Putative roles of cell-ECM interactions in the developing and adult VZ/SVZ niche are often described in terms of adhesion. That is to say, ECM ligands and receptors serve to anchor NSCs/NPCs in a configuration that *supports* niche structure and function, without being directly involved in the regulation of cellular proliferation or fate decisions. For example, the loss of RGC attachment to the pial basal lamina, and the associated disruption of the neuronal migratory scaffold the RGC basal process provides, is thought to produce the severe brain abnormalities observed in patients with secondary dystroglycanopathies and in dystroglycan-deficient mouse models[160,41,246]. The results of the current study, however, suggest that the consequences of dystroglycan loss-of-function during cortical development are far more pervasive than previously assumed. Pial basement membrane discontinuity and radial glial detachment have been observed

in multiple mouse models in which ECM ligand-receptor interactions are abrogated (α6 integrin$^{-/-}$, perlecan$^{-/-}$, laminin γ1 nidogen-binding site mutation), yet none of these mutations have been found to impact embryonic RGC polarity, proliferation or fate [254]. Here I have shown that brain-specific deletion of dystroglycan results in profoundly dysregulated RGC proliferation, with rates well below WT levels at birth. While WT RGCs began to mature between P0 and P3, as evidenced by reduced BLBP expression and decreased proliferation, DAG cKO RGC numbers and proliferation rates remained constant. As the loss of dystroglycan also led to reduced numbers of iOPCs in the SVZ at P0, together these results could reflect a delay in a developmental program in which RGC proliferation increases at the onset of gliogenesis. However, while RGC proliferation did not decrease between P0 and P3 in DAG cKO mice, neither did it increase with the surge of oIPC production witnessed at P3.

In contrast to the RGC proliferative changes I observed, a study in chick retinal neuroepithelial cells showed that RNAi knockdown of dystroglycan by *in ovo* electroporation resulted in hyperproliferation and increased neurogenesis. However, expression of a non-cleavable form of dystroglycan (permanent α-β subunit interaction) led to decreased proliferation and neurogenesis. In this condition, neuroepithelial cell nuclei were found to be concentrated at the basal side, suggestive of disrupted INM [263]. Although these sets of data are somewhat conflicting, it is likely that, like the expression patterns of dystroglycan, the niche response to dystroglycan signaling varies with developmental stage, mode of cell division, local ECM composition and receptor complement, etc. It has been shown that dystroglycan mRNA and protein expression in mammary epithelial cells is cell cycle-dependent and that dystroglycan depletion led to an accumulation of cells in S-phase and decreased differentiation [264]. I found that

Sox2+PCNA+ cells in the P0 DAG cKO SVZ were located more basally than those in the WT, which could suggest a cell cycle stall at S-phase and a subsequent disruption of INM.

There is also some *in vivo* support for a more complex role in the mitotic machinery: dystroglycan has been shown to interact with the Lis1-Nde complex in embryonic RGCs, linking microtubule structures to the actin cytoskeleton and ECM to stabilize the RGC lateral membrane and anchor the mitotic spindle to the cell cortex [124]. Lis1-Nde deficient RGCs had mislocalized and decreased levels of dystroglycan, leading to the randomization of mitotic spindle orientation and an increase in asymmetric divisions. A previous characterization of RGC defects in the Lis1-Nde deficient mouse also reported defects in centrosome duplication [158], a phenotype also observed following dystroglycan depletion in myoblasts [49]. In that system, the β-dystroglycan ICD acts as a nuclear scaffold, interacting with lamin B1 and emerin to regulate their localization and stability at the nuclear envelope. Dystroglycan knockdown resulted in abnormal nuclear morphology, disrupted centrosome-nucleus linkage and the overproduction of centrosomes. Centrosomes are asymmetrically segregated in neurogenic divisions, with the RGC retaining the mother centrosome and the differentiating daughter cell inheriting the daughter centrosome [265]. Centrosomal abnormalities are associated with the loss of cell polarity, defective cell division, and abnormal chromosome segregation (reviewed in [266]. Experiments assessing embryonic/ early postnatal RGC behavior following electroporation with dystroglycan siRNA in combination with live imaging of transfected cells would provide a much clearer picture of the proliferative abnormalities seen in DAG cKO mice. Detailed cell cycle analysis using transgenic constructs that modify, for example, extra- and intracellular cleavage sites, specific ICD interaction sites or the nuclear localization sequence may give additional insight into the functional contribution of different intracellular binding partners.

In an attempt to uncouple the RGC proliferation defects from the increased oligogliogenesis I observed in the DAG cKO SVZ, I performed a single intraventricular injection of antibodies that competitively inhibit dystroglycan-ligand interaction into P2 rats. I found that transient dystroglycan block led to a rapid increase (6 hours post-injection) in the number of oIPCs within the SVZ, with no increase in iOPC proliferation. Dystroglycan block also led to a significant increase in Sox2+ progenitor proliferation, but not until 24 hours post-injection, well after the oIPC numbers had returned to normal. This result indicates that dystroglycan loss-of-function can drive oligodendroglial fate specification in SVZ progenitors without an additional proliferation step and in the absence of previous developmental abnormalities. Several studies have demonstrated that protracted cell cycle progression itself is often sufficient to induce neurogenic divisions and/or cell cycle exit in NECs and RGCs [267,268]. In addition to the aforementioned study, dystroglycan has also been found to localize to the mitotic spindle, cleavage furrow and midbody in a variety of cell types, with depletion resulting in stalled G2/M (REF52, HeLa, Swiss 3T3 cells) or G0/G1 (PC12 cells) transition [269,270]. Given the short time frame in which large numbers of oIPCs were produced, an abrupt exit of SVZ progenitors from the cell cycle and acquisition of the "default" oligodendroglial fate may be the most plausible explanation. Another possibility would a large increase in oligodendrogenic growth factor signaling, such as that of PDGF or EGF. Dystroglycan has been shown to recruit the adaptor protein Grb2 to the cell membrane [45]. Grb2 and dystroglycan have been found to complex with focal adhesion kinase (FAK) [271] in oligodendrocytes [244] and dynamin in CNS cell membrane ruffles, an interaction that has been implicated in the regulation endocytosis [47]. As Grb2 and dynamin are known to participate in the endocytosis of EGFR [272-275] and PDGFaR [276] it would be

interesting to determine if dystroglycan loss-of-function amplified receptor tyrosine kinase signaling via reduced endocytosis of these receptors.

Dystroglycan is also thought to modulate the MEK/ERK pathway, which regulates proliferation, differentiation and cell survival. In different systems it has been proposed to act by competitive inhibition of integrin-mediated ERK activation[277] and by sequestering MEK, preventing it from phosphorylating ERK[43]. Additionally, an ERK binding motif has been identified within the β-dystroglycan ICD itself (ELM prediction) [42]. ERK signaling has been shown to promote the proliferation of NSCs/NPCs *in vivo* and *in vitro* (Li et al., 2001) (Campos et al., 2004) (Matsumoto et al., 2006; Shioda et al., 2008; Staquicini et al., 2009). Preliminary data suggest that neurospheres isolated from perinatal DAG cKO mice may have increased levels of ERK phosphorylation that persists during neural stem and progenitor cell differentiation (not shown). Research from our group has demonstrated that dystroglycan promotes IGF-1-dependent MAPK activation in oligodendrocytes and that loss of dystroglycan prevented MAPK activation and oligodendrocyte differentiation[243].

It is unclear why the lack of dystroglycan during development results in decreased RGC proliferation while the transient block of DG ligand binding leads to an increase. I did not observe proliferative changes in DAG cKO neurosphere cultures or in ependymal cultures following dystroglycan block, suggesting that this phenotype may be niche specific. It has been reported that the the half-life for turnover of cell-surface-expressed dystroglycan is approximately 12 hours[264]. I found that increased Sox2+ cell proliferation was detectable from approximately 12 hours following dystroglycan block and increased significantly by 24 hours post-injection, which could potentially correlate to the timeline of dystroglycan turnover and

restored function. It would be important to determine if exogenously expressed dystroglycan could rescue the decreased RGC proliferation seen in neonatal DAG cKO mice.

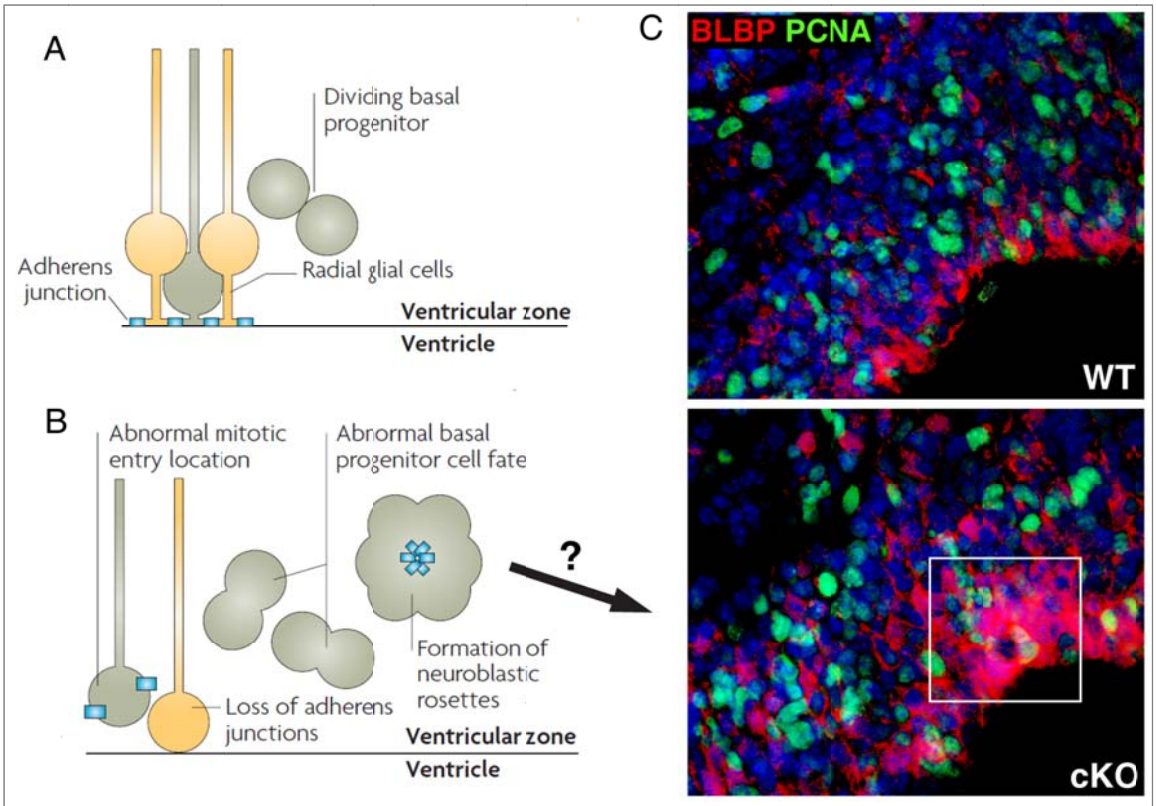


Figure IV-2. Potential outcomes of altered expression or localization of AJ and polarity complex proteins

A) NECs and RGCs interact via adherens junctions (AJs) and divide at the ventricular surface. **B)** Abnormal expression or localization of AJ or polarity proteins can result in ectopic mitosis, hyperproliferation, alterations in basal (intermediate) progenitor fate and the formation of neuro(/glio)blastic rosettes. Actively dividing cells are shown in grey. Adapted from[7]. **C)** Coronal sections from P3 WT and DAG cKO mice. Several of these features were observed in the early postnatal DAG cKO SVZ, including occasional clustering of BLBP+ RGCs into rosette-like structures.

Apico-basal polarity in RGCs is established largely by the response of intracellular polarizing factors to extracellular cues, such as apical cell-cell adhesion and interaction with the ECM, and is integral to proper RGC function. The correct organization of the apical domain is of particular importance for maintaining the balance between self-renewing divisions and those that produce progenitors. One result of cell-cell contact is the specification of apical and basolateral

domains by, respectively, the aPKC-PAR-3-PAR-6 complex and the threonine kinase Par-1 [98]; [99]. During neurogenic RGC divisions, the asymmetric inheritance of Par3 supports the maintenance of stem cell characteristics through its persistent activation of Notch signaling [278]. Conversely, Par-1 has been found to promote epithelial differentiation by both driving asymmetric mitotic spindle orientation [279] and repressing Notch signaling in basal progenitors [280,281]. Par-1 also enables interphase epithelial cells to play a more active role in multicellular niche polarity, through regulating the extracellular assembly of laminin. Importantly, this process is mediated through the stabilization and localized targeting of dystroglycan, and is required for apical domain reconstruction in response to changes in ECM [81]. This finding raises the possibility that dystroglycan contributes to the dynamic regulation of niche polarity and maturation in response to the extensive ECM remodeling we have observed during ependymal niche development (Figure IV-2). Indeed, the expression and proper localization of dystroglycan is required for laminin clustering in a variety of cell types [71,72,75,116,248,82]. In skeletal muscle, where dystroglycan function is best described, the adaptor protein ankyrin3 (ankyrinG) has been identified as one β-dystroglycan binding partner required for its targeted localization [180]. Ank3 is one of few known organizers of ependymal niche construction; its upregulation in the lateral membrane of ependymal-specified RGCs is required for ependymal cell maturation and the formation of niche pinwheel structures [179]. The ependymal niche phenotype resulting from loss of Ank3 is reminiscent of the disturbances I observed following dystroglycan loss-of-function, suggesting that a failure of dystroglycan localization downstream of Ank3 might mediate some of these effects. However, in contrast to the results presented here, Ank3 loss did not impact SVZ proliferation or the downregulation of RGC markers, possibly due to dystroglycan's wider expression patterns and mechanistic diversity. These results do, however, further support the

assertion that the delayed ependymal differentiation and niche construction phenotypes observed in DAG cKO mice are likely not responsible for the proliferation and maturation defects in dystroglycan-deficient RGCs. In concert with my finding that the *in vivo* ependymal maturation defects could be recapitulated *in vitro* by blocking DG-ligand interactions solely during ependymal cell differentiation, the results of the Ank3 study suggest that the RGC and ependymal cell phenotypes I observed are the result of independent mechanistic disturbances.

Extracellular matrix regulation of gap junctional proteins is another potential avenue of interest. Laminin has been shown to modulate the transcription and protein expression of several connexins in a variety of cell types, including neural progenitors and OPCs [282-285]. Upon oligodendroglial specification, oIPCs downregulate neural progenitor-associated connexins and begin to express those associated with mature OL (Cx29, Cx32, Cx47) [286]. Of these, Cx32 has been shown to actively promote OL differentiation, as its loss results in delayed OPC differentiation [287]. When oIPCs derived from postnatal hippocampal neurosphere cultures were grown in the absence of laminin, they expressed Cx32 mRNA but not protein. Exposure to laminin induced Cx32 protein expression and an increase in OL differentiation [282]. On the other hand, Cx36 mRNA is present in NSCs during early neurogenesis but becomes restricted to neuroblasts toward the end of the neurogenic period [288,289]. Intriguingly, exposure to laminin in the same postnatal culture system induced Cx36 protein expression in mature neurons, suggesting that laminin-induced changes in connexin expression patterns may regulate neural/glial fate decisions [282]. Furthermore, the adhesive functions of Cx26 and Cx43 are required for neuronal migration along RGCs during cortical development, where they interact with cytoskeletal elements to stabilize the leading process of the neuroblast and adhere the centrosome to the cell cortex to facilitate nuclear translocation [290], roles that overlap significantly

with those attributed to dystroglycan in the same and similar contexts. In the adult SVZ, gap junctions link type B- and ependymal cells which, like their RGC precursors, express Cx26 and Cx43 [291]. Although the precise function of gap junction adhesion/signaling in ependymal niche cells is not known, it may well play a role in niche assembly and/or the regulation of adult NSC behavior. Given that laminin hubs are found at type B-ependymal cell interfaces in mature pinwheels and that their assembly and relocation occur concurrent with niche building, perhaps laminin interactions contribute to gap junction formation or maintenance mediated, directly or indirectly, by dystroglycan.

In conclusion, we have identified the extracellular matrix receptor dystroglycan as a critical regulator of niche structure and function in the early postnatal VZ/SVZ. It is becoming clear that the spatial and temporal regulation of ECM receptor expression is an important mechanism by which niche cells can modify their relationship to the extracellular environment. However, to our knowledge, this study represents the first time an ECM receptor has been shown to regulate both niche construction and output. Further investigation of dystroglycan function has the potential to reveal novel downstream targets, which could be harnessed to manipulate niche output to aid in tissue repair.

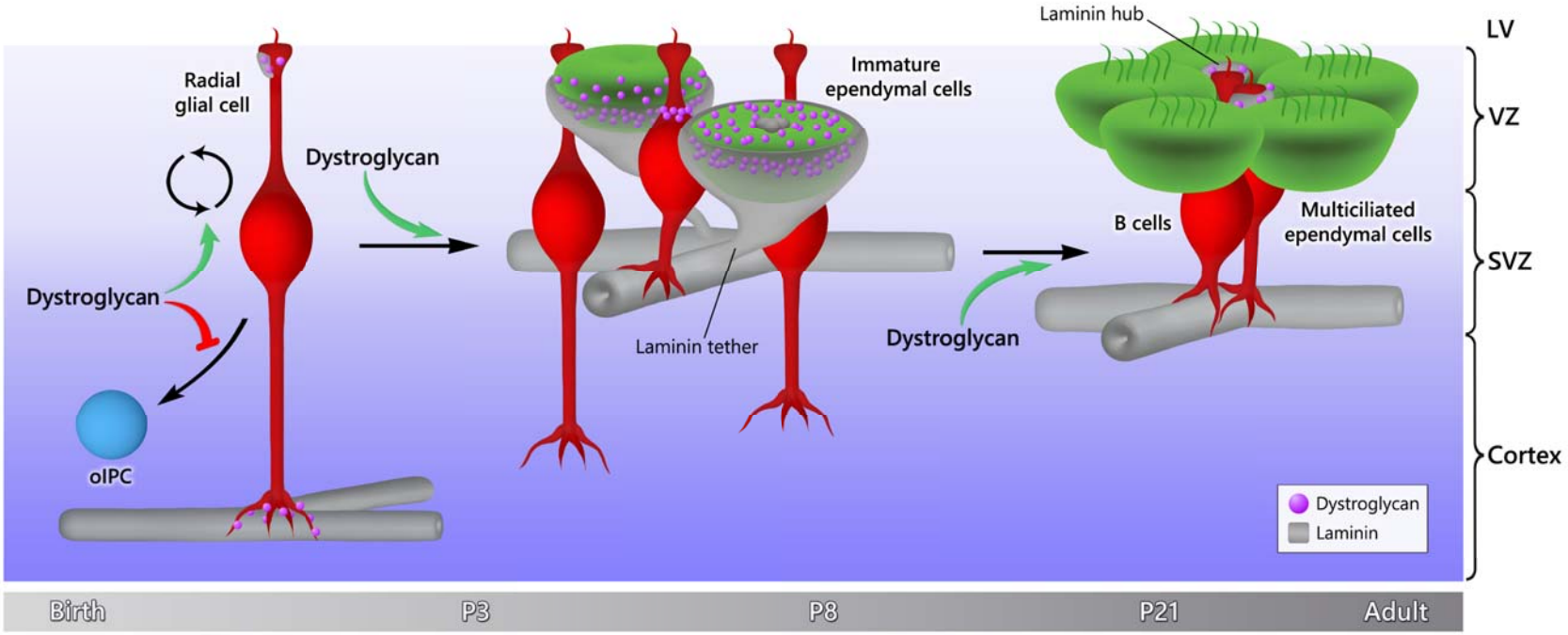


Figure IV-1. Dystroglycan regulates the structural and functional development of the SVZ neural stem cell niche

A) Dystroglycan promotes the assembly of laminin i) on the apico-lateral surface of developing ependymal cells and ii) into extra-vascular ECM tethers that link ependymal cell-associated laminin to the vascular basal lamina. Later in ependymal development, cell surface-associated laminin clusters to form hubs, which are ultimately targeted to the ependymal cell-B cell interface at the center of adult niche pinwheel structures. **B)** Dystroglycan interactions support the controlled proliferation of early postnatal RGCs, their timely maturation into ependymal cells and the reorganization of ependymal- and B cells into pinwheels at the ventricular surface. **C)** Dystroglycan regulates the production of oligodendrogenic precursors by niche stem and progenitor cells and oligodendrocyte precursor cell proliferation in the early postnatal SVZ.

MATERIALS AND METHODS

Generation of Nestin-cre; DAG$^{Flox/Flox}$ mice

The generation of *Nestin-cre; DAG$^{Flox/Flox}$ mice* was previously described [249].

SVZ Wholemount Dissection and Immunohistochemistry

SVZ wholemounts were dissected as described [170]. Briefly, the striatal wall of the lateral ventricle was dissected from Nestin-Cre/DAG-flox mice, their wild-type littermates or FoxJ1-GFP mice, fixed with cold 4% paraformaldehyde in 0.1 M PBS for 12 hours at 4°C and washed with PBS prior to staining.

To visualize antigens at the ventricular surface, wholemounts were blocked in 10% donkey serum with 0.2% Triton-X100 and incubated with primary and secondary antibodies for 24 hours at 4°C. For deeper structures, wholemounts were blocked with 10% donkey serum with 2% Triton-X100 and incubated in primary and secondary antibodies for 48 hours.

Frozen Tissue Processing and Immunohistochemistry

Animals were intra-cardially perfused and brains post-fixed with 4% Paraformaldehyde in 0.1M PBS. Brains from mice younger than P14 were immersion-fixed in 4% PFA. Tissue was cryo-protected with 30% sucrose in PBS, embedded in OCT medium and frozen in dry ice cooled with isopentane. 18 μm sections were prepared on a cryostat. Sections were blocked in 10% donkey serum with 0.1% Triton X-100, incubated with primary antibodies diluted in blocking solution overnight at 4°C and incubated with appropriate fluorophore-conjugated secondary antibodies (Jackson) in blocking solution at room temperature for 2 hours.

Antibodies

The following primary antibodies were used:

Rabbit anti- γ-tubulin (Sigma-Aldrich T5192, 1:500), mouse anti-β-catenin (BD Transduction 610153, 1:500), mouse IgM anti-α-Dystroglycan, clone IIH6C4 (Upstate Cell Signaling 05-593, 1:50- 1:100), mouse anti-PCNA (Cell Signaling Technology, 1:200), rabbit anti-Sox2 (Millipore AB5603, 1:100), chicken anti-Nestin (Aves Labs NES, 1:500), mouse anti-Nestin (Developmental Studies Hybridoma Bank Rat-401, 1:5), rat anti-MBP (Serotec MCA409S, 1:100), mouse anti-CNPase (Sigma C5922, 1:100), rabbit anti-NG2 (Chemicon International AB5320, 1:200), mouse anti-APC (CC-1) (Calbiochem OP80, 1:100), rabbit anti-GFAP (DakoCytomation Z0334, 1:500), rat anti-PDGFRα (CD140a) (BD Pharmingen 558774, 1:100), rabbit anti-PDGFRα (Santa Cruz SC-338, 1:150), rabbit anti-Laminin (Sigma L9393, 1:100), chicken anti-GFP(Aves GFP, 1:500), rat anti-CD24 (BD Biosciences 557436, 1:100-1:200).

Cell Culture

For neurosphere and ependymal cell culture experiments, the lateral ventricular wall was dissected from P0-1 mice and mechanically dissociated in MEM with 25mM HEPES (Lonza) with 1% Pen/Strep (Mediatech).

For neurosphere differentiation assays, cells isolated from Nestin-cre/DAG flox mice and their wild-type littermates were grown in suspension in DMEM/F12 (Thermo) with B27 (GIBCO) and 20 ng/mL EGF and FGF (Peprotech). Neurospheres were passaged at 5 and 10 days *in vitro*. Following the second passage, cells were resuspended in the same media without growth factors, plated at 15,000 cells/cm^2 on Poly-D-Lysine-coated chamber slides and allowed to differentiate for 3 or 10 days. Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature and washed with PBS prior to staining.

Ependymal cell cultures were performed as previously described [179]. Briefly, cells isolated from FoxJ1-GFP mice were resuspended in DMEM-High Glucose (Mediatech) with 10% FBS and 1% Pen/Strep and plated at 100,000 cells/cm^2 on PDL-coated chamber slides. Cells were incubated under normal cell culture conditions until confluent (3-4 days), then media was switched to 2% FBS and cells were incubated for a further 7 days. Dystroglycan-blocking (IIH6C4, Millipore) or IgM control antibodies (Biolegend) were added to culture media at 10 μg/ml and refreshed every 3 days. Cells were fixed with 4% paraformaldehyde in PBS.

In Vivo Antibody Injections

Postnatal day 2 Sprague-Dawley rats were anaesthetized on ice, positioned in a stereotaxic device and given a single 2.5 μL injection of IIH6 antibody or mouse IgM control at 1 mg/mL into the lateral ventricle (1.4 lateral, 2.2 ventral to bregma).

Image Acquisition and Analysis

Coronal sections and SVZ whole mounts were imaged with a Zeiss LSM 510 confocal laser scanning microscope. SVZ whole mount fields were selected at random from anterior dorsal areas of sufficient tissue and staining quality. Images from cell culture preparations were acquired on a Zeiss Axiovert 200M epifluorescent microscope. All images were processed and quantified using ImageJ software. For *in vivo* ependymal cell pinwheel analysis and *in vitro* cluster analysis, pinwheels/clusters were defined as 3 or more ependymal cells immediately adjacent to i. each other and ii. one or more monociliated cells.

Statistics

Sox2+ PCNA+ nuclear distance from ventricle data (Figure III-2.) are presented as box plots +/- 90^{th} and 10^{th} percentiles, respectively. All other data are expressed as mean +/- SEM. Student's t-tests and Wilcoxon rank sum tests were performed using SigmaPlot software.

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