THE ROLE OF DYSTROGLYCAN IN INTERNEURON DEVELOPMENT

By

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

Abbreviation	Expanded name
BAX	bcl2 associated X, apoptosis regulator
CA1	cornu ammonis 1
CAM	cell adhesion molecule
CB1R	cannabinoid receptor 1
CCK	cholecystokinin
CGE	caudal ganglionic eminence
cKO	conditional knockout
CMD	congenital muscular dystrophy
CNS	central nervous system
Dag1	dystroglycan
DGC	dystrophin-glycoprotein complex
ECM	extracellular matrix
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
IN	interneuron
LG	Laminin G
MGE	medial ganglionic eminence
NECAB1	N-terminal EF-hand calcium binding protein 1
NMJ	neuromuscular junction
PyN	pyramidal neuron

PSD	postsynaptic density
PV	parvalbumin
SOM	somatostatin
SO	Stratum oriens
SP	Stratum pyramidale
SR	Stratum radiatum
SLM	Stratum lacunosum moleculare
VGAT	vesicular GABA transporter
VGLUT3	vesicular glutamate transporter 3
VIP	vasoactive intestinal peptide
WT	wild-type
WWS	Walker-Warburg syndrome

ABSTRACT

The brain is a dense network of precisely interconnected cells called neurons. Our thoughts and behaviors require communication between billions of individual neurons that occurs at special cellular junctions called synapses. During brain development, distinct types of neurons must identify each other to form synaptic connections. Although neuroscientists have made progress in understanding how neurons communicate in the adult mammalian brain, how neurons make connections with their synaptic partners during later stages of nervous system development remains largely unknown. One theory for how neurons establish the correct wiring is that they use unique combinations of “recognition tags” on their cell surface called cell adhesion molecules. One cell adhesion molecule, dystroglycan, is prominent at synaptic connections throughout the nervous system. Dystroglycan is a glycosylated protein that links the extracellular and intracellular environment in neural and non-neural tissues. In the brain, dystroglycan has been associated with synapses between excitatory neurons and inhibitory neurons (interneurons), but its role at these synapses has remained elusive. In this dissertation, I use mouse genetic tools to study the role of dystroglycan at inhibitory synapses in the forebrain. In Chapter 2, I show that dystroglycan is critical for a specific type of inhibitory interneuron (cholecystokinin-positive, CCK+) to form appropriate presynaptic connections with excitatory neurons during early postnatal development. In the absence of dystroglycan CCK+ interneurons largely disappear from the forebrain, and the remaining interneurons inappropriately project their axons into the nearby striatum. In Chapter 3, I employ conditional mouse genetics and models of

dystroglycanopathy to study how the loss of dystroglycan affects the development of CCK+ interneurons. I show that CCK+ interneurons are present in the brains of mice that lack dystroglycan from the nervous system, but their axons are distributed abnormally in the hippocampus. Collectively, these results suggest that dystroglycan in the brain is critical for CCK+ interneurons to develop properly and make correct synaptic connections. These findings have important implications for understanding the molecular mechanisms that neurons use to wire together during brain development.

CHAPTER 1: INTRODUCTION

1.1 DYSTROGLYCAN BIOLOGY

Dystroglycan (*Dag1*, dystrophin-associated protein) is a transmembrane cell adhesion molecule found widely throughout metazoans including worms (*dgn-1*), fruit flies (*Dg*), zebrafish (*dag1*), mice (*Dag1*), and humans (*DAG1*) (Adams and Brancaccio, 2015; Bogdanik et al., 2008; Deng et al., 2003; Grisoni et al., 2002). Dystroglycan is a major component of basement membranes in multiple tissues including muscle, brain, peripheral nervous system, kidney, and lung (Durbeej et al., 1998; Górecki et al., 1994; Schofield et al., 1995). Due to its prominent expression in muscle and brain, dystroglycan loss of function causes muscular and nervous system defects in multiple species (Gupta et al., 2011; Parsons et al., 2002). Dystroglycan was identified as an integral membrane protein during the purification of dystrophin from skeletal muscle membranes (Campbell and Kahl, 1989). Subsequently, dystroglycan was identified as a binding partner of Laminin, a major extracellular matrix (ECM) protein component of most basement membranes (Ibraghimov-Beskrovnaya et al., 1992).

Dystroglycan is a core component of the dystrophin-glycoprotein complex (DGC) that functions to link the extracellular matrix to the intracellular cytoskeleton of cells (**Figure 1**; Ervasti and Campbell, 1993; Michele et al., 2002). The gene encoding dystroglycan (*Dag1*) yields a propeptide that is posttranscriptionally cleaved into two subunits, the extracellular alpha dystroglycan (α-DG, 120 kDa) and the transmembrane beta dystroglycan (β-DG, 43 kDa) (Holt et al., 2000; Ibraghimov-Beskrovnaya et al., 1992). These two subunits remain non-covalently bound at the cell membrane where they link other components of the DGC including the intracellular rod-like proteins

dystrophin (Duchenne muscular dystrophy), utrophin and dystrobrevin; membrane proteins including sarcoglycans, sarcospans, and biglycan; and intracellular proteins such as syntrophins and neuronal nitric oxide synthase (Moukhles and Carbonetto, 2001). Dystroglycan is therefore multifunctional due to its ability to bind diverse extracellular ligands and engage multiple downstream intracellular processes (Ervasti and Campbell, 1991; Moore and Winder, 2010).

A striking feature of dystroglycan structure is the extensive glycosylation of the extracellular alpha subunit (α-DG). The sugar chains present on α-DG permit binding of diverse ligands. Although dystroglycan gene is predicted to encode a 67 kDa protein, the apparent molecular weight of α-DG ranges widely from 120-190 kDa (Gesemann et al., 1998; Satz et al., 2010). The amount of Dystroglycan glycosylation varies between tissues and amongst cell types within a single tissue, which may contribute to the diversity of ligands it is capable of binding. For instance, the molecular weight of α-DG varies between the cortex, hippocampus, and

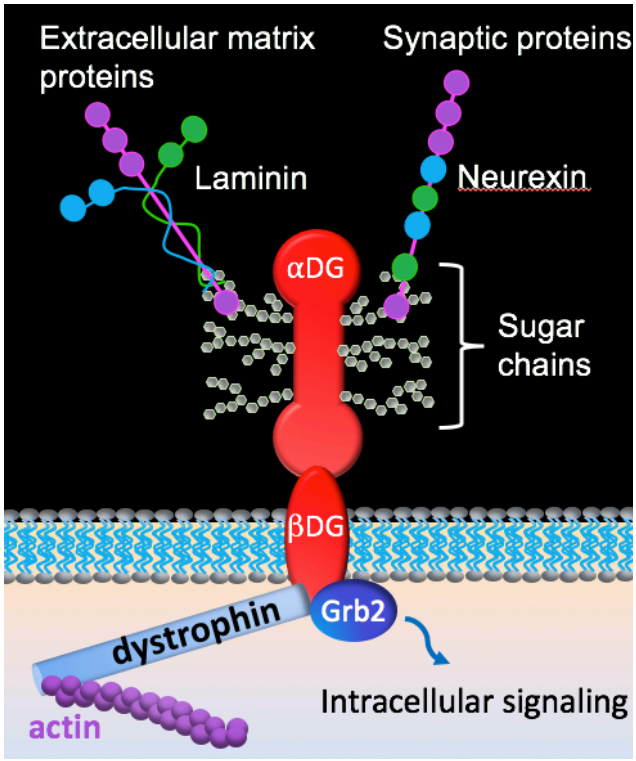

Figure 1. Dystroglycan structure and binding partners. Dystroglycan consists of the extracellular subunit α-DG and the transmembrane subunit β-DG. α-DG is capable of binding multiple ligands including Laminin and Neurexins, through LG-domains that bind sugar chains. The intracellular domain of β-DG contains motifs that allow interaction with the cytoskeleton through dystrophin, and links to the MAP kinase pathwway through Grb2.

cerebellum, and is different in neurons vs glia (Satz et al., 2010). Differences in the molecular weight of dystroglycan may play a role in binding diverse ligands. Dystroglycan binds multiple proteins (Laminins, Perlecan, Agrin, Pikachurin, Slits and Neurexins), which all contain a Laminin G (LG) domain that bind specifically to the O-linked glycan moieties present on mature dystroglycan (**Figure 1**; Briggs et al., 2016; Campanelli et al., 1994; Dempsey et al., 2019; Gee et al., 1994; Ibraghimov-Beskrovnaya et al., 1992; Peng et al., 1998; Sato et al., 2008; Sugita et al., 2001b; Sugiyama et al., 1994; Wright et al., 2012). In many tissues binding of dystroglycan to laminin, perlecan, and extracellular matrix proteins is important for the assembly and integrity of basement membranes (Henry and Campbell, 1998). Reduced glycosylation of dystroglycan due to mutations in multiple enzymes leads to impaired ligand binding, disruption of basement membranes, and results in tissue-specific pathologies. The extent of dystroglycan glycosylation also varies between species. Fruit flies express enzymes that are capable adding O-linked sugars to dystroglycan (Nakamura et al., 2010). Differences in the glycosylation of dystroglycan among species may underlie species-specific functions. For example, in contrast to vertebrates, worm dystroglycan is not expressed in muscle and functions independently of dystrophin (Johnson, Kang, and Kramer, 2006). Dystroglycan is also dynamically regulated in development and in disease. For instance, the glycosylation of α-DG increases in the peripheral and central nervous system during postnatal development (Court et al., 2011).

The intracellular domain of the tramsmembrane subuit β-dystroglycan contains many motifs that allow it to bind adaptor proteins and initiate downstream signaling. In fibroblasts, β-DG acts as a scaffold for multiple components of the ERK-MAP kinase

pathway (Spence et al., 2004). WW motifs on the intracellular domain allow β-DG binding to multiple cytoskeletal proteins such as dystrophin and utrophin (Jung et al., 1995). In addition, β-DG contains a number of signaling motifs (src-homology domains, SH3) that allow it to bind adaptor proteins (Grb2) capable of initiating downstream signaling cascades (Russo et al., 2000). Like α-DG, β-DG undergoes dynamic processing. For instance, the extracellular portion of β-DG can be cleaved by matrix metalloproteases (MMP2/9) in normal and pathological contexts (Court et al., 2011). In experimental models of autoinflammatory diseases, β-DG is cleaved by macrophage-mediated activation of matrix metalloproteases 2 and 9 (MMPs) (Agrawal et al., 2006). Work with cultured muscle cells has also demonstrated that β-DG can be retrogradely trafficked to the nucleus where it interacts with nuclear membrane proteins (González-Ramírez et al., 2008; Gracida-Jiménez et al., 2017). Collectively, these studies demonstrate that dystroglycan is a multifunctional protein, capable of binding a wide variety of ligands and engaging diverse intracellular and signaling cascades depending on the cellular context.

1.2 DYSTROGLYCANOPATHY: HUMAN GENETICS

Loss of dystroglycan function causes a group of rare, clinically heterogeneous set of diseases collectively termed dystroglycanopathy, characterized by variable muscle weakness, brain malformations, cognitive impairments, and seizures (Barresi and Campbell, 2006; Muntoni et al., 2011; Taniguchi-Ikeda et al., 2016). Although muscle pathology is prominent in most cases, dystroglycanopathy is frequently associated with multiple brain defects, including cobblestone lissencephaly,

hydrocephalus, retinal, cerebellar, and white matter pathology (Dobyns et al., 1989). Neuronal migration errors are found in the brains of many dystroglycanopathy patients leading to a form of lissencephaly characterized by a bumpy cortical surface ("cobblestone" features on MRI). Along with reduced dystroglycan function, cobblestone lissencephaly is considered clinically diagnostic for dystroglycanopathy (Devisme, 2012). Dystroglycanopathy is rarely caused by mutations in the dystroglycan gene itself (*DAG1*), termed primary dystroglycanopathy. Only a few cases of primary dystroglycanopathy have been reported in the literature, and can result in mild or severe disease pathology (Dong et al., 2015; Frost et al., 2010; Geis et al., 2013; Hara et al., 2011; Riemersma et al., 2015). Most cases of dystroglycanopathy (secondary dystroglycanopathy) arise due to mutations in any of 18 genes encoding glycosyltransferases, enzymes, and other proteins required for the extensive glycosylation of its extracellular domain (Manya and Endo, 2017). Commonly mutated genes include *LARGE* (like-acetylglucosaminyltransferase), *FKRP* (fukutin related protein), and *ISPD* (isoprenoid synthase domain-containing protein) (**Figure 2**; **Table 1**). Although most cases involve loss of α-DG function, in rare cases, mutations

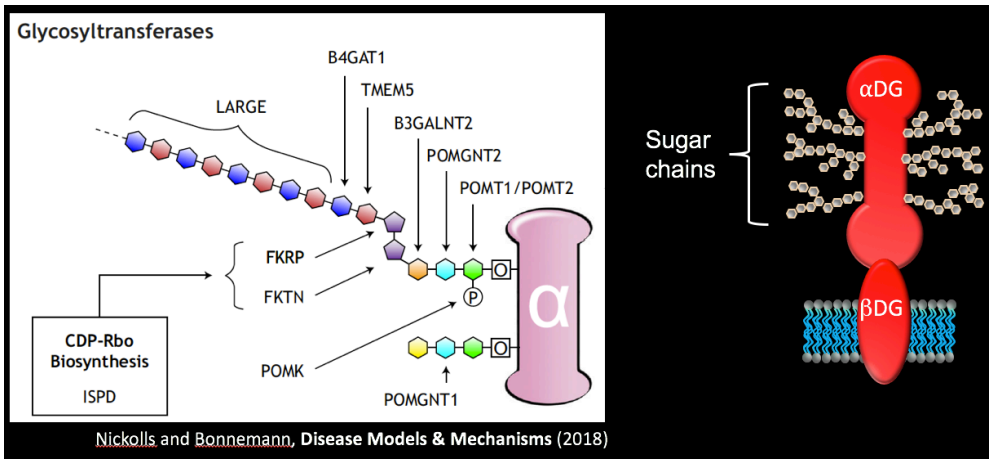

Figure 2. Dystroglycan glycosylation pathway. The extracellular subunit of dystroglycan (α-DG) is heavily glycosylated. At least 18 enzymes participate in the addition and elongation of different sugar moieties on the mucin domain of dystroglycan.

that affect β-DG have also been associated with muscular dystrophy (Salih et al., 1996). Although loss of dystroglycan glycosylation often correlates with disease severity, the precise relationship between glycosylation status and symptom severity or presence of specific pathologies in individuals is not well understood (Jimenez-Mallebrera et al., 2009). Mouse models of dystroglycanopathy involving mutations in the same gene can result in a spectrum of phenotypes (Blaeser et al., 2013). Even identical mutations in a single gene can lead to different clinical outcomes, underscoring the need to better understand how dystroglycan dysfunction leads to various tissue pathologies (Alhamidi et al., 2017).

Mutations that cause severe forms of dystroglycanopathy lead to many clinical manifestations including Walker-Warburg Syndrome (WWS), Muscle Eye Brain Disease (MEB), and Fukuyama Congenital Muscular Dystrophy (FCMD) frequently associated with brain abnormalities. Milder forms of dystroglycanopathy include various forms of limb-girdle muscular dystrophy (LGMD) characterized by variable brain pathology and cognitive impairments (Alhamidi et al., 2017). In patients with milder forms of dystroglycanopathy, cognitive impairments can occur in the absence of detectable brain malformations, suggesting that dystroglycan may have additional roles at later stages of brain development such as synapse formation and maintenance (Godfrey et al., 2007; Mercuri et al., 2009). Loss of dystroglycan function in fruit flies and zebrafish also causes dystroglycanopathy characterized by brain malformations, but whether synapse formation is affected in these models is unknown (Gupta et al., 2011; Parsons et al., 2002; Yatsenko and Shcherbata, 2014; Yatsenko et al., 2014; Yatsekno et al, 2021).

Studying the origins of various brain pathologies will be important for understanding the heterogeneity in cognitive impairments in human dystroglycanopathy.

Table 1. Dystroglycanopathy genes

Gene	Name	References
LARGE	Like-acetylglucosaminyltransferase	Longman et al., 2003
ISPD	Isoprenoid Synthase Domain Containing	Roscioli et al., 2012; Willer et al., 2012
FKRP	Fukutin-related protein	Brockington et al., 2001
FKTN	Fukutin	Kobayashi et al., 1998
B4GAT1 (B3GNT1)	β1,4-glucuronyltransferase1	Buysse et al., 2013; Shaheen et al., 2013; Willer et al., 2014; Praissman et al., 2014; 2016
B3GALNT2	β-1,3-N-acetylgalactosaminyltransferase 2	Stevens et al., 2013
RXYLT1 (TMEM5)	Ribitol xylosyltransferase 1 (Transmembrane protein 5)	Vuillaumier-Barrot et al., 2012
POMK	Protein O-mannose kinase	Jae et al., 2013
POMT1	Protein O-mannosyl transferase 1	Beltrán-Valero de Bernabé, et al., 2002
POMT2	Protein O-mannosyl transferase 2	van Reeuwijk et al., 2005
POMGNT1	Protein O-linked mannose N-acetylglucosaminyltransferase 1	Yoshida et al., 2001
POMGNT2 (GTDC2)	Protein O-linked mannose N-acetylglucosaminyltransferase 1 (glycosyltransferase-like domain containing 2)	Manzini et al., 2012
SGK196		Yoshida-Moriguchi et al., 2013
DPM1	Dolichyl-phosphate mannosyltransferase subunit 1	Yang et al., 2013
DPM2	Dolichyl-phosphate mannosyltransferase subunit 2	Barone et al., 2012
DPM3	Dolichyl-phosphate mannosyltransferase subunit 3	Lefeber et al., 2009
GMPPB	GDP-mannose pyrophosphorylase B	Carss et al., 2013
GOSR2	Golgi SNAP receptor complex member 2	Larson et al., 2018
DOLK	Dolichol kinase	Lefeber et al., 2011
TRAPPC11	Trafficking protein particle complex 1	Larson et al., 2018

1.3 DYSTROGLYCAN IN THE NERVOUS SYSTEM

Dystroglycan is required for the development of the nervous system through its diverse roles in multiple cell types (Reviewed in Nickolls and Bonnemann, 2018). Within the central nervous system, dystroglycan is expressed in neuroepithelial cells, pyramidal neurons, Purkinje neurons, astrocytes, and oligodendrocytes (Tian et al., 1996; Zaccaria et al., 2001). Conditional genetic approaches, which allow targeted gene inactivation from specific tissues or genetically defined cell types, have been invaluable for advancing our understanding of dystroglycan function in the nervous system. Mice lacking both copies of *Dystroglycan* (null) in all tissues die embryonically due to failed formation of a specialized basement membrane called Reichert's membrane, precluding study of dystroglycan function in the nervous system (Williamson et al., 1997). *Dystroglycan* conditionally floxed mice ($Dag1^{Flox}$) were developed to allow tissue-specific deletion of *Dystroglycan*, enabling investigation of muscle and brain phenotypes in dystroglycanopathy (Cohn et al., 2002; Moore et al., 2002). For instance, crossing *Dystroglycan* conditional mice with Nestin-Cre mice permits deletion of *Dystroglycan* specifically from neurons and glia (Tronche et al., 1999; Graus-Porta et al., 2001).

Dystroglycan plays important functions at multiple developmental stages during the formation of the brain, spinal cord, and retina. Mouse models of dystroglycanopathy recapitulate multiple brain abnormalities found in human dystroglycanopathy patients due to loss of dystroglycan function in these regions. Common pathologies include disruptions in the architecture of multiple brain regions due to loss of dystroglycan from neuroepithelium and early glial cells, white matter and myelination defects, impairment in axon guidance, neuronal migration, and synaptic dysfunction (Clements et al., 2017;

Moore et al., 2002; Myshrall et al., 2012; Satz et al., 2008, 2009, 2010; Wright et al., 2012).

Dystroglycan has also been shown to be important for the assembly of an ECM that promotes development of the subventricular zone (SVZ) stem cell niche and proliferation of stem cell progenitors (McClenahan et al., 2016). During early stages of brain development, dystroglycan is also expressed by neuroepithelial cells which gives rise to radial glia and cortical neurons (Moore et al., 2002; Schroder et al., 2007). Dystroglycan is critical for the ability of radial glial endfeet to bind to the cortical pial basement membrane. In the absence of dystroglycan, binding of radial glial endfeet to the Laminin rich cortical basement membrane is impaired, resulting in accumulation of ectopic neurons found in heterotopia throughout the cortex (giving rise to cobblestone lissencephaly). In addition to the cortex, dystroglycan serves as a molecular scaffold in glia required for neuronal migration in the cerebellum (Nguyen et al., 2013). Loss of dystroglycan function in the cerebellum leads to neuronal migration errors and ectopic cells in cerebellar folia, similar to the heterotopia in the cortex. In the developing spinal cord, dystroglycan localizes axon guidance molecules such as Slit (Wright et al., 2012), and serves as a scaffold for axon guidance decisions (Lindenmaier et al., 2019). Loss of dystroglycan function leads to axon crossing failures and stalling of axons at key anatomical choice points. Thus, dystroglycan plays critical roles in building mature neural circuits through its diverse functions in early development.

Dystroglycan has critical functions in multiple central and peripheral nervous system glia including astrocytes, oligodendrocytes, and Schwann cells. In the peripheral nervous system, dystroglycan is present in Schwann cells and is necessary for sodium

channel clustering at Nodes of Ranvier. Loss of *Dystroglycan* from Schwann cells leads to impaired nerve conduction, disruptions in myelination architecture, and motor deficits (Occhi et al., 2005; Saito et al., 2003). In the brain, dystroglycan is expressed on astrocytic endfeet that contact the vascular basement basement membrane through its interaction with a-syntrophin (Moore et al., 2002; Noell et al., 2011). This interaction also requires dystroglycan binding to Laminin (Menezes et al., 2014). Dystroglycan also clusters the water channel aquaporin-4 through intracellular interactions with a-syntrophin (Neely et al., 2001). Loss of dystroglycan from the nervous system also leads to disruption of the blood brain barrier (Menezes et al., 2014). Glial cells are susceptible to multiple bacteria and viruses that use dystroglycan as a receptor. For instance, dystroglycan is the Schwann cell receptor for the causative agent of leprosy, *Mycobaterium leprae* (Rambukkana et al., 1998).

1.4 DYSTROGLYCAN AT SYNAPSES

Dystroglycan is highly expressed at multiple synapses in the CNS regions such as the retina, cortex, hippocampus, and cerebellum (Briatore et al., 2010; Knuesel et al., 1999). In the peripheral nervous system, dystroglycan plays well known roles at the neuromuscular junction, where it functions at basement membranes in concert with Laminin to stabilize acetylcholine receptors (Jacobson et al., 2001; Taniguchi et al., 2006). However, the role of dystroglycan and the DGC at central inhibitory synapses in the hippocampus and cerebellum has remained elusive (Pilgram et al., 2012; Waite et al., 2012). Dystroglycan and dystrophin localize to postsynaptic sites primarily on the soma and proximal dendrites of principle neurons of the CNS, including cortical and

hippocampal PyNs as well as Purkinje neurons (Knuesel et al., 1999; Lidov et al., Zaccaria et al., 2001). Early *in vitro* experiments with hippocampal neurons demonstrated that dystroglycan co-localizes at inhibitory postsynaptic sites in clusters with gephyrin, GABA receptors, and VGAT (Brunig et al., 2002; Levi et al., 2002). These studies showed that dystroglycan is dispensable for the inhibitory synapse formation, raising questions about its function at inhibitory synapses. Interestingly, prolonged increases in neuronal excitation in cultured hippocampal neurons induces the expression of glycosylated dystroglycan at inhibitory synapses along with GABA receptors, suggesting a potential activity-dependent mechanism for dystroglycan in synaptic scaling (Pribiag et al., 2014). Despite *in vitro* evidence that neuronal dystroglycan regulates GABAergic synapse function, the *in vivo* role of dystroglycan at inhibitory synapses has remained elusive.

Dystroglycan is also highly expressed at postsynaptic sites in other CNS regions including the cerebellum and retina. In cerebellar Purkinje neurons, dystroglycan is localized primarily on the soma but is also found throughout the dendritic tree. In the cerebellum, dystroglycan co-localizes with neuroligin 2 (NL2), a neurexin binding partner, at inhibitory postsynaptic sites on Purkinje neurons (Briatore et al., 2020; Patrizi et al., 2008). In the retina, dystroglycan is present at photoreceptor ribbon synapses where it binds pikachurin (Omori et al., 2012; Orlandi et al., 2018).

In the forebrain, dystroglycan is highly concentrated on the cell bodies on principle cells of multiple brain regions including the cortex, amygdala, and hippocampus. Within the hippocampus, dystroglycan is highly concentrated and localized to cell body layers (pyramidal cell layer) CA1-3, as well as the dentate gyrus.

Recently, a role for neuronal dystroglycan at inhibitory synapses was identified using mouse genetic approaches (Fruh et al., 2016). Selective deletion of *Dystroglycan* from postsynaptic excitatory neurons using the *Nex*^{Cre} driver mouse line led to an absence of presynaptic inhibitory terminals and synapses arising from cannabinoid receptor 1 positive, cholecystokinin positive basket interneurons (CCK/CB1R+ INs) in the cortex and hippocampus (Fruh et al., 2016). Strikingly, parvalbumin positive (PV+) interneurons, which also form similar perisomatic basket cell synapses onto the same postsynaptic pyramidal neurons, were unaffected by the loss of dystroglycan. These results demonstrated that dystroglycan is required at a specific subset of inhibitory synapses. This finding is significant because very little is known about the molecules and mechanisms involved in orchestrating the formation of specific types of inhibitory synapses (Krueger-Burg et al., 2017).

1.5 INTERNEURON DIVERSITY AND FUNCTION

Inhibitory GABAergic interneurons (INs) are a large and diverse group of cells that share the ability to release the inhibitory neurotransmitter GABA (gamma aminobutyric acid). In the adult mammalian cortex, INs make up 20% of the neuronal population and play critical roles in the formation and maturation of function neural circuits that underlie cognition (Le Magueresse and Monyer, 2013). Forebrain INs are comprised of multiple neurochemically, morphologically, and functionally distinct IN subtypes (Ascoli et al., 2008; Huang et al., 2007; Kepecs and Fishell, 2014; Miyoshi et al., 2010). Many IN subtypes are defined neurochemically by expression of neuropeptides (Vasoactive Intestinal Peptide, VIP; Neuropeptide Y, NPY;

Cholecystokinin, CCK; and Somatostatin, SOM), calcium binding proteins (Calretinin, CR; Calbindin, CB; Parvalbumin, PV), and retrograde signaling mediators (Cannabinoid receptor 1, CB1R; Nitric oxide synthase, nNOS) (Freund and Buzsaki, 1996; Pelkey et al., 2017; Sultan et al., 2013). INs also exhibit diversity in their preference for targeting specific subcellular domains of PyNs such as the distal dendrites (SOM), cell bodies (CCK, PV), or onto other INs (CR, VIP). The diversity of IN connectivity allows for exquisite control of the electrophysiological activity and output of ensembles of excitatory pyramidal neurons (PyNs). For example, the CA1 region of the hippocampus is estimated to have at least 20 distinct types of INs exhibiting diverse molecular, morphological, and functional properties that support different neural circuit activities (Klausberger and Somogyi, 2008). The diversity of INs has been postulated to give rise to brain oscillations (theta, gamma) that underlie different forms of cognition and memory (Antonoudiou et al., 2020). For instance, CCK+ and PV+ basket cells, both of which synapse onto the cell bodies of PyNs, exhibit markedly different physiological properties that profoundly influence the information content carried by PyNs between brain regions (Bartos and Elgueta, 2012). Impairments in the function of specific subtypes of INs have been observed in a wide range of neurodevelopmental and neurological conditions including autism, schizophrenia, seizure disorders, and neurological diseases such as Alzheimers', Parkinsons', and multiple sclerosis (Chao et al., 2010; de Lanerolle et al., 1989; Lewis et al., 2005; Verret et al., 2012). Although the physiological contributions of interneurons have been intensely studied, the molecular mechanisms that control how interneurons develop, particularly during early postnatal ages are largely unknown.

1.6 INTERNEURON DEVELOPMENT

INs originate from multiple transient embryonic ventral forebrain regions, the medial (MGE) and caudal ganglionic eminences (CGE), as well as other areas such as the preoptic area (POA) (Wonders and Anderson, 2006; Yozu et al., 2005). The majority (~60%) of INs are derived from the MGE, which produces multiple types of IN subtypes including parvalbumin (PV) and somatostatin (SOM). CGE-derived interneurons, including cholecystokinin (CCK), calretinin (CR), reelin (RLN), make up ~30% of the total interneuron population (Miyoshi et al., 2010). In mice, INs migrate tangentially from ganglionic eminences to populate the cortex and hippocampus as early as embryonic day 15.5 (E15.5) (Calvigioni et al., 2017; Pleasure et al., 2000). INs take multiple migratory routes to reach their final forebrain destinations such as the cortex and hippocampus (Tricoire et al., 2011; Touzot et al., 2016). During the first two postnatal weeks, INs become morphologically mature and begin to express unique, subtype-specific profiles of cell adhesion molecules, transcription factors, neuropeptides, and ion channels (Lang and Frotscher, 1990; Miyoshi et al., 2015; Pelkey et al., 2020).

The final number of INs is tightly regulated during early postnatal development, with excess INs eliminated by *Bax*-dependent apoptosis (Carriere et al., 2020; Mancia Leon et al., 2020; Priya et al., 2018; Southwell et al., 2012). This wave of developmentally programmed, *Bax*-dependent cell death occurs in the first two postnatal weeks, peaking around P9 for CGE-derived INs, and P7 for MGE-derived INs. The final number of INs in the brain is scaled up or down by the activity of their postsynaptic targets (excitatory neurons) during the period of synapse formation (Wong et al, 2018), demonstrating the complex interplay between INs and their targets.

Activity-dependent mechanisms regulate the migration, survival, and morphology of most INs in the early postnatal brain (De Marco García et al., 2011; Priya et al., 2018; Wong et al., 2018). A subset of INs (VIP+ INs) may not require significant neuronal activity, although this appears to be an exception.

1.7 WIRING OF INHIBITORY INTERNEURONS

The proper formation of neural circuits requires many distinct stages including (among many others) proliferation of neurons from progenitors, migration and guidance of cell types and their axons to their final anatomical destinations, and the formation and maintenance of functional synapses. One of the least understood aspects of synapse development is synaptic partner recognition and wiring, the process by which one subtype of neuron forms connections with another subtype (or itself) (Huang et al., 2007; Sanes and Zipursky, 2020). How specific synaptic connectivity is established is not well understood for organisms such as *C. elegans* where the exact wiring diagram and number of genes encoding cell adhesion molecules are known (Kim and Emmons, 2017; Philbrook et al., 2018). Many mechanisms have been described that control the specific wiring of neurons with their afferent and efferent targets, but the molecules necessary for synaptic connectivity in vertebrates are largely unknown (Sanes and Yamagata 2009; Shen and Scheiffele, 2010; Waites et al., 2005; Williams et al., 2010).

Many different classes of cell adhesion molecules have been implicated in various aspects of synapse development including neurexins, neuroligins, cadherins, protocadherins, DSCAMs, Slitrks, protein tyrosine phosphatases (PTMs), leucine-rich repeat transmembrane proteins (LRRTMs), and latrophilins (Anderson et al., 2017;

Favuzzi et al., 2019; Linhoff et al., 2009; Sando et al., 2019; Tai et al., 2019; Weiner et al., 2005). A large number of *trans*-synaptic protein complexes have been identified that organize excitatory or inhibitory synapses, or both (Biederer and Scheiffele, 2007; Chen et al., 2017; Linhoff et al., 2009; Takahashi et al., 2012; Siddiqui et al., 2013). Many of these molecules are broadly expressed and have been found to act cooperatively during synaptogenesis (de Nardo et al., 2012; Lu, Bromley-Coolidge and Li, 2017).

GABAergic INs form synapses onto distinct subcellular domains of their target cells, including the cell body, proximal dendrites, distal dendrites, and the axon initial segment (Klausberger and Somogyi, 2008). In the CNS, perisomatic inhibition (cell body and proximal dendrites) is mediated by CCK+ and PV+ basket INs and serves to control the integration and coordinated output of groups of PyNs in multiple brain regions (Bartos and Elgueta, 2012; Vereczki et al., 2016; Veres et al., 2017). Interestingly, subcellular targeting of IN axons to different compartments of PyNs appears largely independent of activity-dependent mechanisms, suggesting that genetically encoded cell surface molecules play a larger role in this process (Di Cristo et al., 2004). Recent work has sought to identify molecules required for the establishment of the subcellular targeting specificity of different molecularly defined IN classes. Using genetic tools to achieve IN type-specific mRNA profiling, one group identified a number of cell surface and secreted molecules that exhibit distinct expression in different cell types (Favuzzi et al., 2019). Other studies have identified regulators of perisomatic or dendritic inhibition, but much of this evidence comes from physiological experiments. For instance, the establishment of perisomatic inhibition by PV+ INs is regulated by PTEN suppression of

repulsive cues such as ephrins, but whether this affects other perisomatic or dendritic targeting IN subtypes was not examined (Baohan et al., 2016).

In a few cases, proteins that contribute to the unique subcellular targeting of INs have been identified. For example, members of the L1 family of immunoglobulin (Ig) cell adhesion molecule (L1CAMs) including neurofascin-186 (NF186) in cerebellar Purkinje neurons and L1CAM in PyNs, both located postsynaptically at the axon initial segment (AIS), were found to determine the unique subcellular targeting of basket interneurons and Chandelier cells (ChCs), respectively (Ango et al., 2004; Tai et al., 2019). Although some examples of molecules controlling axon targeting specificity have been described, INs frequently target multiple cell types, and often include subtypes that target different domains. More work is necessary to determine which of the differentially expressed molecules are required to achieve the specificity of IN targeting, and whether these act redundantly or not.

1.8 CCK/CB1R INTERNEURONS

CCK+ INs exhibit substantial molecular, morphological, and functional diversity (Fuzik et al., 2016; Szabo et al., 2014). The most well studied subtype are perisomatic targeting (cell body and proximal dendrite targeting) CCK+ INs, which, along with perisomatic targeting PV+ INs are a major class of IN due to their unique ability to control the action potential firing of their postsynaptic PyN targets. Nearly all CCK+ INs in the rodent hippocampus express cannabinoid receptor-1 (CB1R) in their axon terminals (Katona et al., 1999). Cannabinoid receptors (CB1Rs) are G-protein-coupled receptors ($G_{i/o}$) highly expressed throughout the developing and adult brain. CB1Rs are

one of the most abundant GPCRs in the brain (Herkenham et al., 1991) found on both pyramidal neurons and interneurons, and although PyNs are more numerous than INs, the majority of CB1R protein in the brain is contained in INs (Steindel et al., 2013). In the postnatal brain, the majority of CB1R protein arises from axonal processes deriving from cholecystokinin (CCK) interneurons (Tsou, 1998; Katona et al., 1999; Marsicano and Lutz, 1999; Leterrier et al., 2006; Gutierrez-Rodriguez, 2017). CCK+ INs also exhibit molecular diversity, expressing either vasoactive intestinal peptide (VIP) or vesicular glutamate transporter 3 (VGLUT3) (Kawaguchi and Kubota, 1997; Somogyi et al., 2004). Nearly half of hippocampal CCK+ INs express VGLUT3, which allows these INs to release glutamate in addition to GABA (del Pino, 2017; Fasano et al., 2017). CCK+VGLUT3+ INs themselves are comprised of different morphological subtypes that target different domains of PyNs, including dendrite targeting, Schaffer-collateral associated, Mossy fiber associated, etc (Pelkey et al., 2020). CCK+ INs also consist of multiple morphological subtypes including polar, multipolar, and tufted (Hendry, Jones and Beinfeld, 1983; Nunzi et al., 1985). Recent transcriptomic profiling studies have revealed additional CCK+ IN diversity (Harris et al., 2018; Paul et al., 2017).

The study of CCK+ INs has lagged that of other INs such as parvalbumin (PV+) and VIP INs due to lack of genetic tools for selective targeting and manipulation of this subtype. In addition to CCK+ INs, excitatory neurons also express the cannabinoid gene (*Cnr1*) but at lower levels (Katona et al., 2006; Kawamura et al., 2006; Monory et al., 2006). Although VGLUT3 marks a subset of CCK+ INs, the onset of expression in CCK/CB1R-INs increases postnatally during the first two weeks and peaks between P30-P60, limiting the usefulness of this marker for targeting CCK+ INs for

developmental studies. Recently, NECAB1 was identified as a calcium binding protein highly and selectively expressed in CCK+ INs, but whether it is expressed at early ages is unknown (Sugita, Ho and Sudhof, 2002; Paul et al., 2017).

During postnatal development, CCK+ IN cell bodies and their CB1R+ axon terminals undergo dramatic laminar specific rearrangements in the cortex and hippocampus (Deshmukh et al., 2007; Morozov and Freund, 2003a; Morozov and Freund, 2003b). At birth, CCK+ IN axons are largely restricted to the stratum radiatum, where the immature dendrites of pyramidal neurons reside. In the first postnatal week, CB1R+ axon density increases in the pyramidal cell body layers where they begin to form characteristic perisomatic “basket” synapses. The morphology of the CCK+ INs appears mature beginning around P13 (Pelkey et al., 2020). In the mature hippocampus, the density of CB1R innervation is greatest in the pyramidal cell body layer (CA1-3) reflecting the powerful control these INs have over PyN activity (Lenkey et al., 2015; Morozov, Torii and Rakic, 2009).

Many neurochemically defined IN subtypes, including CCK+ INs, are capable of targeting different domains of PyNs as well as multiple postsynaptic target cells, implying that the diversity of wiring must be controlled by distinct molecular mechanisms. Although progress has been made in identifying differentially expressed molecules in INs, we lack an understanding of how INs form connections with their targets. Dystroglycan was recently identified as a critical regulator of perisomatic CCK+ IN, but not perisomatic PV+ IN synapse formation. The work in this dissertation explores the role of the cell adhesion molecule dystroglycan in CCK+ IN development and axon targeting.

CHAPTER 2

Neuronal Dystroglycan regulates postnatal development of CCK/cannabinoid receptor-1 interneurons

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2.1 ABSTRACT

Background: The development of functional neural circuits requires the precise formation of synaptic connections between diverse neuronal populations. The molecular pathways that allow GABAergic interneuron subtypes in the mammalian brain to initially recognize their postsynaptic partners remain largely unknown. The transmembrane glycoprotein Dystroglycan is localized to inhibitory synapses in pyramidal neurons, where it is required for the proper function of CCK+ interneurons. However, the precise temporal requirement for *Dystroglycan* during inhibitory synapse development has not been examined. **Methods:** In this study, we use NEX^{Cre} or $Camk2a^{CreERT2}$ to conditionally delete *Dystroglycan* from newly-born or adult pyramidal neurons, respectively. We then analyze forebrain development from postnatal day 3 through adulthood, with a particular focus on CCK+ interneurons. **Results:** In the absence of postsynaptic *Dystroglycan* in developing pyramidal neurons, presynaptic CCK+ interneurons fail to elaborate their axons and largely disappear from the cortex, hippocampus, amygdala, and olfactory bulb during the first two postnatal weeks. Other interneuron subtypes are unaffected, indicating that CCK+ interneurons are unique in their requirement for postsynaptic Dystroglycan. *Dystroglycan* does not appear to be required in adult pyramidal neurons to maintain CCK+ interneurons. *Bax* deletion did not rescue CCK+ interneurons in *Dystroglycan* mutants during development, suggesting that they are not eliminated by canonical apoptosis. Rather, we observed increased innervation of the striatum, suggesting that the few remaining CCK+ interneurons re-directed their axons to neighboring areas where *Dystroglycan* expression remained intact. **Conclusion:** Together these findings show that Dystroglycan functions as part of

a synaptic partner recognition complex that is required early for CCK+ interneuron development in the forebrain.

2.2 BACKGROUND

Proper function of neural circuits requires precise connections between specific populations of excitatory pyramidal and inhibitory neurons. GABAergic interneurons are a highly diverse group of neurons that control brain function by synchronizing and shaping the activity of populations of excitatory pyramidal neurons (PyNs) (Harris et al, 2018; Kepecs and Fishell, 2014; Lim et al., 2018; Paul et al., 2017; Pelkey et al., 2017). In mice and humans, the majority of interneurons in the cortex and hippocampus are produced in the medial and caudal ganglionic eminences (MGE and CGE) of the ventral forebrain, and migrate long distances to their final destinations (Anderson et al., 1997; Tamamaki et al., 1997; Zecevic et al., 2010). The importance of interneurons for brain function is underscored by their involvement in a wide variety of neurodevelopmental and neurological disorders including autism, schizophrenia, seizures, and Alzheimer's disease (Chao et al., 2010; de Lanerolle et al., 1989; Lewis et al., 2005; Verret et al., 2012).

The proper integration of inhibitory interneurons into neural circuits during development relies on multiple processes such as proliferation, migration, axon guidance, cell death, synaptic target selection, synapse formation (synaptogenesis) and synaptic maintenance. Although much progress has been made in identifying candidate molecules that regulate inhibitory synaptogenesis, our understanding of how molecularly defined subtypes of inhibitory interneurons initially identify specific

postsynaptic target cells is lacking (Sanes and Zipursky, 2020; Sudhof, 2018). One prominent hypothesis for explaining how diverse interneuron subtypes recognize one another during synapse development is the “molecular code” hypothesis, whereby different cell types use unique pairs or complexes of cell adhesion molecules to select their target cells (de Wit and Ghosh, 2016; Foldy et al., 2016; Krueger-Burg et al., 2017; Lu et al., 2017). Cell adhesion molecules are ideally suited to regulate synaptic target recognition due to their large diversity and presence at pre- and postsynaptic membranes. Several recent studies support the idea that cell adhesion molecules are key players in regulating subcellular targeting and synaptic specificity (Anderson et al., 2017; Favuzzi et al., 2019; Sando et al., 2019; Tai et al., 2019). Although many families of cell adhesion molecules have been implicated in controlling synapse development, they are often involved in multiple aspects of neural circuit development, making it difficult to determine their precise role in mediating synaptic specificity.

Dystroglycan is a cell adhesion molecule widely expressed throughout the body including the developing and adult brain. Dystroglycan is extensively glycosylated, and mutations in at least 19 genes that participate in synthesizing and elongating specific O-mannose sugar chains on Dystroglycan result in a form of congenital muscular dystrophy called dystroglycanopathy, characterized by muscle weakness and neurological defects of varying severity (Barresi and Campbell, 2010; Manya and Endo, 2017; Taniguchi-Ikeda et al., 2016). *Dystroglycan* (*Dag1*) is expressed by multiple cell types in the developing nervous system, including neuroepithelial cells, astrocytes, oligodendrocytes, and excitatory neurons (Zaccaria et al., 2001; Nickolls and Bonnemann, 2018). Loss of *Dystroglycan* function in the nervous system phenocopies

the most severe forms of dystroglycanopathy, and causes multiple structural brain and retinal abnormalities due to its indirect role in regulating neuronal migration and axon guidance (Clements et al., 2017; Lindenmaier et al., 2019; Moore et al., 2002; Myshrall et al., 2012; Satz et al., 2010; Wright et al., 2012). However, some individuals with milder forms of dystroglycanopathy exhibit cognitive impairments even in the absence of detectable brain malformations, suggesting a possible role for *Dystroglycan* at later stages of brain development including synaptogenesis (Godfrey et al., 2007; Mercuri et al., 2009). In PyNs, Dystroglycan is highly concentrated on the cell body and proximal dendrites where it is a major postsynaptic component of inhibitory synapses (**Fig. 1A**; Brunig et al., 2002; Levi et al., 2002; Uezu et al., 2019; Zaccaria et al., 2001). However, because of its importance in early aspects of brain development, the role of Dystroglycan at synapses has remained obscure. Using a mouse genetic approach to selectively delete *Dystroglycan* from PyNs, a recent study showed that *Dystroglycan* is required for the formation and maintenance of CCK+ interneuron (CCK+ IN) synapses in adult animals, but its specific role in the early development of these interneurons has not been examined (Fruh et al., 2016).

In this study, we show that postsynaptic Dystroglycan on PyNs is required for the proper development of presynaptic CCK+ INs throughout the forebrain. In mice lacking *Dystroglycan* in PyNs, CCK+ INs fail to elaborate their axons during the first postnatal week and are largely absent by P10. CCK+ INs were not rescued by genetic deletion of *Bax* suggesting that CCK+ INs may undergo *Bax*-independent cell death or fail to differentiate in the absence of Dystroglycan. Some remaining CCK+ INs retarget their axons into the striatum, where Dystroglycan expression is retained, suggesting that

Dystroglycan functions to allow CCK+ INs to recognize their synaptic partners. Collectively, these results demonstrate that Dystroglycan is a critical regulator of CCK+ IN development.

2.3 RESULTS

CCK+ interneurons are largely absent in mice lacking *Dystroglycan* from pyramidal neurons

To investigate the role of neuronal Dystroglycan in forebrain development, we used a conditional genetic approach to delete *Dystroglycan* selectively from pyramidal neurons (PyNs). We crossed *Dystroglycan* conditional mice ($Dag1^{Flox/Flox}$) with Nex^{Cre} driver mice to delete *Dystroglycan* in all postmitotic excitatory neurons of the forebrain except Cajal-Retzius cells, beginning at E12.5 (Schwab et al., 1998; Goebbels et al., 2006; Belvindrah et al., 2007; Wu et al., 2005). Control ($Nex^{Cre};Dag1^{F/+}$) and conditional knockout mice ($Nex^{Cre};Dag1^{F/-}$) are hereafter referred to as $Dag1^{Control}$ and $Dag1^{cKO}$ mice, respectively (**Fig. 1B**). We verified the recombination specificity of the Nex^{Cre} line by crossing it with a reporter mouse that expresses mCherry in the nuclei of *Cre*-recombined cells ($R26^{LSL\text{-}H2B\text{-}mCherry}$; Peron et al., 2015). mCherry+ nuclei were detected in excitatory neurons of the forebrain including the cortex, hippocampus, amygdala, and nucleus of the lateral olfactory tract (nLOT) (**Fig. S1A)**. Importantly, mCherry+ nuclei did not overlap with markers for interneurons (CB_1R, PV, Calbindin) or astrocytes (GFAP), confirming the specificity of the Nex^{Cre} mouse (**Fig. S1B, C)**. In $Dag1^{Control}$ mice, Dystroglycan staining was observed as puncta concentrated primarily on the cell bodies and proximal dendrites of PyNs, as well as blood vessels (**Fig. 1C**). In $Dag1^{cKO}$ mice,

Dystroglycan staining was absent from PyNs but was still present on blood vessels, confirming the specificity of the conditional knockout.

Deletion of *Dystroglycan* from neuroepithelial cells results in disrupted neuronal migration, axon guidance, and dendrite development in the brain, spinal cord and retina (Clements et al., 2017; Lindenmaier et al., 2019; Moore et al., 2002; Myshrall et al., 2012; Satz et al., 2010; Wright et al., 2012). In contrast, deletion of *Dystroglycan* from PyNs with Nex^{Cre} did not affect overall brain architecture, consistent with previous results (Satz et al., 2010). Cortical lamination in $Dag1^{cKO}$ mice was normal based on CUX1 immunostaining of layer 2-4 PyNs and labeling of layer 5-6 and hippocampal PyNs with a $Thy1^{GFP\text{-}H}$ transgenic line (**Fig. 1D-F**). Therefore, neuronal Dystroglycan is not required for PyN migration in the forebrain**.**

Forebrain interneurons (INs) are a remarkably diverse population, with multiple molecularly and morphologically distinct IN subtypes forming synapses onto different subcellular domains of PyNs (Huang et al., 2007; Kepecs and Fishell, 2014; Miyoshi et al., 2010). Since Dystroglycan is localized to inhibitory synapses on the soma and dendrites of PyNs, we examined whether IN development is affected in $Dag1^{cKO}$ mice. We performed immunostaining with a panel of molecular markers that label IN subpopulations in the hippocampus of adult mice (**Fig. 2**). In $Dag1^{Control}$ mice, parvalbumin (PV) and somatostatin (SOM) positive INs, which label the majority of interneurons that originate from the medial ganglionic eminence (MGE), were abundant throughout the hippocampus. The distribution of PV+ and SOM+ cell bodies and their synaptic targeting patterns appeared the same in $Dag1^{cKO}$ mice, suggesting these populations are unaffected by the loss of *Dystroglycan* (**Fig. 2A, B**).

We next stained the hippocampus for IN subtypes that originate from the caudal ganglionic eminence (CGE). The distribution and synaptic targeting of Calretinin interneurons, which target other INs as well as PyN dendrites, appeared normal (**Fig. 2C**; Gulyas et al., 1996; Urban et al., 2002). In contrast, we found a dramatic reduction in cannabinoid receptor-1 (CB_1R) staining in the hippocampus, which labels the axon terminals of cholecystokinin (CCK)+ INs (**Fig. 2D**; Katona et al., 1999; Marsicano and Lutz, 1999; Tsou et al., 1998). CB_1R+ terminals were significantly reduced in all CA subregions (**Fig. 2E, F**). In both the CA1 and CA3, the magnitude of the reduction varied by layer. CB_1R+ terminals were most strongly reduced (>95%) in the *stratum pyramidale* (SP) where CCK+ INs form basket synapses onto PyN cell bodies, and more moderately reduced in the *stratum radiatum* (SR) and *stratum oriens* (SO) where CCK/CB_1R+ INs synapse onto PyN dendrites (**Fig. 2E, F**). In contrast, CB_1R+ terminals were abundant in the dentate gyrus of $Dag1^{cKO}$ mice (**Fig. S2**). This is likely because Nex^{Cre} recombination is restricted to the outer third of granular layer neurons (**Fig. S1C**; Goebbels et al., 2006).

The loss of CB_1R staining in the hippocampus of $Dag1^{cKO}$ mice could reflect either downregulation of CB_1R expression or a loss of CCK+ INs. To distinguish between these possibilities, we examined whether other independent markers of CGE-derived CCK+ INs were similarly reduced. These include NECAB1, a calcium binding protein that specifically labels CCK+ IN cell bodies **(Fig. 3A)** (Miczan et al., 2021), and VGLUT3, a vesicular glutamate transporter enriched at CCK+ IN synapses **(Fig. 3C)** (del Pino et al., 2017; Pelkey et al., 2020; Somogyi et al., 2003). Both NECAB1+ cell bodies and VGLUT3+ synaptic terminals were reduced in the hippocampus of $Dag1^{cKO}$

mice (**Fig. 3B, D**). Based on the loss of all three markers, we conclude that CCK+ INs are largely absent from the hippocampus of *Dag1*^{cKO} mice.

In addition to the hippocampus, Dystroglycan is present in PyNs of the cortex, amygdala, and nucleus of the lateral olfactory tract (nLOT) (Zaccaria et al., 2001), which all receive extensive innervation from CCK+ INs (Herkenham et al., 1990, 1991; Katona et al., 2001). Therefore, we assessed whether deletion of *Dystroglycan* from PyNs affects CCK+ INs and their terminals in these forebrain regions. We first performed immunostaining for CB_1R on sagittal sections from *Dag1*^{Control} and *Dag1*^{cKO} mice. CB_1R terminals were largely absent throughout the entire forebrain of *Dag1*^{cKO} mice (**Fig. 4A, B**). Next, we stained P30 *Dag1*^{Control} and *Dag1*^{cKO} mice for NECAB1 and CB_1R to label the cell bodies and terminals of CCK+ INs, respectively (**Fig. 4C-E**). In *Dag1*^{Control} mice, NECAB1+ cell bodies were numerous and CB_1R innervation was extensive in the cortex, amygdala, and nLOT. In contrast, NECAB1+ cell bodies were dramatically reduced, and CB_1R staining was almost completely absent in all regions of *Dag1*^{cKO} mice (**Fig. 4C-E**). In each region, a few NECAB1+ cell bodies remained in *Dag1*^{cKO} mice, and these co-localized with CB_1R. Therefore, Dystroglycan expressed in PyNs is required broadly in the developing forebrain for the proper integration of CCK+ INs.

Postnatal development of CCK+ interneurons is impaired in the forebrains of *Dag1*^{cKO} mice

Our results showing that deletion of *Dystroglycan* from PyNs leads to a reduction in CCK+ IN innervation is consistent with previous work (Fruh et al., 2016). However, the temporal onset of this phenotype has not been determined. During embryonic

development, CCK+ INs generated in the caudal ganglionic eminence (CGE) begin populating the hippocampus around E14.5 (Calvigioni et al., 2017; Tricoire et al., 2011) (**Fig. 5A**). At postnatal ages, CCK+ INs settle into their final positions within the hippocampus and initially extend axons throughout the hippocampal layers before refining their projections to form characteristic basket synapses onto PyN somas (**Fig. 5B, D**) (Morozov et al., 2003a; 2003b; 2009). We first examined the development of CB_1R+ terminals in *Dag1*^{Control} mice during the first two postnatal weeks (P3-P15), as CB_1R staining is largely absent from CCK+ INs before birth (Berghuis et al., 2007; Eggan et al., 2010; Mulder et al., 2008; Vitalis et al., 2008). At early postnatal ages (P3-P5), the majority of CB_1R+ terminals were observed in the *stratum radiatum* (SR) layer of the hippocampus, where immature PyN dendrites are located (**Fig. 5B, D**). Between P5 and P10, CB_1R+ terminals increased in the *stratum pyramidale* (SP) where PyN cell bodies are located. From P15 through adulthood (15 months), CB_1R+ terminals became progressively concentrated in the SP.

Next, we examined CB_1R+ terminal development in *Dag1*^{cKO} mice. At P3, the earliest age we were able to conclusively identify CCK+ INs, CB_1R+ staining was already reduced in the hippocampus of *Dag1*^{cKO} mice. This reduction persisted throughout the period of postnatal development and into adulthood, as late as 15 months (**Fig. 5C, E, F**). To further confirm this finding, we stained the hippocampus for VGLUT3, an independent synaptic marker for CCK+ IN terminals that is upregulated during early postnatal ages (**Fig. S3A**). In *Dag1*^{Control} mice, VGLUT3+ terminals increased in the hippocampus during the first two postnatal weeks, and showed a similar pattern of innervation as CB_1R+ staining **(Fig. S3B)**. In contrast, VGLUT3+

terminals were reduced at all ages examined in *Dag1*^{cKO} mice (**Fig. S3B**). PV staining, which increases between P10 and P15 (del Rio et al., 1994), was unaltered in *Dag1*^{cKO} mice compared with controls (**Fig. S3C**).

We next determined whether the reduction of CCK+ INs in the cortex, amygdala, and nLOT followed the same developmental time course as the hippocampus. In *Dag1*^{Control} mice, CB_1R+ terminals gradually increased in density in all regions between P3 and P15, and remained stable beyond this age into adulthood (15 months) (**Fig. 6**). In contrast, CB_1R+ terminals in *Dag1*^{cKO} mice failed to elaborate during the first two postnatal weeks, and remained sparse in adult animals. Collectively, these results demonstrate that Dystroglycan in PyNs is critical during the first two postnatal weeks for the development and integration of CCK+ INs throughout the forebrain.

Post-developmental maintenance of CCK+ interneurons does not require Dystroglycan

Inhibitory synaptogenesis increases between P5-P15, and is largely complete by P30 (Favuzzi et al., 2019; Pelkey et al., 2020; Tai et al., 2019). Therefore, we wanted to assess whether deletion of *Dystroglycan* after inhibitory synapse formation impairs the maintenance of CCK+ INs. To achieve temporal control of *Dystroglycan* deletion from PyNs, we generated mice expressing tamoxifen-inducible *Cre* recombinase under the control of an excitatory neuron-specific promoter *Camk2a*, (Calcium/calmodulin-dependent protein kinase II alpha; Madisen et al., 2010). Control (*Camk2a*^{CreERT2}*;DG*$^{F/+}$*;Ai9*) or inducible-cKO (*Camk2a*^{CreERT2}*;DG*$^{F/-}$*;Ai9*) mice were administered tamoxifen at P23 via oral gavage, which induced recombination in the

majority of PyNs in the hippocampus (**Fig. 7A, B**). We then analyzed CB_1R+ innervation six weeks later at P65. No differences were found between the *Dag1* inducible-cKO and controls, suggesting that *Dystroglycan* is not required for the post-developmental maintenance of CCK+ INs (**Fig. 7C, D**).

Blocking *Bax*-dependent cell death does not rescue CCK+ interneurons in *Dag1^{cKO}* mice

The number of PyNs and INs in the forebrain is tightly regulated during early postnatal development, with excess or inappropriately connected neurons pruned by *Bax*-dependent apoptosis (Carriere et al., 2020; Mancia Leon et al., 2020; Priya et al., 2018; Southwell et al., 2012). PyN apoptosis is largely complete by P5, followed by IN apoptosis which peaks at P7-P9. We hypothesized that in the absence of PyN Dystroglycan, CCK+ INs are unable to recognize their postsynaptic targets and are therefore eliminated by apoptosis. We tested this hypothesis by generating *Dag1^{Ctrl}* and *Dag1^{cKO}* mice that lack either one (*Dag1^{Ctrl};Bax^{Ctrl}* and *Dag1^{cKO};Bax^{Ctrl}*) or both copies of *Bax* (*Dag1^{Ctrl};Bax^{KO}* and *Dag1^{cKO};Bax^{KO}*) to block apoptosis (**Fig. 8A**). Deletion of *Bax* from control mice (*Dag1^{Ctrl};Bax^{KO}*) did not alter CB_1R+ innervation in the CA1 subregion of the hippocampus (**Fig. 8B-C, F)**. In line with our previous results, *Dag1^{cKO};Bax^{Ctrl}* mice lacking one copy of *Bax* had a similar reduction in CB_1R+ terminals as *Dag1^{cKO}* mice (**Fig. 8D, F**). Surprisingly, we found that complete deletion of *Bax* in *Dag1^{cKO}* mice (*Dag1^{cKO};Bax^{KO}*) was not sufficient to rescue CB_1R+ innervation (**Fig. 8E, F**). Staining for an additional CCK+ IN synapse marker (VGLUT3) further confirmed this result (**Fig. S4**). Finally, we examined whether deletion of *Bax* could rescue CB_1R+ innervation in

the cortex, amygdala, and the nucleus of the lateral olfactory tract (nLOT) of *Dag1*^{cKO} mice (**Fig. S5**). In all regions examined, CB_1R+ terminals were reduced in mice lacking *Dystroglycan* (*Dag1*^{cKO}*;Bax*^{Ctrl}). Similar to our observations in the hippocampus, deleting both copies of *Bax* (*Dag1*^{cKO}*;Bax*^{KO}) was not sufficient to rescue CB_1R+ innervation in the cortex, amygdala, or nLOT (**Fig. S5**). Collectively, these results suggest that loss of CB_1R+ innervation in the absence of PyN Dystroglycan is not due to CCK+ INs undergoing *Bax*-dependent apoptosis.

CCK+ interneurons inappropriately innervate the striatum of *Dag1*^{cKO} mice

During embryonic development, CCK+ INs are produced in and migrate through the caudal ganglionic eminence (CGE), one of two ventral forebrain regions that ultimately develop into the striatum. Expression of *Dystroglycan* in striatal neurons is retained in *Dag1*^{cKO} mice, as they are not targeted by *Nex*^{Cre} (**Fig. 4A-B;** Fruh et al., 2016; Goebbels et al., 2006). We therefore examined CB_1R innervation of the striatum in *Dag1*^{Control} and *Dag1*^{cKO} mice. In *Dag1*^{Control} mice, CB_1R innervation in the striatum was present, but sparse compared with neighboring regions of the cortex (**Fig. 9A**) (Davis et al., 2018; Van Waes, et al., 2012). In contrast, CB_1R innervation in the striatum of *Dag1*^{cKO} mice was noticeably increased (**Fig. 9C, I**). The lateral regions of the striatum closest to the cortex exhibited dense CB_1R innervation, which decreased towards the medial striatum. Global deletion of *Bax* from *Dag1*^{Control} or *Dag1*^{cKO} mice did not alter the pattern of CB_1R innervation in the striatum (**Fig. 9B, D**).

Examination of the developmental timecourse of CB_1R+ innervation in the striatum revealed sparse CB_1R+ terminals at P10 in both *Dag1*^{Control} and *Dag1*^{cKO} mice

(**Fig. 9E**), which increased in *Dag1*^{cKO} mice compared with controls between P15 and P30 (**Fig. 9F-G**). This coincides with the period of CB_1R+ innervation of forebrain targets in *Dag1*^{Control} mice. Occasionally, CB_1R+ cell bodies could be seen in the cortex near the striatal boundary, with their axon terminals projecting into the striatum (**Fig. 9H**). These results suggest that some CCK+ INs in the cortex of *Dag1*^{cKO} mice may redirect their axons into the neighboring regions of the striatum that retain Dystroglycan.

2.4 DISCUSSION

Dystroglycan plays a critical role in maintaining the integrity of the neuroepithelial scaffold during early stages of brain development, which has made it difficult to assess its function within neurons at subsequent stages. In the current study, we show that Dystroglycan in pyramidal neurons regulates the development of a subset of their pre-synaptic partners. When *Dystroglycan* is selectively deleted from PyNs, CCK+ INs throughout the entire forebrain fail to properly integrate, and largely disappear during the first postnatal week. Surprisingly, we found that deletion of *Bax* did not rescue CCK+ INs in *Dag1*^{cKO} mice, suggesting their disappearance is not due to apoptotic cell death. The few remaining CCK+ INs redirect their axons into neighboring regions of the brain in which Dystroglycan is still present, suggesting that Dystroglycan functions as a part of a synaptic partner recognition complex.

What stage of CCK+ interneuron development requires Dystroglycan?

The localization of Dystroglycan to inhibitory synapses in forebrain pyramidal neurons has been described by multiple studies, while its function at these synapses

has remained obscure (Brunig et al., 2002; Levi et al., 2002; Pribiag et al., 2014; Uezu et al., 2019). Recently, it was found that Dystroglycan is required for the formation and function of CCK+ inhibitory basket synapses, but not PV+ basket synapses onto the same PyNs (Fruh et al., 2016). This finding is significant, because very little is known about the molecules and mechanisms involved in orchestrating the formation of specific subtypes of inhibitory synapses (Krueger-Burg et al., 2017). However, since the earliest timepoint examined in this previous study was P21, it was unclear what stage of synapse development requires Dystroglycan.

During neural circuit development, neurons must first migrate and direct their axons to their appropriate targets, then recognize the appropriate synaptic partners from a myriad of potential choices, then finally form functional synapses (Sanes and Zipursky, 2020). Our data suggest that Dystroglycan is required for the least understood of these processes: synaptic partner recognition. This is supported by the observation that CCK+ INs are present at the earliest stages they can be conclusively identified in the forebrain of *Dag1*^{cKO} mice (P3), but then fail to elaborate their axons and integrate into these circuits during the first postnatal week (**Figs. 5, S3, 6**). Interestingly, the few remaining CCK+ INs appear to project their axons into regions that continue to express *Dystroglycan* in *Dag1*^{cKO} mice **(Fig. 5, 9)**. Taken together, this data suggests that CCK+ INs in *Dag1*^{cKO} mice fail to recognize their normal postsynaptic PyN targets in the hippocampus and cortex during early postnatal development, and instead re-route to neighboring *Dag1*+ neurons, discussed further below.

The process of synaptic partner recognition in mammals has been difficult to study due to our inability to precisely identify and genetically manipulate the specific

neuronal populations during development. Determining whether loss of Dystroglycan impairs CCK+ IN development before birth is technically challenging due to the lack of immunohistochemical and genetic tools for detecting CCK+ INs prenatally (Calvigioni et al., 2017). The cannabinoid receptor-1 (*Cnr1*) and cholecystokinin (*Cck*) genes are both expressed in PyNs at prenatal timepoints, limiting their usefulness for detecting CCK+ INs. Transcription factors such as Prox1 are also of limited usefulness due to its broad expression in multiple CGE-derived IN subtypes (Miyoshi et al., 2015). VGLUT3, which labels a subset of CCK+ INs, does not increase in expression until the first postnatal week (Pelkey et al., 2020). Other IN subtypes exhibit delayed expression of selective molecular markers as well. For instance, MGE-derived Parvalbumin INs do not begin to express Parvalbumin until P10, well after the period of initial synaptic partner recognition (Carlen et al., 2012; del Rio et al., 1994).

What happens to CCK+ interneurons in the absence of Dystroglycan?

Our results show that deletion of *Dystroglycan* from PyNs resulted in a loss of all of the markers we used to identify CCK+ INs in the forebrain (**Figs. 2, 3, 4**). What happens to these neurons in the absence of PyN Dystroglycan? One possibility that we examined is that CCK+ INs undergo apoptosis. During the first two weeks of development (P5-P10), a significant number of excitatory and inhibitory neurons are pruned by *Bax*-dependent apoptotic cell death (Carriere et al., 2020; Mancia Leon et al., 2020; Priya et al., 2018; Southwell et al., 2012). This ensures the proper number of neurons and removes neurons that fail to integrate into the developing circuit. Whereas *Bax*-dependent developmental cell death has been described for most MGE and CGE-

derived interneuron subtypes, whether CCK+ INs normally undergo the same process has not been directly examined (Priya et al., 2018; Southwell et al., 2012). We tested whether the loss of CCK+ INs in *Dag1*^{cKO} mice could reflect premature or amplified developmental apoptosis, which peaks around P9 for other IN subtypes. However, constitutive deletion of *Bax*, which is sufficient to block developmental apoptosis in other neuronal populations, did not rescue CCK+ INs (**Figs. 8, S4, S5**). This suggests that canonical apoptosis is not responsible for the loss of CCK+ INs in *Dag1*^{cKO} mice. It is possible that CCK+ INs are eliminated in a *Bax*-independent manner, similar to some populations of Cajal-Retzius cells in the cortex and astrocytes in the developing retina (Ledonne et al., 2016; Punal et al., 2019).

CCK+ INs comprise a molecularly and morphologically diverse group of cells that include both cell body targeting (perisomatic) and multiple dendrite targeting subtypes (Cope et al., 2002; Pelkey et al., 2020; Szabo et al., 2014). In the hippocampus, CCK+ INs frequently express one of two non-overlapping markers, VGLUT3 (~45%) and VIP (~16%) (del Pino et al., 2017). In *Dag1*^{cKO} mice, all synaptic and cell body markers selective for CCK+ INs (CB_1R, VGLUT3, NECAB1) that we examined were reduced at the onset of their expression. While it is formally possible that Dystroglycan in PyNs is required for CCK+ INs to fully differentiate into their mature, molecularly defined subtype, we consider this unlikely. In this situation, Dystroglycan present on PyNs would be required to transmit a retrograde signal to CCK+ INs to direct their differentiation. We are unaware of any cell adhesion molecules that function in this manner. Rather, fate switching or failure to fully differentiate is usually observed upon cell-autonomous loss of specific transcription factors (Guillemot, 2007).

Our data also indicate that Dystroglycan is not required to maintain CCK+ INs after the period of synapse formation (**Fig. 7**). This is in contrast to a previous study that showed a gradual reduction in the number of Vglut3+ puncta when *Dag1* was deleted in adult mice using AAV-Cre (Fruh et al., 2016). Aside from the different approaches used for adult deletion, this difference may arise from the level of analysis: in our study, we saw no difference in the cellular organization of CCK+ INs following adult deletion, whereas the previous study was focused specifically on presynaptic puncta. It is possible that in our inducible-cKO (*Camk2a*^{CreERT2}*;DG*$^{F/-}$*;Ai9*) mice, synaptic inputs from CCK+ INs are reduced without altering the survival of these neurons. Alternatively, there may still be some residual Dystroglycan protein remaining in *Camk2a*^{CreERT2}*;DG*$^{F/-}$*;Ai9* mice, at levels sufficient to support CCK+ IN maintenance.

Although CCK+ INs and their terminals were dramatically reduced throughout the brains of *Dag1*^{cKO} mice, some CCK+ IN terminals were still present, particularly along the cortico-striatal boundary and in the upper dendritic layers of the cortex (layer 1) and hippocampus. Importantly, striatal neurons and Cajal-Retzius cells, which are located in superficial cortical layers during postnatal development, are not targeted by *Nex*^{Cre}(del Rio et al., 1995). This suggests that in the absence of *Dystroglycan* on their normal postsynaptic targets (PyNs), CCK+ INs may direct their axons to secondary synaptic targets that retain *Dystroglycan* expression.

A number of studies have indicated that synaptic partner recognition and targeting may be “stringent” or “flexible”, depending on the cell type involved. Studies in the *Drosophila* visual system have shown that synaptic cell adhesion molecules such as DIP/Dprs can promote either stringent or flexible outcomes among synaptic partners

depending on the cellular context and the molecules involved. For instance, postsynaptic Dm8 neurons containing the receptor DIP-γ undergo cell death if not innervated by a matching R7 photoreceptor containing the cognate ligand Dpr11 (Courgeon and Desplan, 2019). In contrast, loss of DIP-β from L4 neurons does not impair synapse formation or cause cell death, but instead leads to ectopic synapses onto alternative synaptic partners (Xu et al., 2019). Synaptic partner recognition "flexibility" and "stringency" has also been demonstrated in the mammalian nervous system. In the developing retina, On-alpha retinal ganglion cells will re-wire to increase inputs from neighboring bipolar cell types when their normal presynaptic inputs (Type 6 bipolar cells) are genetically ablated (Tien et al., 2017). In contrast preGABA INs in the developing spinal cord retract their processes when their primary targets (proprioceptor axons) are not present, rather than forming synapses onto secondary targets (Betley et al., 2009). Despite retracting their axons, preGABA INs do not undergo cell death, suggesting that loss of neurons is not a necessary consequence of losing synaptic partners. In *Dag1*^{cKO} mice, CCK+ INs may stringently require *Dystroglycan* for their ability to recognize their primary synaptic targets and die in a *Bax*-independent manner in its absence. The observation that some CCK+ INs near the cortico-striatal boundary survive and innervate the striatum suggests that they may exhibit some degree of flexibility to make contacts onto secondary targets. Determining whether the remaining CCK+ INs in *Dag1*^{cKO} mice exhibit normal morphological and physiological properties will require fate mapping these neurons, which is difficult with currently available genetic tools.

Why are CCK+ interneurons selectively affected in *Dag1*^{cKO} mice?

CCK+ INs appear to be the only interneuron subtype affected by deletion of *Dystroglycan* from PyNs. Compared to other IN populations, CCK+ INs express high levels of CB_1Rs, which can play important roles in neuronal proliferation, migration, and axon outgrowth (Gaffuri et al., 2012). *In utero* exposure to exogenous cannabinoids results in a specific loss of CCK+ INs through unknown mechanisms (Vargish et al., 2017). However, conditional deletion of the cannabinoid receptor-1 gene *Cnr1* from CCK+ INs does not affect interneuron migration or neurochemical specification, but rather increases the number of perisomatic VGLUT3+ inhibitory synapses on cortical PyNs (Berghuis et al., 2007). In addition, CB_1R signaling is not necessary for the survival of CCK+ INs (Albayram et al., 2016). Therefore, it is unlikely that alterations in CB_1R activity underlie the selective loss of CCK+ INs in *Dag1*^{cKO} mice.

One possible explanation for this selective loss is that Dystroglycan interacts with specific molecules on presynaptic CCK+ INs compared with other IN subtypes. Dystroglycan is highly glycosylated, and unique matriglycan moieties present on its extracellular domain bind to proteins containing Laminin G (LG) domains (Yoshida-Moriguchi and Campbell, 2015). Proteins that bind Dystroglycan through their LG domains include extracellular matrix proteins (Agrin, Laminins, Perlecan), axon guidance molecules (Slits, Celsr3), as well as synaptic proteins (Neurexin, Pikachurin) (Campanelli et al., 1994; Gee et al., 1994; Ibraghimov-Beskrovnaya et al., 1992; Peng et al., 1998; Sato et al., 2008; Sugita et al., 2001; Wright et al., 2012; Lindenmaier et al., 2019). Several other putative synaptic proteins also contain LG domains (ie: Cntnap1-6), although their binding to Dystroglycan has not been examined.

Biochemical experiments have identified α-dystroglycan as a major interaction partner of α- and β-neurexins in whole brain lysates, and these interactions are dependent upon the lack of splice inserts in LNS2 and LNS6 of neurexin (Sugita et al., 2001; Boucard et al., 2005; Reissner et al., 2014; Fucillo, et al., 2015). Conditional deletion of all three *Neurexins* from interneurons revealed distinct outcomes depending on the IN population examined (Chen et al., 2017). Deletion of all *Neurexin* isoforms from PV+ INs results in a significant decrease in the number of PV+ synapses in the cortex, whereas it does not affect inhibitory synapse numbers when deleted from SST+ INs. While PV+ IN numbers were not affected by conditional deletion of *Neurexins*, this could reflect the timing of deletion, which is unlikely to occur before three weeks of age based on the onset of *Cre* expression (Carlen et al., 2012; del Rio et al., 1994). Nrxn1α and Neurexin 3α/β are expressed at significantly higher levels in CCK+ INs than in PV+ INs, and CCK+ INs predominantly express Neurexin isoforms lacking splice inserts in LNS6 (Fucillo, et al., 2015). Therefore, CCK+ INs may show a larger degree of Nrxn:Dystroglycan interaction than other IN subtypes. Mice harboring a mutation in *Dystroglycan* that exhibits reduced glycosylation, and thus Neurexin binding capacity (*Dag1*^{T190M}), showed no impairments in CCK+ IN terminal development (Fruh et al., 2016; Hara et al., 2011). However, these mice do not display the cortical migration phenotypes associated with a complete loss of *Dystroglycan*, suggesting that Dystroglycan retains some residual function, which may be sufficient for CCK+ IN terminal development. Whether Neurexins are required cell autonomously in CCK+ INs for their development has not been directly tested, in part due to a lack of genetic tools.

Limitations in studying CCK+ interneuron development

Our understanding of CCK+ IN development and function has lagged behind other interneuron subtypes (PV, SOM, VIP, etc) due in part to the lack of viral and mouse genetic tools available for selectively labeling and manipulating CCK+ INs. All major markers of CCK+ INs (CCK, CB1R, and VGLUT3) are also expressed at lower levels in PyNs, limiting the usefulness of single promoter/recombinase approaches for targeting CCK+ INs (Dimidschstein et al., 2016; Tasic et al., 2016; Zeisel et al., 2015). Specific targeting of CCK+ INs therefore requires dual recombinase-based intersectional approaches, including CCK-Cre;Dlx5/6-Flp double transgenic mice (Nguyen et al., 2020; Taniguchi et al., 2011; Rovira-Esteban et al., 2019; Whissell et al., 2015; Whissell et al., 2019), dual CCK-dsRed;GAD67-GFP reporter mice (Calvigioni et al., 2017), or *VGLUT3*^{Cre} mice which label approximately half of CCK+ INs (Fasano et al., 2017; Pelkey et al., 2020). Other reporter lines (*5HT3AR*^{EGFP}) target the entire CGE-derived interneuron population, of which CCK+ INs only comprise ~10% (Chittajallu et al., 2013; Lee et al., 2010). A recently developed *Sncg*^{FlpO} mouse line appears to provide selective genetic access CCK+ basket cells by taking advantage of the fact that *Sncg* is specifically expressed in CCK+ INs (Dudok et al., 2021). However, it is not clear when the onset of recombination occurs in this line, and whether it will be useful for studying the early development of CCK+ INs. Indeed, many of the genes used for targeting IN subtypes are not significantly expressed until after the first postnatal week in mice, when much of the process of synaptic partner recognition and initial synapse formation has already occurred (**Fig. S3**; Carlen et al., 2012; del Rio et al., 1994; Pelkey et al., 2020).

2.5 CONCLUSION

In this study, we identified a critical role for excitatory neuron Dystroglycan in regulating the development of forebrain CCK+ interneurons during the first postnatal week. Given the emerging role for CCK+ INs and cannabinoid signaling in controlling neural circuit activity, *Dag1*^{cKO} mice may be useful for studying the consequences of losing a major IN population.

2.6 MATERIALS AND METHODS

Animal husbandry

All animals were housed and cared for by the Department of Comparative Medicine (DCM) at Oregon Health and Science University (OHSU), an AAALAC-accredited institution. Animal procedures were approved by OHSU Institutional Animal Care and Use Committee (Protocol # IS00000539) and adhered to the NIH *Guide for the care and use of laboratory animals*. Animals older than postnatal day 6 (P6) were euthanized by administration of CO_2, animals <P6 were euthanized by rapid decapitation. Animal facilities are regulated for temperature and humidity and maintained on a 12 hour light-dark cycle and animals were provided food and water *ad libitum*.

Mouse strains and genotyping

The day of birth was designated postnatal day 0 (P0). Ages of mice used for each analysis are indicated in the figure and figure legends. Mice were maintained on a C57BL/6 background and have been previously described or obtained from JAX (**Table**

1): *Dystroglycan* conditional mice (*Dag1*^{Flox}) (Cohn et al., 2002; Moore et al., 2002), *Nex*^{Cre} (Schwab et al., 1998; Goebbels et al., 2006), *VGLUT3*^{Cre} (Grimes et al., 2011), *Bax*$^{-/-}$ (Knudson et al., 1995; White et al., 1998), *Camk2a*^{CreERT2} (Madisen et al., 2010), *Ai9*$^{LSL\text{-}tdTomato}$ (Madisen et al., 2010), and *R26*$^{LSL\text{-}H2B\text{-}mCherry}$ (Peron et al., 2015). Loss of BAX protein in *Bax*$^{-/-}$ mouse brains was validated by western blot in a previous study (Slupe et al., 2021). Genomic DNA extracted from tissue samples (Quanta BioSciences) was used to genotype animals. The presence of the Cre allele in *Nex*^{Cre} mice and *Camk2a*^{CreERT2} mice was detected using generic Cre primers (JAX).

Table 1. Mouse strains

Common name	Strain name	Reference	Stock #
Dag1$^{-/-}$	*B6.129-Dag1*^{tm1Kcam}*/J*	Williamson et al., 1997	006836
Dag1^{Flox}	*B6.129(Cg)-Dag1*$^{tm2.1Kcam}$*/J*	Cohn et al., 2002	009652
Nex^{Cre}	*NeuroD6*$^{tm1(cre)Kan}$	Goebbels et al., 2006	MGI:4429523
Vglut3^{Cre}	*Tg(Slc17a8-icre)1Edw*	Grimes et al., 2011	018147
Ai9$^{LSL\text{-}tdTomato}$	*B6.Cg-Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$*/J*	Madisen et al., 2010	007909
Camk2a^{CreERT2}	*B6.Tg(Camk2a-cre/ERT2)1Aibs/J*	Madisen et al., 2010	012362
R26$^{LSL\text{-}H2B\text{-}mCherry}$	*B6.Gt(ROSA)26Sor*$^{tm1.1Ksvo}$	Peron, et al., 2015	023139
Bax$^{-/-}$	*B6.129X1-Bax*^{tm1Sjk}*/J*	Knudson et al., 1995	002994

Tamoxifen administration

Tamoxifen (Sigma; Cat# T5648-1G) was dissolved 1:10 in sunflower seed oil. Each mouse was orally gavaged with 200 μL of tamoxifen at a final concentration of 5 mg/ml tamoxifen.

Perfusions and tissue preparation

Brains from mice younger than P15 were dissected and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight for 18-24 hrs at 4°C. Mice P15 and older were deeply anesthetized using CO2 and transcardially perfused with ice cold

0.1M PBS for two minutes to clear blood from the brain, followed by 15 mL of ice cold 4% PFA in PBS. After perfusion, brains were dissected and post-fixed in 4% PFA for two hours. Brains were rinsed with PBS, embedded in 4% low-melt agarose (Fisher: Cat# 16520100), and 50 μm sections were cut on a vibratome (VT1200S, Leica Microsystems Inc., Buffalo Grove, IL).

Immunohistochemistry and antibodies

Single and multiple immunofluorescence detection of antigens was performed as follows: Free-floating vibratome sections (50 μm) were briefly rinsed with PBS, then blocked for 1 hr in PBS containing 0.2% Triton-X (PBST) plus 10% normal donkey serum. Sections were incubated with primary antibodies (**Table 2**) diluted in PBST at 4°C overnight (18-24 hrs) or for 3 days for Dystroglycan staining. The following day, sections were rinsed briefly with PBS, then washed with PBST three times for 20 min each. Sections were then incubated with a cocktail of secondary antibodies (1:1000, Alexa Fluor 488, 546, 647; Fisher) in PBST for 90 min at room temperature. Sections were washed with PBS three times for 20 min each and counterstained with Hoechst 33342 (Life Technologies, Cat# H3570) for 10 min to visualize nuclei. Finally, sections were mounted on slides using Fluoromount-G (Fisher; SouthernBiotech) and sealed using nail polish.

Table 2. Primary antibodies used for immunohistochemistry

Target	Host	Dilution	Source	Catalog #	RRID
α-Dystroglycan (IIH6C4)	Mouse	1:200	Millipore	05-593	AB_309828
Calbindin	Rabbit	1:4000	Swant	CB38	AB_10000340
Calretinin	Rabbit	1:4000	Swant	CG1	AB_2619710
CB1R	Guinea pig	1:2000	Synaptic Systems	258-104	AB_2661870

Cux1	Rabbit	1:250	Santa Cruz Biotech	sc-13024	AB_2261231
GFAP	Mouse	1:1000	Millipore	MAB360	AB_2109815
NECAB1	Rabbit	1:2000	Sigma	HPA023629	AB_1848014
Parvalbumin	Goat	1:2000	Swant	PVG-213	AB_2650496
Somatostatin	Rabbit	1:2000	Peninsula Labs	T-4103	AB_518614
tdTomato	Goat	1:1000	Biorbyt	orb182397	AB_2687917
VGlut3	Rabbit	1:2000	Synaptic Systems	135-203	AB_887886

Microscopy

Imaging was performed on a Zeiss Axio Imager M2 fluorescence upright microscope equipped with an Apotome.2 module for structured illumination microscopy. The microscope uses a metal halide light source (HXP 200 C), Axiocam 506 mono camera, and 10X/0.3 NA EC Plan-Neofluar, 20X/0.8 NA Plan-Apochromat objectives. Z-stack images were acquired and processed as maximum projection images using Zeiss Zen Imaging software, and analyzed offline in ImageJ/FIJI (Schindelin et al., 2012). Images used for quantification between genotypes were acquired using the same exposure times. Brightness and contrast were adjusted in FIJI to improve visibility of images for publication. Figures were composed in Adobe Illustrator CS6 (Adobe Systems).

Quantification

Quantification of CB_1R terminals in the hippocampus (CA1, CA3, Dentate gyrus) and caudal striatum was performed on 5 μm z-stacks acquired using a 20X objective. Six to twelve sections per animal (technical replicates) from at least three animals per genotype (biological replicates) were used for analysis, except where noted in the text and figure legends. Sections were taken from equivalent rostro-caudal positions including the dorsal hippocampus (Bregma between -1.48 to -1.94 mm) using coordinates from the mouse brain atlas (Franklin and Paxinos, 1997). All images used

for quantification were processed identically. Briefly, background subtraction (Rolling ball radius = 50) and mean filtering (Smooth function in FIJI) were applied to each image to enhance the detection of CB_1R terminals by thresholding. To measure CB_1R signal in specific regions of interest (ROIs), a threshold was manually set and applied equally across images to detect only CB_1R signal. Separate regions of interest (ROIs) were used to quantify CB_1R pixels in CA1 and CA3 layers: stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR). Three separate ROIs were used to analyze Dentate gyrus layers: Outer molecular layer (OML), Inner molecular layer (IML), and Granule cell layer (GCL). Hoechst signal in the SP (CA regions) and GCL (Dentate regions) were used to align the ROIs consistently for each image. Raw integrated density values from each ROI were averaged across all images for each animal and normalized to the mean intensity of the control group (set to 100% for each ROI).

Experimental Design and Statistical Analysis

All phenotypic analyses were conducted using tissue collected from at least three mice per genotype from at least two independent litters unless otherwise noted. The number of mice used for each analysis (“n”) are indicated in the figures and figure legends. No specific power analyses were performed, but sample sizes were similar to our previous work and other published literature (Wright et al., 2012; Clements et al., 2017; Lindenmaier et al., 2019). Male and female mice were analyzed together. In many cases, highly penetrant phenotypes revealed the genotypes of the mice and no blinding could be performed. Significance between groups was determined using unpaired two-tailed Student’s t-test. Data are presented as mean ± standard error of the mean (s.e.m)

and statistical significance was set at alpha = 0.05 ($P < 0.05$). Graphical representations of data and statistical analyses were performed in GraphPad Prism 8 (San Diego, CA).

2.7 Acknowledgments

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2.8 FIGURES

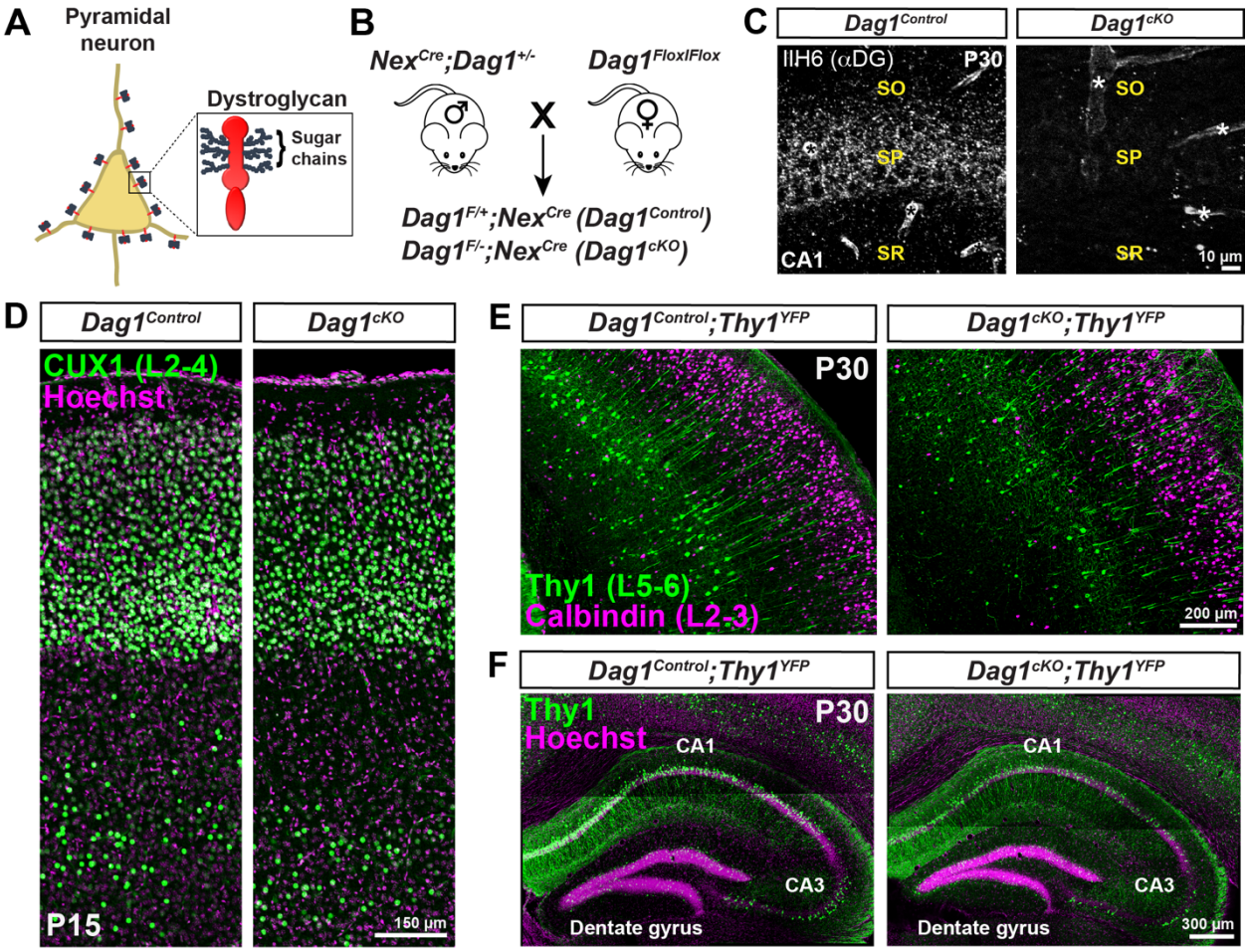

Figure 1. Neuronal *Dystroglycan* is not required for pyramidal neuron migration. **(A)** Schematic of Dystroglycan on pyramidal neurons. Inset shows the structure of Dystroglycan and sugar chain moieties present on the extracellular subunit. **(B)** Mouse breeding scheme for generating pyramidal neuron-specific *Dag1* conditional knockout mice using Nex^{Cre} driver mice. **(C)** Immunostaining for Dystroglycan in the hippocampal CA1 region of P30 $Dag1^{Control}$ mice (left panel) shows punctate Dystroglycan protein on the soma and proximal dendrites of pyramidal neurons, whereas $Dag1^{cKO}$ mice (right panel) lack perisomatic staining. Asterisks denote Dystroglycan staining on blood vessels which is retained in $Dag1^{cKO}$ mice. **(D)** Coronal sections from P15 $Dag1^{Control}$ and $Dag1^{cKO}$ cortex were immunostained for upper layer marker CUX1 (L2-4). **(E)** Coronal sections of the cortex from P30 $Dag1^{Control}$ and $Dag1^{cKO}$ mice crossed with a $Thy1^{YFP}$ reporter mouse to sparsely label layer 5-6 pyramidal neurons (green) and stained for Calbindin (magenta) to label layer 2-3 pyramidal neurons. **(F)** Coronal sections of the hippocampus from P30 $Dag1^{Control}$ and $Dag1^{cKO}$ mice crossed with a $Thy1^{YFP}$ reporter mouse to label excitatory neurons (green) in the CA regions and dentate gyrus.

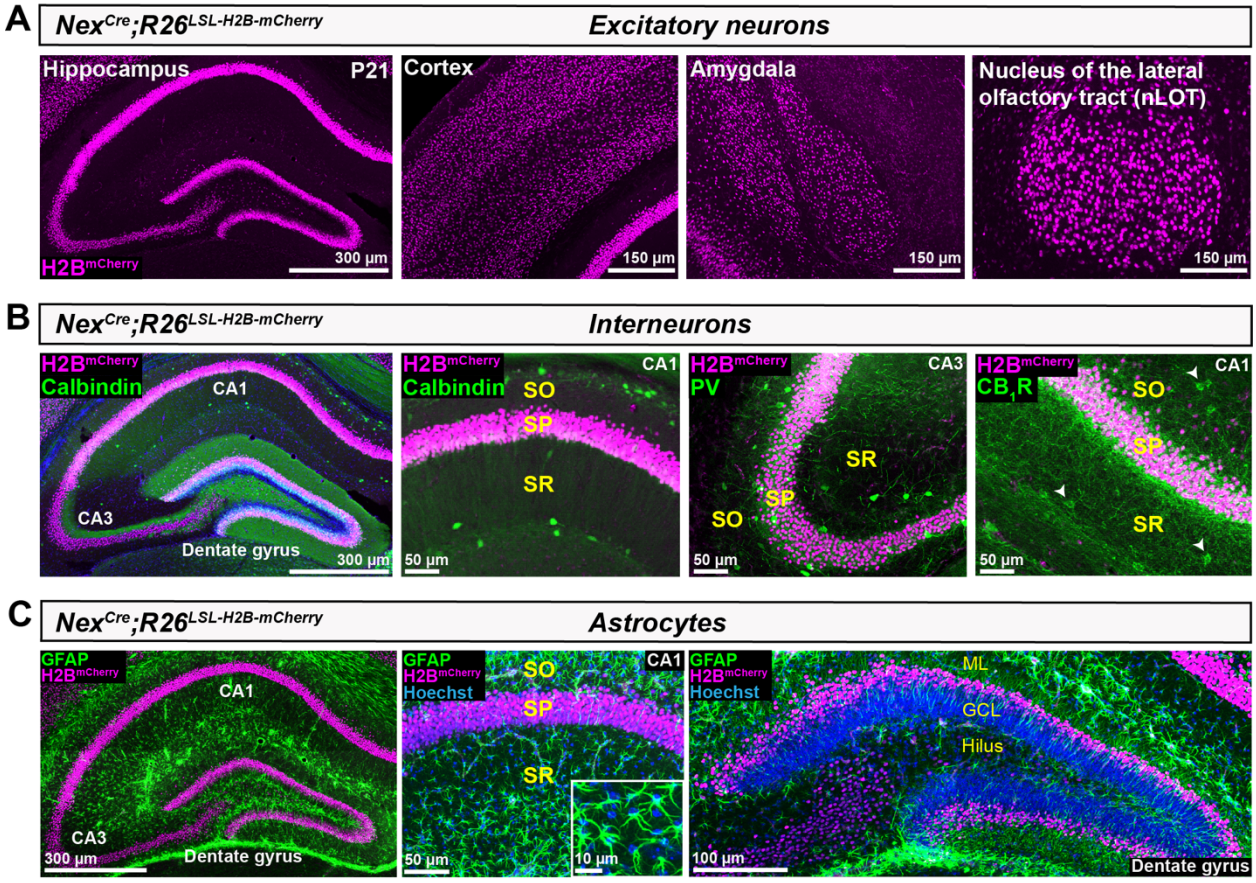

Figure S1. *Nex*^{Cre} drives recombination in forebrain pyramidal neurons but not interneurons or glia. **(A)** Coronal sections from *Nex*^{Cre};*R26*$^{LSL\text{-}H2B\text{-}mCherry}$ reporter mice at P21 show mCherry+ nuclei (magenta) of pyramidal neurons in the hippocampus, cortex, amygdala, and nucleus of the lateral olfactory tract (nLOT). **(B)** Hippocampal sections from *Nex*^{Cre};*R26*$^{LSL\text{-}H2B\text{-}mCherry}$ reporter mice immunostained for interneuron markers (green) Calbindin (left panels), Parvalbumin (middle panel), and CB_1R (right panel) show no overlap of interneuron cell bodies with mCherry+ nuclei. White arrowheads indicate CB_1R+ cell bodies. SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*. **(C)** The astrocyte marker GFAP (green) shows no overlap with mCherry+ nuclei in the hippocampal CA regions or dentate gyrus (left and middle panels). Inset (middle panel) shows a magnified view of astrocyte nuclei (blue). mCherry+ nuclei occupy the outer third of the dentate gyrus granule cell layer (right panel). ML, molecular layer; GCL, granule cell layer.

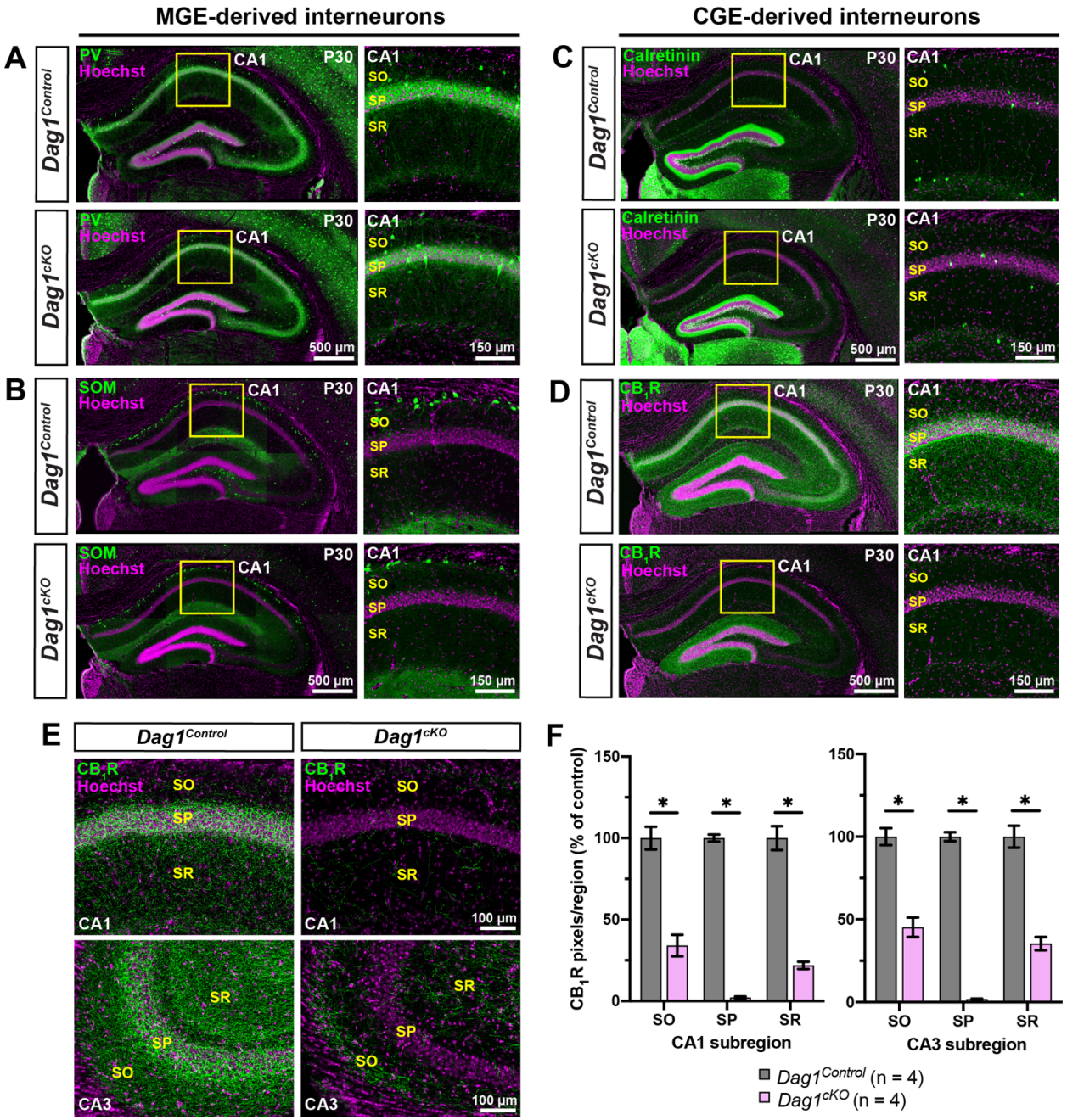

Figure 2. CCK+ interneurons are selectively reduced in mice lacking *Dystroglycan* from pyramidal neurons. (A-B) Immunostaining for medial ganglionic eminence (MGE)-derived interneuron markers (green) parvalbumin (PV) **(A)** and somatostatin (SOM) **(B)** show normal innervation of the hippocampus in P30 *Dag1*^{Control} and *Dag1*^{cKO} mice. Insets (yellow boxed regions) show enlarged images of the CA1. **(C-D)** Immunostaining for caudal ganglionic eminence (CGE)-derived interneuron markers (green) Calretinin **(C)**, and CB_1R **(D)** show normal innervation of Calretinin interneurons in *Dag1*^{Control} and *Dag1*^{cKO} mice, whereas CB_1R is largely absent from the CA regions of *Dag1*^{cKO} mice. Insets (yellow boxed regions) show enlarged images of the CA1. **(E)** Immunostaining for CB_1R in hippocampal CA1 (top) and CA3 (bottom) of P30 *Dag1*^{Control}

and *Dag1*^{cKO} mice. **(F)** Quantification of CB_1R pixels for each CA layer of the CA1 and CA3 shows a significant reduction in CB_1R staining in *Dag1*^{cKO} mice (**P* < 0.05, unpaired two-tailed Student's t-test; n = 4 mice/genotype). Data are presented as mean values ± s.e.m. Data are normalized to *Dag1*^{Control} signal in each CA layer. CA layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

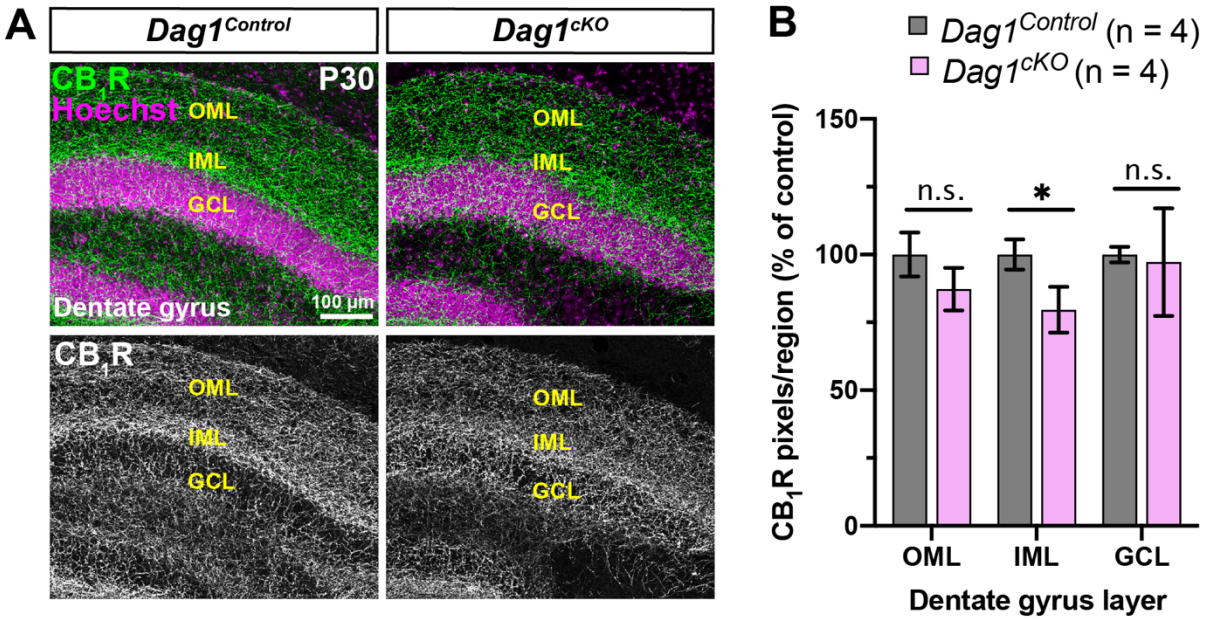

Figure S2. CCK+ interneuron innervation of the dentate gyrus is minimally altered in *Dag1*^{cKO} mice. (A) Immunostaining of CB_1R in the dentate gyrus from P30 *Dag1*^{Control} (left panels) and *Dag1*^{cKO} mice (right panels). Single channel images of CB_1R (gray) are shown below. **(B)** Quantification of CB_1R pixels for each dentate gyrus layer (*$P < 0.05$, unpaired two-tailed Student's t-test; n = 4 mice/genotype). Data are presented as mean values ± s.e.m. Data are normalized to *Dag1*^{Control} signal in each dentate gyrus layer. OML, outer molecular layer; IML, inner molecular layer; GCL, granule cell layer.

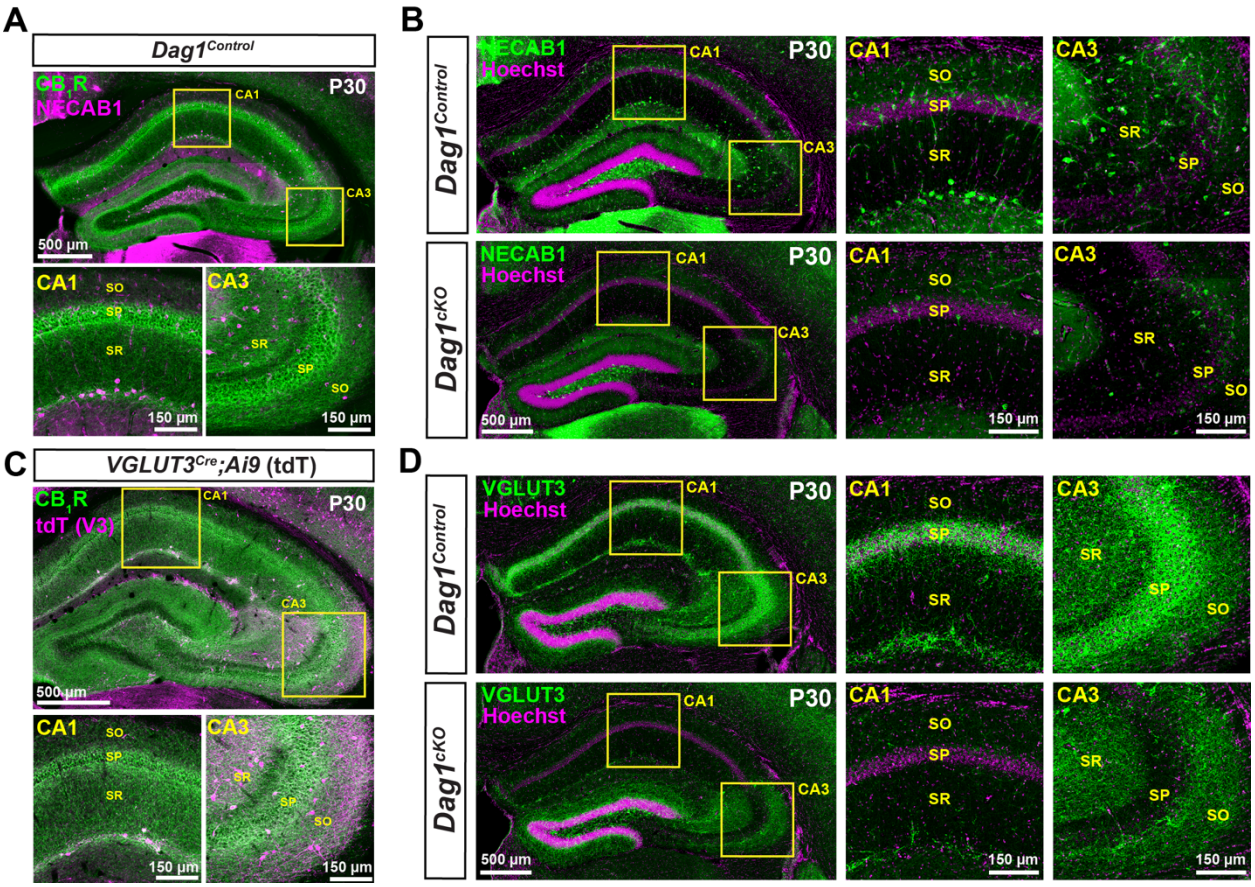

Figure 3. Cell body and synaptic markers for CCK+ interneurons are reduced in *Dag1*^{cKO} mice. (A) Immunostaining showing the co-localization of CB_1R (green) and NECAB1 (magenta) in CCK+ interneurons. Insets (yellow boxed regions) show enlarged images of the CA1 and CA3. **(B)** Immunostaining for NECAB1 (green) shows a reduction of NECAB1+ interneurons in the hippocampus of P30 *Dag1*^{cKO} mice. Insets (yellow boxed regions) show enlarged images of the CA1 and CA3. **(C)** Immunostaining of hippocampal sections from *VGLUT3*^{Cre} mice crossed with a Lox-STOP-Lox-tdTomato (Ai9) reporter mouse showing the co-localization of CB_1R (green) and VGLUT3 (magenta) in a subset of CCK+ interneurons. Insets (yellow boxed regions) show enlarged images of the CA1 and CA3. **(D)** Immunostaining for VGLUT3 (green) shows a reduction of CCK+ interneuron synaptic terminals in the hippocampus of P30 *Dag1*^{cKO} mice. Insets (yellow boxed regions) show enlarged images of the CA1 and CA3. CA layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

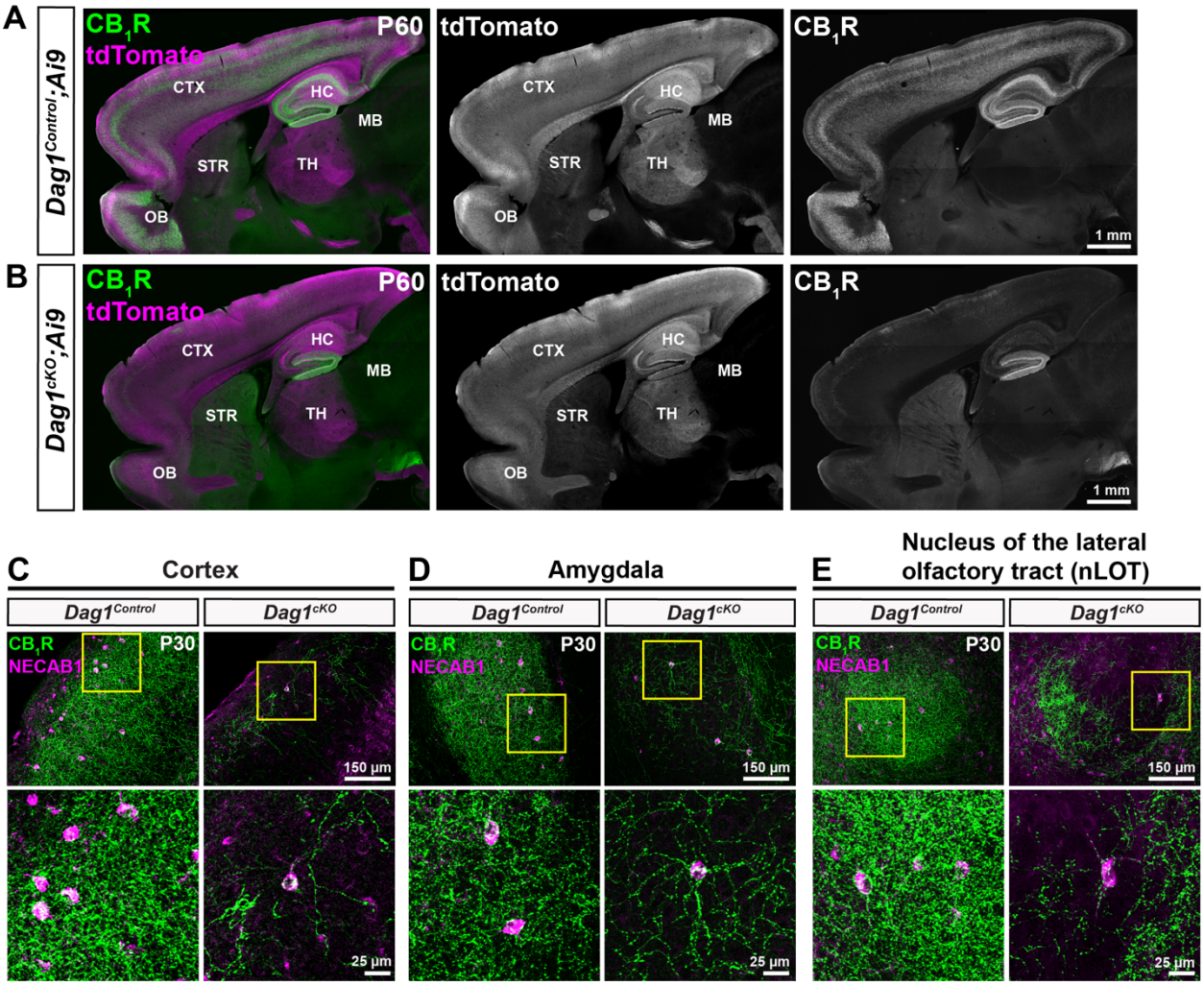

Figure 4. CCK+ interneurons are reduced throughout the forebrain of mice lacking *Dystroglycan* from pyramidal neurons. (A-B) Sagittal sections from P60 *Dag1*^{Control}*;Ai9* **(A)** and *Dag1*^{cKO}*;Ai9* mice **(B)** immunostained for CB_1R (green; right panels) and tdTomato/Ai9 (magenta; middle panels). In *Dag1*^{cKO}*;Ai9* mice, CB_1R staining is lacking in all the forebrain regions where *Nex*^{Cre} drives recombination in excitatory neurons (tdTomato expression, middle panels) including the cortex (CTX), hippocampus (HC), and olfactory bulb (OB). Note the absence of tdTomato signal in the striatum (STR) and midbrain (MB), which are not targeted by *Nex*^{Cre}. **(C-E)** Immunostaining for CB_1R (green) and NECAB1 (magenta) in the cortex **(C)**, amygdala **(D)**, and nucleus of the lateral olfactory tract **(E)** shows the reduction of CCK+ interneuron markers in the forebrain of P30 *Dag1*^{cKO} mice (right panels). Enlarged images (yellow boxed regions) show individual NECAB1+ cell bodies (magenta) co-localized with CB_1R (green).

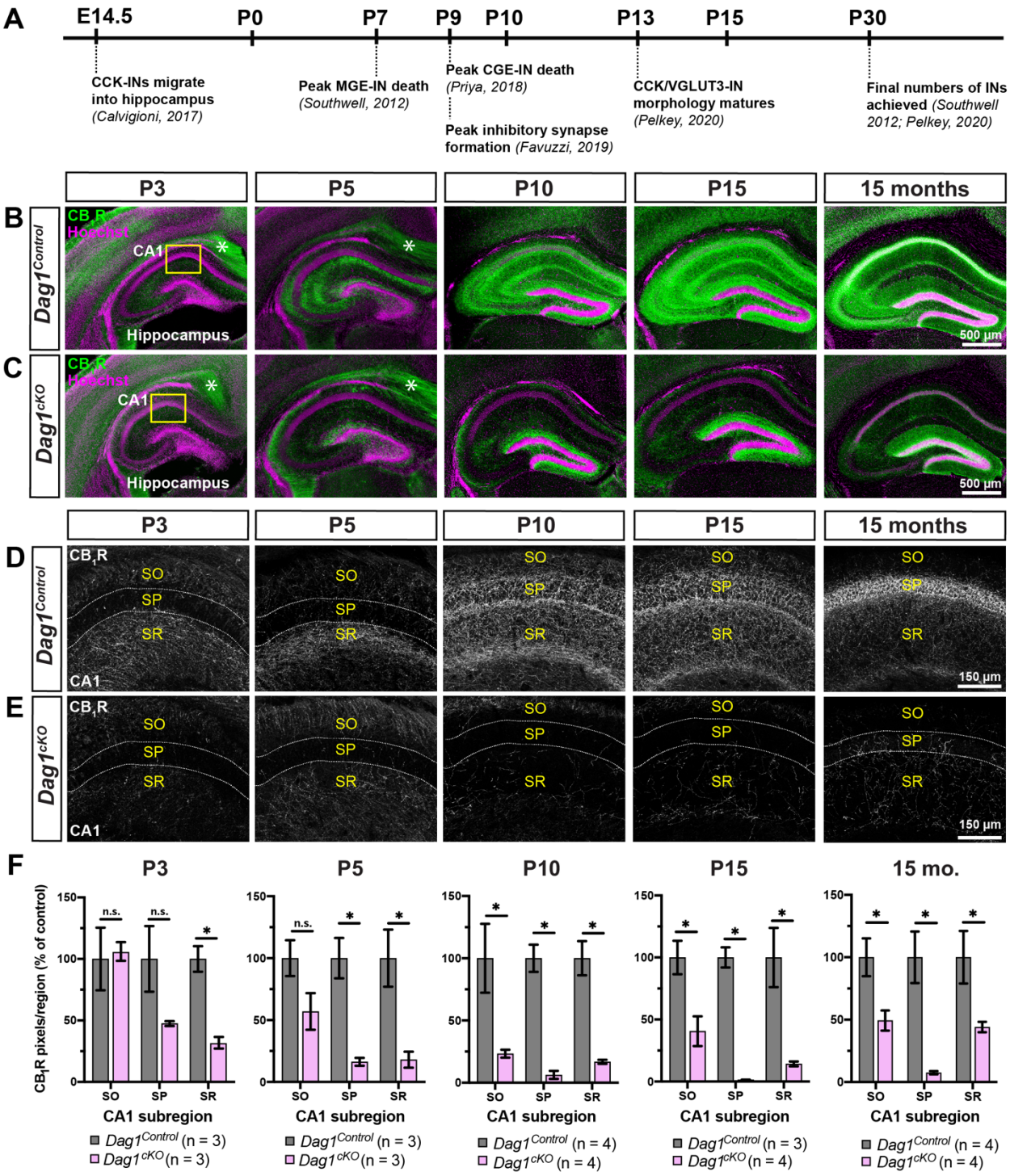

Figure 5. Postnatal development of CCK+ interneurons is impaired in the hippocampus of *Dag1*^{cKO} mice. (A) Timeline of interneuron developmental milestones including interneuron migration, cell death, and inhibitory synapse formation. **(B-C)** Immunostaining for CB_1R (green) in the hippocampus of *Dag1*^{Control} mice (**B**) shows a progressive increase in CCK+ interneuron axon terminals from P3-P15. In contrast,

CB_1R+ axon terminals are diminished at all ages in *Dag1*^{cKO} mice (**C**). Asterisks (P3 and P5) denote the presence of CB_1R immunoreactivity in pyramidal neuron axons at early postnatal ages. Yellow boxes **(B, C)** indicate approximate locations of high magnification images in **(D-E).** High magnification (20X), single channel images (gray) of CB_1R+ axon terminals in the CA1 of *Dag1*^{Control} (**D**) and *Dag1*^{cKO} mice (**E**) from P3-15 months. Dotted white lines indicate the position of the pyramidal cell layer (SP). SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*. **(F)** Quantification of CB_1R pixels in hippocampal CA1 layers from *Dag1*^{Control} (gray) and *Dag1*^{cKO} (pink) mice shows significantly reduced CB_1R staining at all ages examined (**P* < 0.05, unpaired two-tailed Student's t-test; n = 3-4 mice/genotype). Data are presented as mean values ± s.e.m. Data are normalized to *Dag1*^{Control} signal in each CA layer.

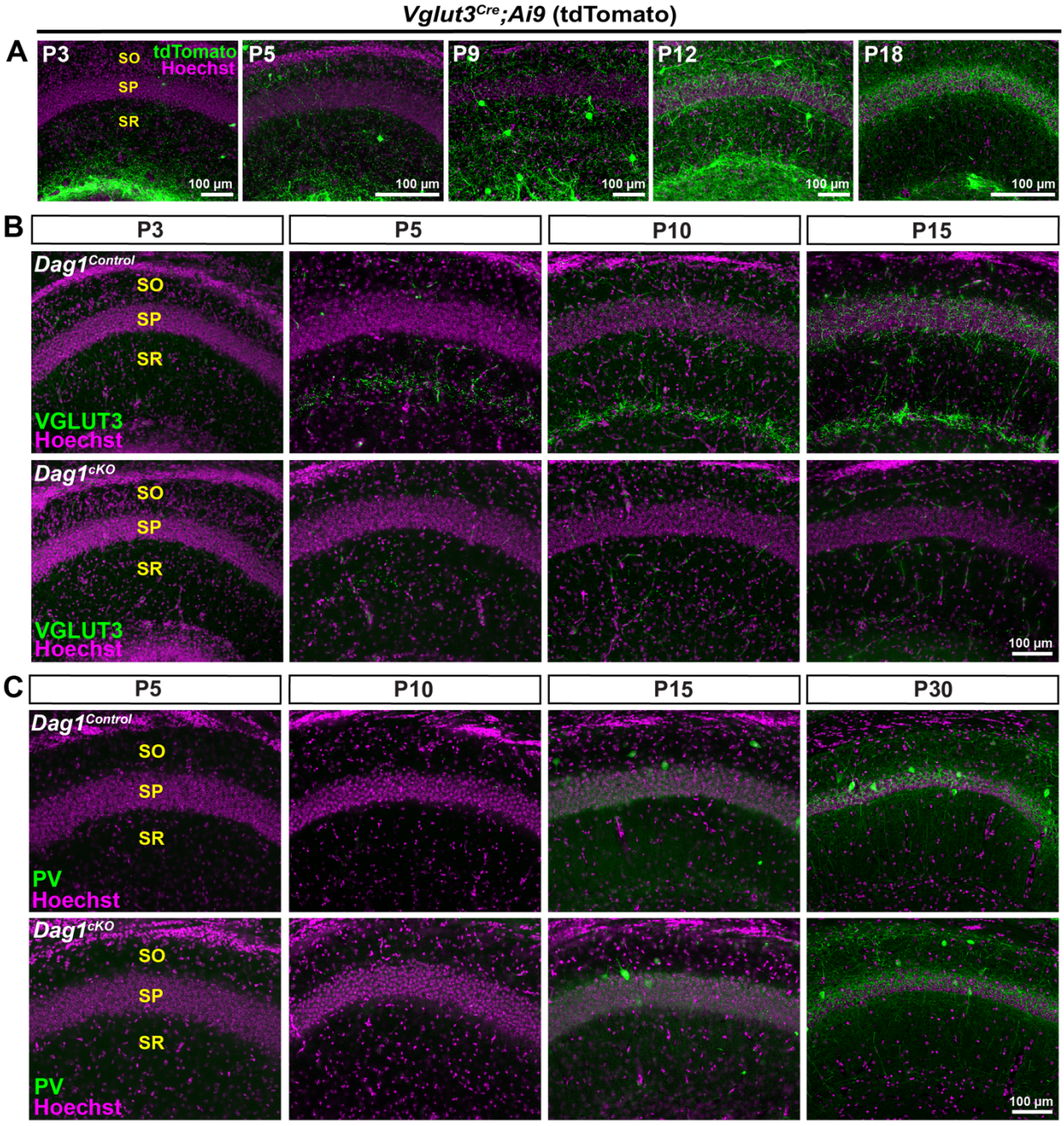

Figure S3. CCK+ interneuron markers are reduced postnatally in *Dag1*^{cKO} mice. **(A)** Images of hippocampal CA1 from *VGLUT3*^{Cre}*;Ai9* mice from P3-P18. Immunostaining for tdTomato (green) shows progressive increase in VGLUT3 expression in the pyramidal cell layer (SP, magenta). **(B)** Immunostaining for VGLUT3 in the CA1 of *Dag1*^{Control} (top panels) and *Dag1*^{cKO} mice (bottom panels) from P3-P15. Note the lack of VGLUT3 expression at all ages in *Dag1*^{cKO} mice. **(C)** Parvalbumin (PV) labeling is similar in the CA1 of *Dag1*^{Control} (top panels) and *Dag1*^{cKO} mice (bottom panels) from P5-P30.

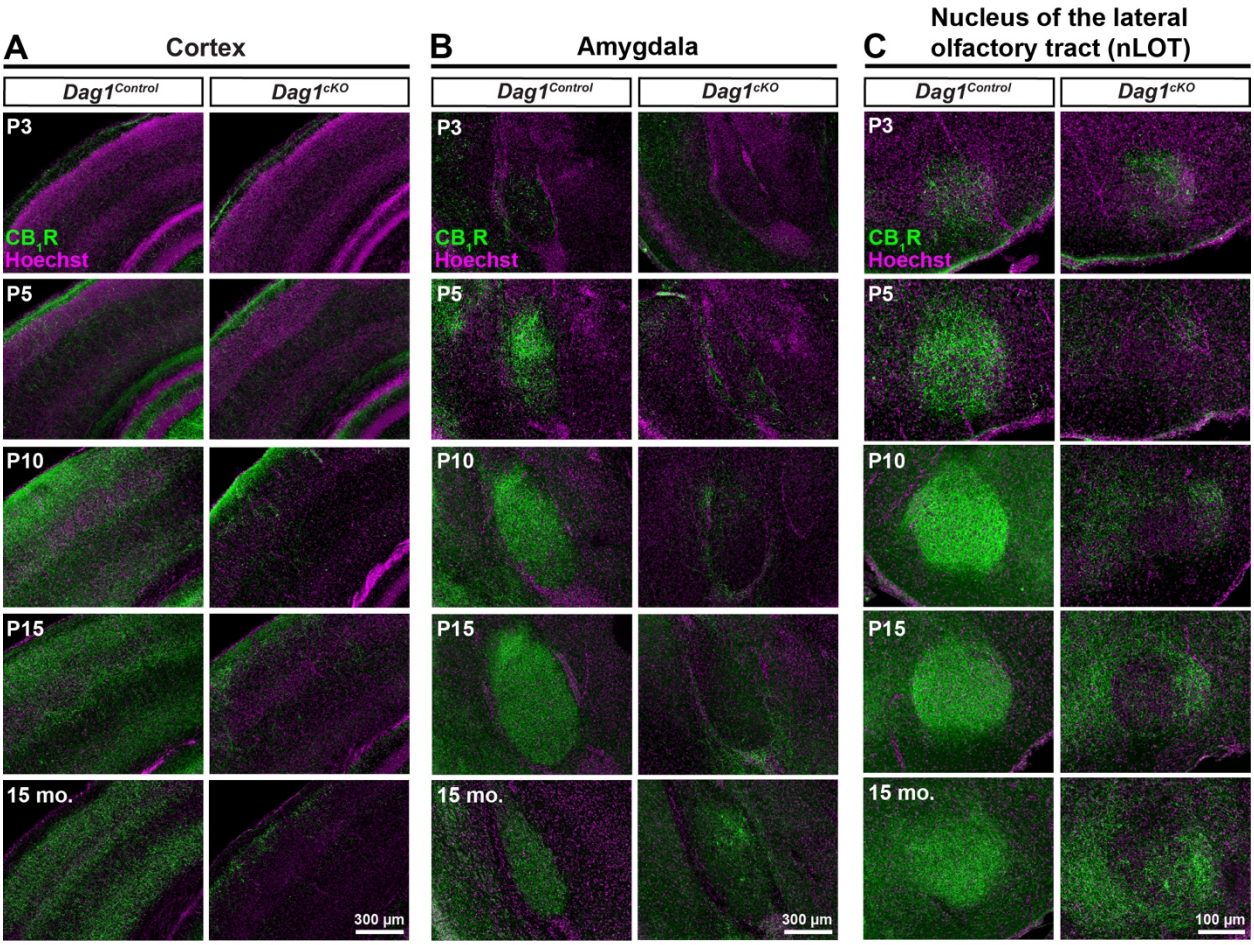

Figure 6. Postnatal development of CCK+ interneurons is impaired in the forebrain of *Dag1*^{cKO} mice. (A-C) Immunostaining for CB_1R (green) and Hoechst (magenta) shows the progressive innervation of the cortex **(A)**, amygdala **(B)**, and nucleus of the lateral olfactory tract **(C)** of *Dag1*^{Control} (left panels) mice by CCK+ interneurons from P3-P15. CB_1R staining is decreased in all regions of *Dag1*^{cKO} mice (right panels) at all ages examined from P3-15 months.

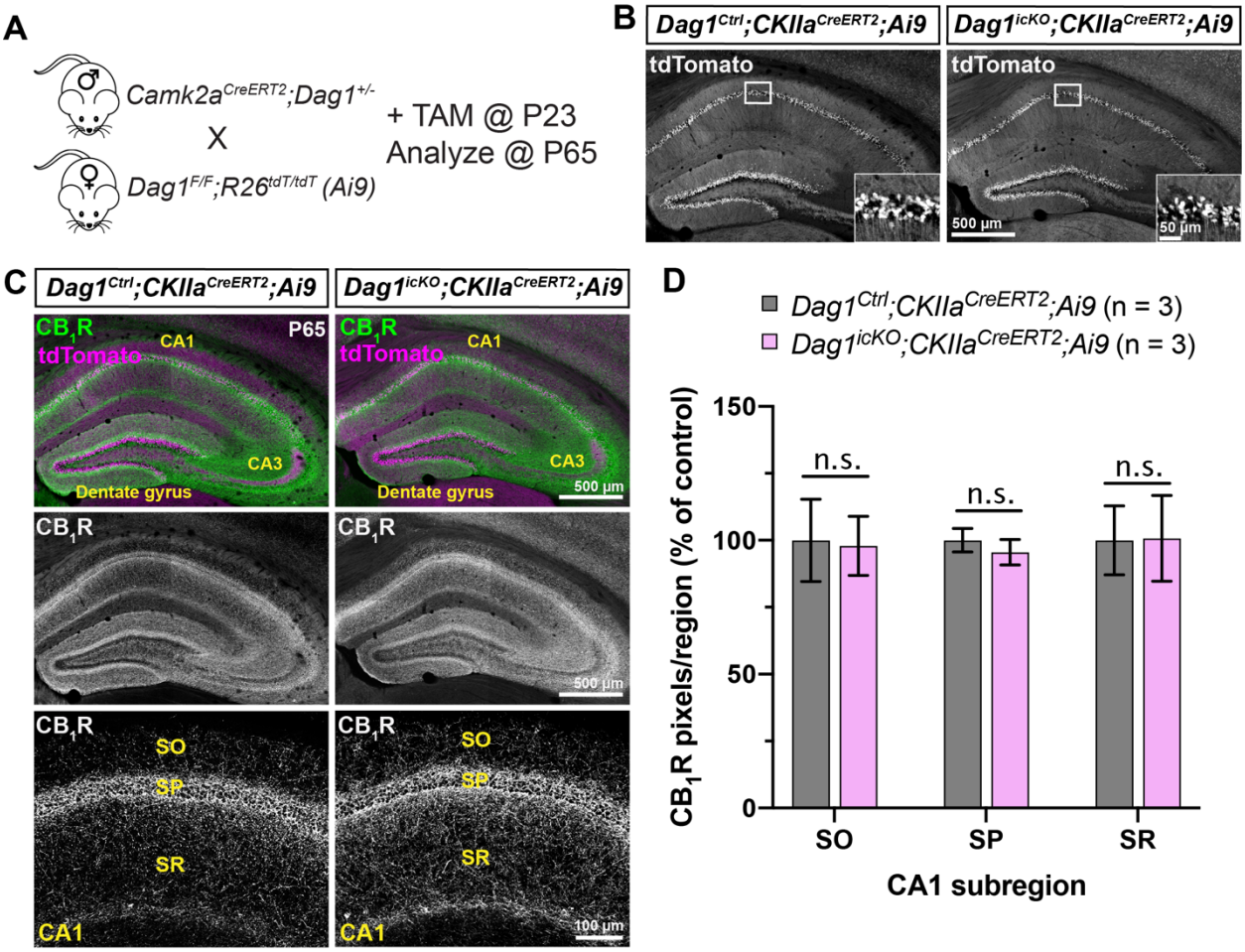

Figure 7. Post-developmental maintenance of CCK+ interneurons does not require Dystroglycan. (A) Breeding scheme and experimental approach for generating tamoxifen-inducible *Dystroglycan* conditional knockout mice. *Dag1*^{Ctrl}*;Camk2a*^{CreERT2}*;Ai9* and *Dag1*^{icKO}*;Camk2a*^{CreERT2}*;Ai9* mice were treated with tamoxifen (5 mg/ml) at P23 and brains were collected for immunohistochemistry six weeks later at P65. **(B)** Single channel images of tdTomato staining in the hippocampus show the recombination pattern in PyNs. Insets show enlarged view of tdT+ pyramidal neurons in the CA1. **(C)** Immunostaining for CB_1R+ terminals (green) and tdTomato signal (magenta) in the hippocampus of P65 *Dag1*^{Ctrl}*;Camk2a*^{CreERT2}*;Ai9* (left panels) and *Dag1*^{icKO}*;Camk2a*^{CreERT2}*;Ai9* mice (right panels) shows that the deletion of *Dystroglycan* in adult PyNs does not affect CB_1R+ terminal maintenance. **(D)** Quantification of CB_1R pixels in hippocampal CA1 of *Dag1*^{Ctrl}*;Camk2a*^{CreERT2}*;Ai9* (gray) and *Dag1*^{icKO}*;Camk2a*^{CreERT2}*;Ai9* (pink) mice (n.s. = not significant, unpaired two-tailed Student's t-test; n = 3 mice/genotype). Data are presented as mean values ± s.e.m. Data are normalized to *Dag1*^{Control} signal in each layer. SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

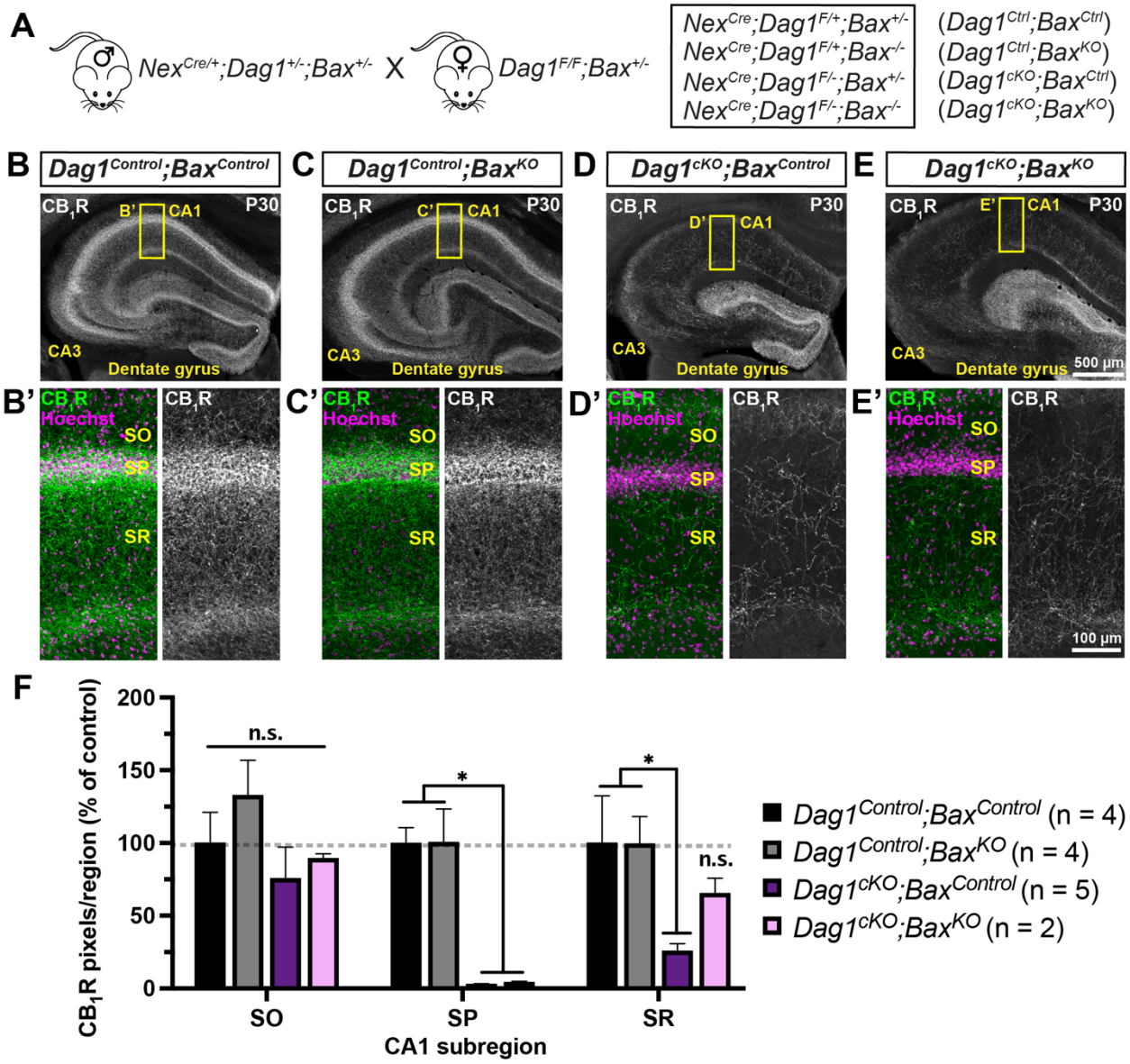

Figure 8. Constitutive deletion of *Bax* does not rescue CB_1R+ terminals in the hippocampus. (A) Breeding scheme for deletion of *Bax* in *Dag1*^{Control} and *Dag1*^{cKO} mice; the four genotypes analyzed and their abbreviations are shown to the right. **(B-E)** Coronal sections of the hippocampus stained for CB_1R (gray) from **(B)** *Dag1*^{Control};*Bax*^{Control}, **(C)** *Dag1*^{Control};*Bax*^{KO}, **(D)** *Dag1*^{cKO};*Bax*^{Control} and **(E)** *Dag1*^{cKO};*Bax*^{KO} mice. **(B'-E')** Enlarged images of the CA1 (yellow boxed regions) stained for CB_1R (green; Right, gray single channel images) and Hoechst (magenta). **(F)** Quantification of CB_1R pixels in hippocampal CA1 layers from *Dag1*^{Control};*Bax*^{Control} (black bars), *Dag1*^{Control};*Bax*^{KO} (gray bars), *Dag1*^{cKO};*Bax*^{Control} (purple bars), and *Dag1*^{cKO};*Bax*^{KO} (pink bars) shows that deleting *Bax* fails to rescue the loss of CB_1R in *Dag1*^{cKO} mice (n.s. = not significant; *$P < 0.05$, unpaired two-tailed Student's t-test; n = 2-5 mice/genotype). Data are presented as mean values ± s.e.m. Data are normalized

to *Dag1*^{Control}*;Bax*^{Control} signal in each CA1 layer. SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

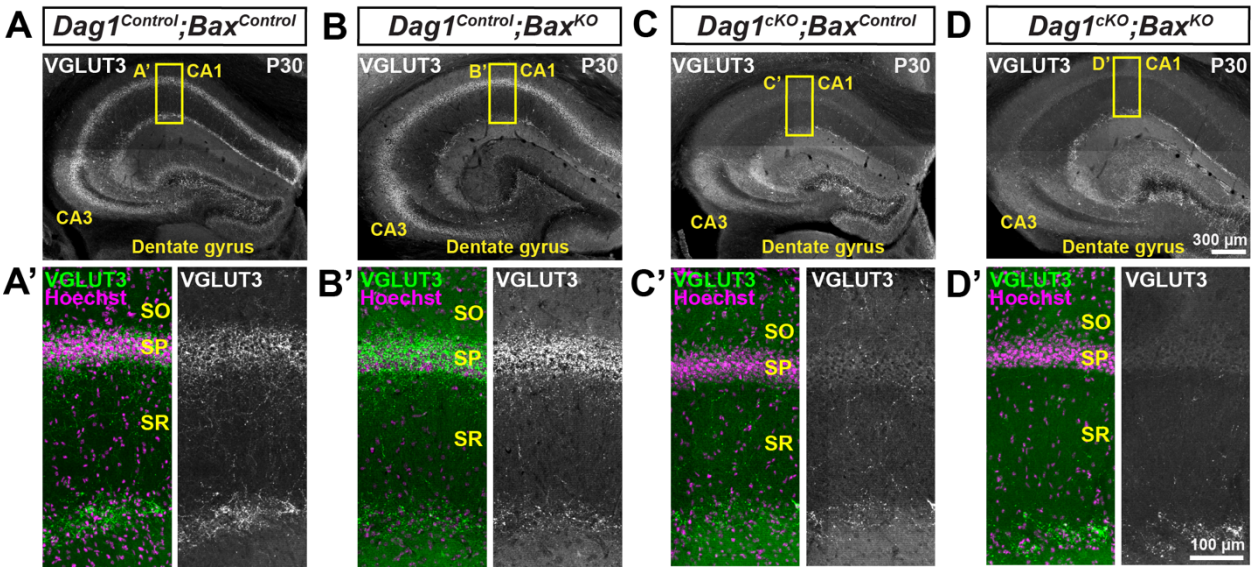

Figure S4. Constitutive deletion of *Bax* in *Dag1*^{cKO} mice does not rescue VGLUT3+ terminals. (A-D) Coronal sections of the hippocampus stained for VGLUT3 (gray) from P30 **(A)** *Dag1*^{Control};*Bax*^{Control}, **(B)** *Dag1*^{Control};*Bax*^{KO}, **(C)** *Dag1*^{cKO};*Bax*^{Control} and **(D)** *Dag1*^{cKO};*Bax*^{KO} mice. **(A'-D')** Magnified images of the CA1 (yellow boxed regions) stained for VGLUT3 (green; Right, gray single channel images) and Hoechst (magenta) to stain the pyramidal cell layer (SP). SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

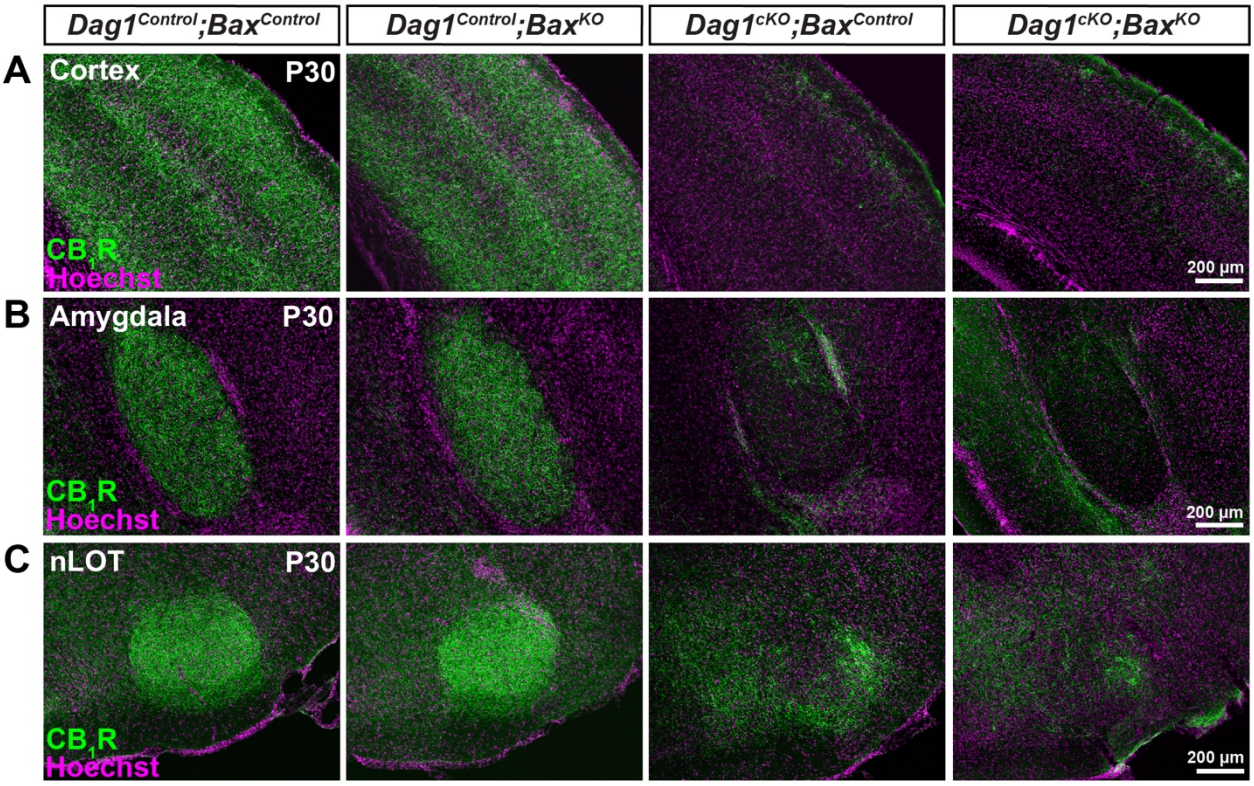

Figure S5. Constitutive deletion of *Bax* in *Dag1^{cKO}* mice does not rescue CB$_1$R+ terminals in the forebrain. (A-C) Coronal sections immunostained for CB$_1$R (green) and Hoechst (magenta) in the cortex **(A)**, amygdala **(B)**, and nucleus of the lateral olfactory tract **(C)** of P30 *Dag1^{Control};Bax^{Control}*, *Dag1^{Control};Bax^{KO}*, *Dag1^{cKO};Bax^{Control}* and *Dag1^{cKO};Bax^{KO}* mice.

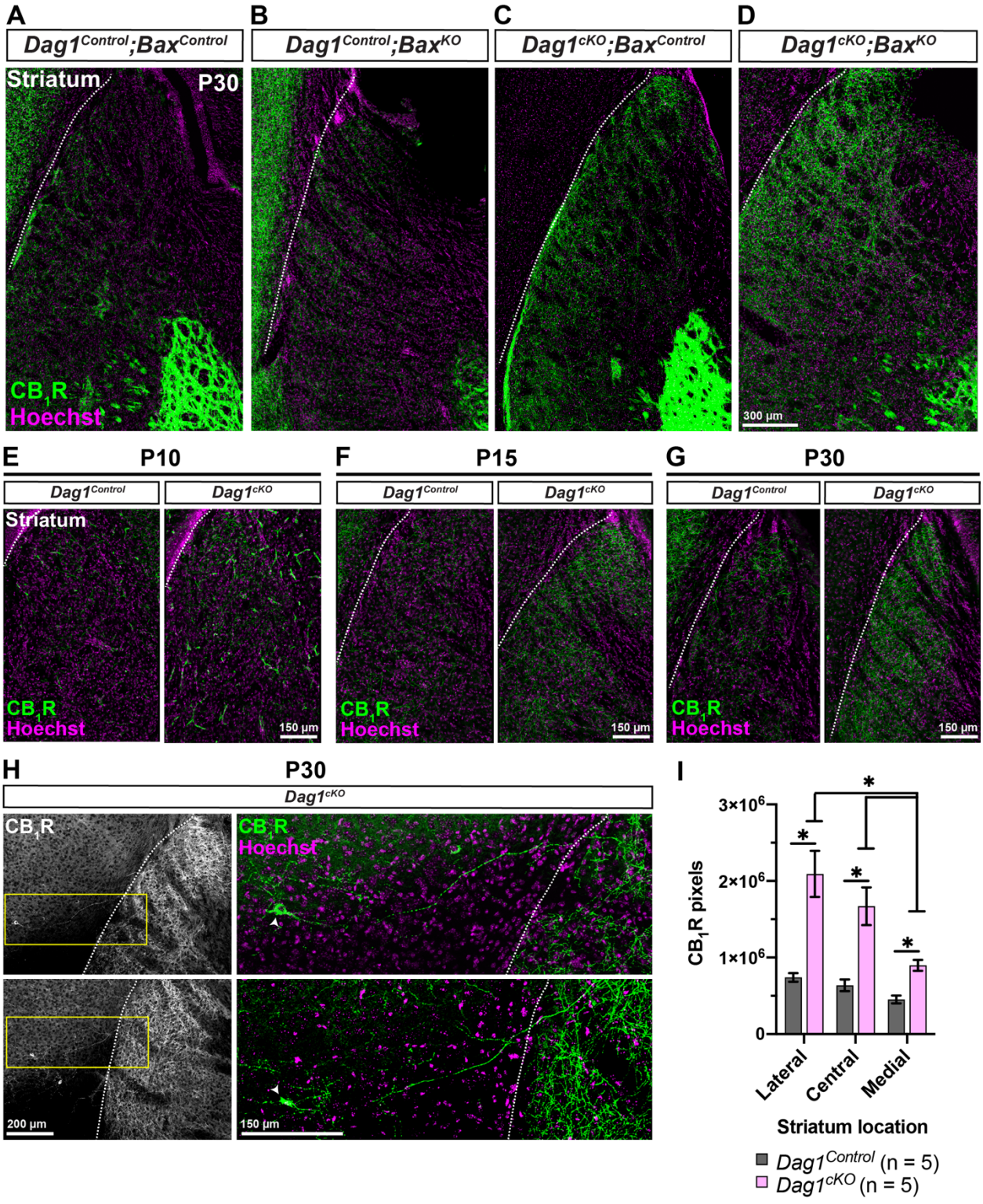

Figure 9. CCK+ interneurons inappropriately innervate the striatum of *Dag1*^{cKO} mice. (A-D) Immunostaining for CB_1R (green) and Hoechst (magenta) shows minimal CB_1R innervation in the striatum of P30 **(A)** *Dag1*^{Control};*Bax*^{Control} and **(B)** *Dag1*^{Control};*Bax*^{KO} mice. Striatal innervation by CB_1R+ axons is abnormally increased in

(C) *Dag1*^{cKO};*Bax*^{Control} and **(D)** *Dag1*^{cKO};*Bax*^{KO} mice. **(E-G)** Immunostaining for CB_1R (green) and Hoechst (magenta) in the striatum of *Dag1*^{Control} and *Dag1*^{cKO} mice at P10 **(E)**, P15 **(F)**, and P30 **(G**), showing that the inappropriate CB_1R innervation in the striatum of *Dag1*^{cKO} mice increases gradually between P10-P30. **(H)** Low magnification images (10X) of CB_1R+ cell bodies and their axons (Left panels, gray) near the cortico-striatal boundary from two separate *Dag1*^{cKO} mice at P30. Yellow boxed regions (right panels) show high magnification (20X) images of individual CB_1R+ cell bodies (arrowheads, green) and their axons projecting from the cortex into the striatum. White dotted lines **(A-H)** indicate the approximate boundary between the cortex and striatum. **(I)** Quantification of CB_1R pixels in the caudal striatum from P30 *Dag1*^{Control} (black bars) and *Dag1*^{cKO} (pink bars) mice shows increased CB_1R staining in *Dag1*^{cKO} (**P* < 0.05, unpaired two-tailed Student's t-test; n = 5 mice/genotype). Data are presented as mean values ± s.e.m.

CHAPTER 3

Dystroglycan is required for proper CCK/CB1R axon targeting in mouse models of dystroglycanopathy

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3.1 ABSTRACT

Dystroglycan is a cell adhesion molecule expressed in muscle and brain that links the extracellular matrix to the actin cytoskeleton through two subunits, a glycosylated extracellular subunit alpha dystroglycan (α-DG), and a transmembrane subunit beta-dystroglycan (β-DG). Dystroglycan performs critical functions during muscle and brain development, and mutations in multiple enzymes required for its glycosylation result in a spectrum of neuromuscular diseases characterized by mild to severe muscle weakness, brain defects, and cognitive impairments. Whereas dystroglycan's role in early muscle and brain development are fairly well studied, much less is known about the role of dystroglycan at later stages of neural circuit development including synapse targeting and refinement. Recent studies have shown that dystroglycan is necessary for the development of inhibitory synapses arising from GABAergic interneurons expressing the neuropeptide cholecystokinin (CCK). However, the molecular mechanism of dystroglycan at CCK+ interneuron synapses is unknown. In this study, we use *Dystroglycan* conditional mice and mouse models of dystroglycanopathy to investigate how dystroglycan regulates CCK+ interneuron development during early postnatal ages. Mice lacking *Dystroglycan* throughout the CNS recapitulate neuronal migration defects observed in human dystroglycanopathy. Although CCK+ interneurons were capable of migrating into the forebrains of dystroglycan mutant mice, they exhibited impaired axonal targeting of pyramidal cell bodies by the first postnatal week. CCK+ interneuron axon targeting was largely intact in mutant mice with reduced dystroglycan glycosylation of α-DG (*B4gat1*, *Fkrp*) and mice lacking the intracellular domain of β-DG. Collectively, these data suggest that

dystroglycan is critical for the proper laminar targeting of pyramidal neurons by presynaptic CCK+ interneurons.

3.2 BACKGROUND

The formation of neural circuits is a multistep process involving stem cell proliferation, neuronal migration, axon guidance, neurochemical and morphological maturation of neuronal subtypes, and establishment of functional synaptic connections between neurons. Cell adhesion molecules (CAMs) play important roles during multiple stages of neural circuit development, but their functions in synapse formation and function are largely unknown. Many families of CAMs have been identified at excitatory and inhibitory synapses, including Neurexins/Neuroligins, Cadherins/Protocadherins, Slitrks, SynCAMs, LRRs (leucine rich repeat)/Netrin-G, Ephrins/Ephs, among others (Chanda et al., 2017; Chen et al., 2018; Dalva et al., 2007; Giagtzoglou et al., 2009; Pettem et al., 2003; Takahashi et al., 2012; Krueger-Burg et al., 2017). An emerging theme from these studies is that synaptic CAMs act in parallel and redundantly to ensure the fidelity of synapse formation and function. Whether CAMs are important for determining initial aspects of synapse development including target recognition/synaptic specificity and laminar or subcellular synaptic targeting is not well understood. Recently, dystroglycan, a CAM widely expressed in muscle and brain, was identified as necessary for the development of a subset of central inhibitory synapses arising from interneurons expressing the neuropeptide CCK (CCK+ interneurons) (Fruh et al., 2016; Miller and Wright, 2021). However, the specific role of dystroglycan at these inhibitory synapses during development remains unclear.

Dystroglycan is a central component of the dystrophin-glycoprotein complex (DGC) known primarily for its role in the etiology of neuromuscular diseases including Duchenne muscular dystrophy (DMD), Limb-girdle muscular dystrophy (LGMD), and Congenital muscular dystrophy (CMD). The gene encoding *Dystroglycan* (*Dag1*) yields two subunits, the extracellular alpha dystroglycan (α-DG) and the transmembrane beta dystroglycan (β-DG) which allow it to bind diverse extracellular ligands as well as interact with cytoskeletal and signaling proteins (Ervasti and Campbell, 1991; Holt et al., 2000; Ibraghimov-Beskrovnaya et al., 1992; Moore and Winder, 2010). The extracellular subunit of dystroglycan (α-DG) binds multiple extracellular proteins in the nervous system including Laminins, Perlecan, Agrin, Pikachurin, Slits and Neurexins that contain Laminin G (LG) domains that interact with dystroglycan's sugar moieties (Campanelli et al., 1994; Dempsey et al., 2019; Gee et al., 1994; Ibraghimov-Beskrovnaya et al., 1992; Peng et al., 1998; Sato et al., 2008; Sugita et al., 2001b; Sugiyama et al., 1994; Wright et al., 2012). The glycosylation of dystroglycan is required for ligand binding, and over 18 enzymes participate in synthesis of glycan chains on α-DG (Manya and Endo, 2017). Mutations in genes encoding these enzymes lead to reduced ligand binding, impaired dystroglycan function, and dystroglycanopathy, a collection of rare genetic diseases characterized by mild to severe muscle and brain defects. Human patients with severe forms of congenital muscular dystrophy (CMD) frequently present with structural brain abnormalities associated with seizures and cognitive impairments (Barresi and Campbell, 2006; Muntoni et al., 2011; Taniguchi-Ikeda et al., 2016). Interestingly, patients without detectable brain malformations often exhibit cognitive impairments

suggesting that dystroglycan may function at later stages of neural circuit formation such as synapse formation or maintenance (Godfrey et al., 2007; Clement et al., 2008).

Within the brain, dystroglycan is expressed in pyramidal neurons, radial glia, astrocytes, and oligodendrocytes where it plays important roles in the formation of basement membranes during early brain development. At later developmental stages, dystroglycan has been identified at multiple synapses throughout the peripheral and central nervous system, including at photoreceptor ribbon synapses in the retina (Omori et al., 2012; Orlandi et al., 2018), inhibitory synapses in the cerebellum (Patrizi et al., 2008), and at a subset of GABAergic inhibitory synapses onto pyramidal neurons (Brunig et al., 2002; Levi et al., 2002). In mice lacking *Dystroglycan* from the CNS, cortical neurons fail to migrate properly and cortical layering is severely disrupted due to early disruption of dystroglycan function in the neuroepithelium and radial glia (Myshrall et al., 2012). Despite the disruption of cortical architecture, GABAergic interneurons, which migrate tangentially through the cortex during embryonic development, are abundant in the brains of *Dag1*-cKO mice. Although interneurons are present in the cortex, whether they are capable of migrating into the hippocampus or innervating their synaptic targets are unknown. Recent studies have demonstrated that cholecystokinin interneurons (CCK+ INs) fail to populate the brain or extend cannabinoid receptor-1 (CB_1R) axons in mice lacking *Dystroglycan* from pyramidal neuron (PyNs) (Fruh et al., 2016; Miller and Wright, 2021). Therefore, we sought to determine whether loss of dystroglycan function in mouse models of dystroglycanopathy affects the postnatal development of interneurons.

3.3 RESULTS

CNS-deletion of *Dystroglycan* results in cortical migration defects resembling cobblestone lissencephaly

To study interneuron development in the context of dystroglycanopathy, we first generated two independent conditional knockout (cKO) mouse lines lacking *Dystroglycan* from the CNS (collectively referred to as CNS-$Dag1^{cKO}$). *Dystroglycan* floxed mice ($Dag1^{F/F}$) (Cohn et al., 2002) were crossed with either $Nestin^{Cre}$ or $Emx1^{Cre}$ mice which drive recombination in CNS progenitors (Tronche et al., 1999; Gorski et al., 2002; Graus-Porta et al., 2001; Guo et al., 2000) (**Fig. 1A)**. Recombination of floxed alleles in $Nestin^{Cre}$ mice begins around embryonic day 11.5 (E11.5) and is complete throughout the CNS by birth (P0). Recombination in $Emx1^{Cre}$ mice begins in the dorsal forebrain at E10.5 and is complete by E12.5 (Liang et al., 2012). Differences in the timing of recombination between $Nestin^{Cre}$ and $Emx1^{Cre}$ mice allowed us to model the clinical heterogeneity seen in dystroglycanopathy (Sudo et al., 2018). In CNS-$Dag1^{Control}$ mice, dystroglycan staining was observed prominently on blood vessels throughout the brain, as well as puncta concentrated on the cell bodies and proximal dendrites of pyramidal neurons (PyNs) (**Fig. 1B**). In contrast, dystroglycan staining was absent from PyNs and blood vessels in CNS-$Dag1^{cKO}$ mice. In agreement with previous work, $Nestin^{Cre}$;$Dag1^{cKO}$ mice exhibited severe disruption of normal cortical architecture revealed by both immunohistochemical staining for layer markers and sparse genetic labeling of layer 5 pyramidal neurons using the $Thy1^{YFP}$ reporter line (**Fig. 1C;** Satz et al., 2010; Myshrall et al., 2012). CNS-deletion of *Dystroglycan* using $Emx1^{Cre}$ resulted in similar neuronal migration defects, revealed by immunostaining for cortical layer

markers CUX1 (upper layers, 2-4) and CTIP2 (deep layers, 5-6) (**Fig. 1D**). In both CNS-*Dag1*^{cKO} lines, the loss of normal cortical layering and presence of heterotopia recapitulate the cobblestone lissencephaly phenotype observed in human patients with severe dystroglycanopathy.

Dystroglycan **is required non-cell autonomously for CCK+ interneuron axon targeting**

Whether INs migrate into the hippocampus or properly innervate their synaptic targets in mouse models of dystroglycanopathy are unknown. In contrast with the cortex, hippocampal architecture and layers are largely unaffected in CNS-*Dag1*^{cKO} mice, allowing us to assess whether dystroglycan loss affects synaptic targeting. Recent studies have demonstrated that cholecystokinin interneurons (CCK+ INs) fail to populate the brain and extend cannabinoid receptor-1 (CB_1R) axons in mice lacking *Dystroglycan* from pyramidal neurons (PyNs) (Fruh et al., 2016; Miller and Wright, 2021). We first assessed whether CCK+ INs are present in *Nestin*^{Cre}*;Dag1*^{cKO} mice. In *Nestin*^{Cre}*;Dag1*^{Control} mice, CCK+ IN axon terminals expressing CB_1R were abundant throughout the forebrain including the hippocampus (**Fig. 2A**). In the hippocampus, the density of CB_1R+ axon terminals was greatest in the cell body layers (CA1-3) where they are known to form characteristic basket synapses onto PyNs (**Fig. 2A'-A''**). Surprisingly, CCK+ INs were present in the brains of *Nestin*^{Cre}*;Dag1*^{cKO} mice, but their CB_1R+ axon terminals were noticeably reduced in the PyN cell body layer (SP, *stratum pyramidale*) of the CA1 and CA3 (**Fig. 2B**). To confirm the loss of CCK+ IN axons from the SP, we performed immunostaining for VGLUT3, an independent synaptic marker for

CCK+ IN terminals (**Fig. S3**). Similar to CB_1R, the density of VGLUT3+ axon terminals was reduced in the SP, suggesting that the innervation of PyNs by CCK+ INs is impaired. These results demonstrate that CCK+ INs are capable of migrating, differentiating, and establishing axons in *Nestin^{Cre};Dag1^{cKO}* mice, but fail to concentrate in the PyN cell body layer.

During embryonic development, *Nestin^{Cre}* drives recombination in the ventral forebrain (ganglionic eminences) which produces most INs, including CCK+ INs. Many cell types in the brain express *Dystroglycan* including PyNs, astrocytes, and oligodendrocytes, but whether INs also express *Dystroglycan* is unknown (Zaccaria et al., 2001). Because of this, we sought to determine whether *Dystroglycan* plays a cell autonomous role in CCK+ IN axon targeting. Due to the lack of specific Cre driver lines for targeting CCK+ INs, we instead examined *Emx1^{Cre};Dag1^{cKO}* mice that lack *Dystroglycan* in neurons, astrocytes, and oligodendrocytes, but not interneurons (Guo et al., 2000; Gorski et al., 2002). We confirmed the specificity of Cre-mediated recombination in *Emx1^{Cre}* mice by crossing it with Rosa26-Lox-STOP-Lox-H2B-mCherry reporter mice to label the nuclei of recombined cells. H2B-mCherry signal was present throughout the forebrain, including the cortex, hippocampus, and amygdala (**Fig. S1A**). Immunohistochemical staining for cell type-specific markers revealed a high degree of overlap between the H2B-mCherry signal with neurons and astrocytes, but not interneurons, confirming the specificity of this mouse line (**Fig. S1, B-E**). Similar to *Nestin^{Cre};Dag1^{cKO}*, CCK+ IN axon terminals were present in the forebrain of *Emx1^{Cre};Dag1^{cKO}* mice but were reduced in the cell body layer (SP) of CA1 and CA3

(**Fig. 2D**). These results suggest that *Dystroglycan* functions in a non-cell autonomous manner to regulate CCK+ IN axon targeting.

Dystroglycan **is required for proper CCK+ IN axon targeting during early postnatal development**

We next sought to identify the onset of CCK+ IN axon targeting defects in CNS-*Dag1*^{cKO} mice. During early postnatal ages, CCK+ IN axons undergo dramatic laminar rearrangements to preferentially target PyN cell bodies in the hippocampus (Miller and Wright, 2021; Morozov et al., 2003a; 2003b; 2009). As CB_1R staining is largely absent from CCK+ IN axons before birth, we examined the development of CCK+ IN axons beginning at P5 when CB_1R+ axons are visible in the hippocampus (Berghuis et al., 2007; Eggan et al., 2010; Mulder et al., 2008; Vitalis et al., 2008). We first characterized the development of CCK+ IN axons in *Emx1*^{Cre}*;Dag1*^{Control} mice **(Fig. 3A, C)**. At postnatal day 5 (P5), CB_1R+ axons were predominantly found in the *stratum radiatum* (SR) of the hippocampus where immature PyN dendrites are located (**Fig. 3C, F**). Between P10 to P30, the density of CB_1R+ axons became significantly reduced in the SR, coinciding with a progressive increase in the pyramidal cell body layer (SP). Next, we examined CB_1R+ axon development in *Emx1*^{Cre}*;Dag1*^{cKO} mice **(Fig. 3B, D)**. Compared with controls, the density of CB_1R+ axons in the hippocampus of *Emx1*^{Cre}*;Dag1*^{cKO} mice was significantly reduced in the SP layer at all ages examined, beginning at P5 (**Fig. 3E**). By P30, after IN synapse formation and targeting are largely complete, the density of CB_1R+ axons increased in the PyN dendrite layers (SO, SR) compared with controls. In *Emx1*^{Cre}*;Dag1*^{cKO} mice at P30, the density of CB_1R+ axons in

the SP was nearly identical to the SO and SR (**Fig. 3D, F**). Collectively, these results demonstrate that *Dystroglycan* is critical during the first two postnatal weeks for the proper laminar distribution of CCK+ IN axons in the hippocampus.

Glycosylation of dystroglycan is not required for CCK interneuron development

Dystroglycan is capable of binding ligands through extensive glycosylation of its extracellular subunit, α-dystroglycan (α-DG). To assess whether glycosylation of α-DG is required for CCK+ IN axon targeting, we examined mice with varying degrees of dystroglycan glycosylation. We first assessed the integrity of CCK+ IN axons in the brains of mice that exhibit a complete loss of dystroglycan glycosylation due to a point mutation in the gene encoding *Ispd*, an enzyme essential for the extension of sugar chains onto dystroglycan (Wright et al., 2012). $Ispd^{L79*}$ mutant mice exhibit a complete loss of dystroglycan glycosylation, resulting in severe congenital muscle and brain defects similar to human patients with Walker-Warburg syndrome (**Fig. 4A**). Due to the loss of glycosylation and dystroglycan function in multiple tissues, *Ispd* mutant mice ($Ispd^{L79*/L79*}$) die at birth, likely due to respiratory or heart defects. Examination of the cortex of *Ispd* mutant mice revealed breakdown of the pial basement membrane and severe disruptions in cortical architecture (**Fig. 4B**). Immunostaining for cortical layer marker CTIP2 revealed severe cortical dysplasia, with ectopic CTIP2+ neurons present in heterotopia characteristic of neuronal migration defects seen in human dystroglycanopathy (**Fig. 4C**). We next performed immunostaining for CCK+ IN axon terminals in the hippocampus. At P0, the latest time point that could be examined, CB_1R+ axons were present in the SR layer of the hippocampus (**Fig. 4D**). Although

these results suggest that glycosylation of dystroglycan may not be required for CCK+ IN axon targeting, we could not definitively conclude this due to the fact that *Ispd* mutant mice die before CCK+ IN axon targeting is mature.

We next examined CCK+ IN axon targeting in hypomorphic mice that exhibit partial reduction of dystroglycan glycosylation and mild forms of dystroglycanopathy. Mice with mutations in β-1,4-glucuronyltransferase *B4gat1* (previously known as *B3gnt1*) exhibit a 65% reduction in dystroglycan glycosylation, mild muscular dystrophy and no discernable neuronal migration defects (**Fig. 5A**; Wright et al., 2012). Compared with control mice (*B4gat1*$^{M155T/+}$), *B4gat1* mutant mice (*B4gat1*$^{M155T/M155T}$) have normal cortical layers as shown by immunohistochemical staining for layer marker CUX1 (**Fig. 5B**). CCK+ IN axons were largely unaffected the hippocampus of *B4gat1* mutant mice except for a modest reduction of CB1R axons in the SP (**Fig. 5C, D**).

We also examined mice with a mutation in the gene *Fkrp* (fukutin related protein) (Chan et al., 2010). Similar to *B4gat1* mutant mice, *Fkrp* mutant mice (*Fkrp*$^{P448L/P448L}$) exhibited no neuronal migration defects. CCK+ IN axons were largely unaffected the hippocampus of *Fkrp* mutant mice (**Fig. 6C, D**). CCK+ IN axons in Fkrp and B4gat1 mutant mice were also grossly normal in other forebrain brain regions including the cortex, amygdala, and nucleus of the olfactory tract (**Fig. S5**). Collectively, these results suggest that glycosylation of dystroglycan is largely dispensable for proper targeting of CCK+ IN axon.

Deletion of the cytoplasmic domain of β-dystroglycan leads to minor alterations in CCK+ interneuron axon targeting

In addition to binding extracellular ligands, dystroglycan is capable of transducing signals and binding cytoskeletal proteins through the intracellular domain of β-DG. We next asked whether signaling through β-DG was required for CCK+ INs to establish axon terminals in the hippocampus. To answer this question we generated mice lacking the intracellular domain of β-DG (**Fig. 7A**). The generation and characterization of these mice was previously described (Satz et al., 2009; Satz et al., 2010). Immunostaining for cortical layer marker CUX1 (L2-4) revealed normal brain architecture in β-DG mutant mice (DG-/βcyt) (**Fig. 7B**). To determine whether CCK+ INs establish axon terminals in the hippocampus, we performed immunostaining for CB1R (**Fig. 7C**). Similar results were found with VGLUT3, an independent synaptic marker of CCK+ IN axon terminals **(Fig. 7D)**. Examination of other forebrain regions in β-DG mutant mice revealed abundant CCK+ IN terminals throughout the cortex, amygdala, and nucleus of the lateral olfactory tract (**Fig. S5**). The density of CCK+ axon terminals appeared grossly indistinguishable from control mice. Collectively, these results suggest that the intracellular domain of β-DG is not required for CCK+ IN innervation of PyNs. These results are consistent with previous studies showing that β-DG is not required for lamination in other regions of the CNS (Satz et al., 2009).

3.4 DISCUSSION

The study of interneurons at early postnatal ages has been hampered by lack of genetic tools for targeting and manipulating specific subtypes. For instance, CCK+ interneurons have proved challenging as CCK (*Cck*) and CB1R (*Cnr1*) are also expressed in pyramidal neurons. The lack of tools has made it challenging to study how

individual interneuron subtypes may be affected in mouse models of disease, especially during the first two postnatal weeks when interneuron-specific molecular markers are beginning to be expressed. Therefore, much of our understanding of interneuron defects in various neurological diseases involves examination of adult animals, or has required using reporter mice that label large groups encompassing multiple subtypes of interneurons during development (MGE vs CGE).

Previous work has demonstrated that genetic deletion of dystroglycan from pyramidal neurons using Nex^{Cre} leads to profound loss of CCK+ interneuron terminals from the forebrain (Fruh et al., 2016). It remains unclear why CB1R terminals fail to populate the brain in PyN-specific DG-cKO mice (Nex-Cre), whereas combined loss of DG from both PyNs and glia results in a CB1R targeting defect (Nestin/Emx-Cre). One explanation for the synaptic defect in $Nestin^{Cre};Dag1^{cKO}$ mice is that deletion of *Dystroglycan* is delayed due to inefficient Cre-mediated recombination in neural progenitors of $Nestin^{Cre}$ mice compared with $Emx1^{Cre}$ (Liang et al., 2012; Sudo et al., 2018). Small amounts of dystroglycan protein are sufficient to mediate ligand binding (Kanagawa et al., 2009), suggesting that residual dystroglycan in $Nestin^{Cre}$ and hypoglycosylation mutant mice may be sufficient to allow for CB1R+ synapse formation.

Another explanation is that glial DG plays a role in targeting CB1R synapses. Both the $Nestin^{Cre}$ and $Emx1^{Cre}$ driver mice lead to recombination in multiple populations of glia during development, including radial glia, astrocytes, and oligodendrocytes (Nickolls and Bonnemann, 2018). Whether dystroglycan in astrocytes plays a synaptic role has not been directly examined. Astrocytes have been shown to prune excess

synapses during postnatal development and may be involved in sculpting synaptic connectivity.

Our results reveal a critical role for dystroglycan in the correct targeting of CB1R+ axon terminals. Despite the importance of CCK+ INs and CB1R signaling in the brain, relatively little is known about factors required to specify the connectivity of these interneurons. Interestingly, early work showed that conditional deletion of CB1Rs from CCK+ interneurons themselves leads to increased probability of hippocampal pyramidal neurons receiving perisomatic inputs, and an increase in the amount of VGLUT3+ terminals (Berghuis et al., 2007). More recent work used an intersectional genetic approach to manipulate CCK+ interneurons, taking advantage of the fact that the neuregulin receptor ErbB4 is expressed by interneurons. CCK-Cre mice bred with ErbB4 conditional mice allow genetic access to the CCK+ interneuron population, and loss of ErbB4 in CCK+ interneurons lead to defects in CCK+ interneuron wiring in the hippocampus and impairments in spatial coding (del Pino et al., 2017).

Dystrophin is also highly concentrated at postsynaptic sites in the brain (Lidov et al., 1990). Intriguingly, mice lacking dystrophin, a core member of the dystrophin-glycoprotein complex (DGC), exhibit specific defects in the distribution of CCK interneuron synapses in the hippocampus (Krasowska et al., 2014). Mice lacking dystrophin exhibit a reduction in perisomatic VGLUT3/VGAT/NL2 synapses, accompanied by an increase in these markers in the stratum radiatum. The altered distribution of synapses resembles the Nestin/Emx-cKO mice. Dystroglycan is required for the localization/concentration of dystrophin at inhibitory synapses in vitro and in vivo,

raising the possibility that the DGC complex is involved in correct synaptic targeting of CB1R+ interneurons (Fruh et al., 2016; Levi et al., 2002).

3.5 CONCLUSION

We demonstrate that dystroglycan is critical for the development of CCK+ interneuron axon targeting in the postnatal brains of mice lacking dystroglycan in the CNS (modeling dystroglycanopathy). Dystroglycan functions non-cell autonomously, suggesting that it is part of a transsynaptic complex important for presynaptic CCK+ axon targeting. Glycosylation of dystroglycan and the intracellular domain are important for proper CCK+ interneuron targeting. These results suggest that CCK+ interneuron axon targeting defects may contribute to cognitive impairments in dystroglycanopathy.

3.6 MATERIALS AND METHODS

Animal husbandry

All animals were housed and cared for by the Department of Comparative Medicine (DCM) at Oregon Health and Science University (OHSU), an AAALAC-accredited institution. Animal procedures were approved by OHSU Institutional Animal Care and Use Committee (Protocol # IS00000539) and adhered to the NIH *Guide for the care and use of laboratory animals*. Animals older than postnatal day 6 (P6) were euthanized by administration of $CO_{2,}$ animals <P6 were euthanized by rapid decapitation. Animal facilities are regulated for temperature and humidity and maintained on a 12 hour light-dark cycle and animals were provided food and water *ad libitum*.

Mouse strains and genotyping

The day of birth was designated postnatal day 0 (P0). Ages of mice used for each analysis are indicated in the figure and figure legends. Mouse strains used in this study have been previously described and were obtained from JAX: *Dystroglycan* conditional mice *Dag1*^{Flox} (JAX #009652; Cohn et al., 2002; Moore et al., 2010), *Dag1*$^{-/-}$ (JAX #006836; Williamson et al., 1997), *Dag1*$^{\Delta cyto}$ (Satz et al., 2009), *Emx1*$^{IRES\text{-}Cre}$ (JAX #005628; Gorski et al., 2002; Guo et al., 2000), *Nestin*^{Cre} mice (JAX #003771; Tronche et al., 1999; Graus-Porta et al., 2001; Dubois et al., 2006). The generation and genotyping of mutant *B4gat1*^{M155T} and *Ispd*^{L79*} mice was previously described (Wright et al., 2012). The *R26*$^{LSL\text{-}H2B\text{-}mCherry}$ reporter mouse (JAX #023139; Peron et al., 2015) was used to conditionally express red fluorescent protein in nuclei upon Cre-mediated deletion of a STOP cassette. All mice were maintained on a C57BL/6 background. To generate control and *Dystroglycan* conditional knockout mice, *Nestin*$^{Cre/+}$*;Dag1*$^{+/-}$ or *Emx1*$^{Cre/+}$*;Dag1*$^{+/-}$ mice were bred with *Dystroglycan* homozygous floxed mice (*Dag1*$^{Flox/Flox}$). Cre positive age-matched littermates were used as controls. Genomic DNA extracted from toe or tail samples (Quanta BioSciences) was used to genotype animals. The presence of the Cre allele in *Nestin*^{Cre} and *Emx1*^{Cre} mice was detected using generic Cre primers (JAX). Floxed alleles were detected as previously described (Michele et al., 2002).

Table 1. Mouse strains

Common name	Strain name	Reference	Stock #
Dag1$^{-/-}$	*B6.129-Dag1*^{tm1Kcam}*/J*	Williamson et al., 1997	006836
Dag1^{Flox}	*B6.129(Cg)-Dag1*$^{tm2.1Kcam}$*/J*	Cohn et al., 2002	009652
Ispd^{L79*}	*C3.B6-Ispd*^{m1Ddg}*/J*	Wright et al., 2012	022019

B4gat1^{M155T}	*B6(C3)-B4gat1*^{m1Ddg}*/J*	Wright et al., 2012	022018
Fkrp^{P448L}	*C57BL/6NJ-Fkrp*^{em1Lgmd}*/J*	Chan et al., 2010	034659
R26$^{LSL-H2B-mCherry}$	*B6.Gt(ROSA)26Sor*$^{tm1.1Ksvo}$	Peron, et al., 2015	023139
Emx1^{Cre}	*B6.129S2-Emx1*$^{tm1(cre)Krj}$*/J*	Gorski et al., 2002	005628
Nestin^{Cre}	*B6.Cg-Tg(Nes-cre)*^{1Kln}*/J*	Tronche et al., 1999	003771

Perfusions and tissue preparation

Brains from mice younger than P15 were dissected and fixed in 5 mls of 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight for 18-24 hrs at 4 degrees C. Mice P15 and older were deeply anesthetized using CO2 and transcardially perfused with ice cold 0.1M PBS for two minutes to clear blood from the brain, followed by 15 mls of ice cold 4% PFA in PBS. After perfusion, brains were dissected and post-fixed in 4% PFA for two or 24 hours. Brains were rinsed with PBS, embedded in 4% low-melt agarose (Fisher: Cat# 16520100), and sectioned at 40 um using a vibratome (VT1200S, Leica Microsystems Inc., Buffalo Grove, IL) into 24-well plates containing 1 ml of 0.1M PBS.

Immunohistochemistry

Single and multiple immunofluorescence detection of antigens was performed as follows: Free-floating vibratome sections (40 μm) were briefly rinsed with PBS, then blocked for 1 hr in PBS containing 0.2% Triton-X (PBST) plus 10% normal donkey serum. Sections were incubated with primary antibodies (**Table 2**) diluted in PBST at 4 degrees C overnight (18-24 hrs). For staining of dystroglycan synaptic puncta, an antigen retrieval step was performed prior to incubation in primary antibody. Briefly, sections were incubated in sodium citrate solution for 15 min at 95 degrees in a water bath. Following incubation in primary antibody, sections were rinsed briefly with PBS

then washed with PBST three times for 20 min each. Sections were then incubated with a cocktail of secondary antibodies (1:1000, Alexa Fluor 488, 546, 647; Fisher) in PBST for 90 min at room temperature. Sections were washed with PBS three times for 20 min each and counterstained with Hoescht 33342 (Life Technologies, Cat# H3570) for 10 min to visualize nuclei. Finally, sections were mounted on slides using Fluoromount-G (Fisher; SouthernBiotech) and sealed using nail polish.

Table 2. Primary antibodies used for immunohistochemistry

Target	Host	Dilution	Source	Catalog #	RRID
α-Dystroglycan (IIH6C4)	Mouse	1:200	Millipore	05-593	AB_309828
CB1R	Guinea pig	1:2000	Synaptic Systems	258-104	AB_2661870
Ctip2	Rat	1:500	Abcam	ab18465	AB_2064130
Cux1	Rabbit	1:250	Santa Cruz Biotech	sc-13024	AB_2261231
GFAP	Mouse	1:1000	Millipore	MAB360	AB_2109815
GFP	Chicken	1:1000	Abcam	13970	AB_300798
Iba1	Rabbit	1:1000	Wako	27030	AB_2314667
Laminin	Rabbit	1:1000	Sigma	L9393	AB_477163
NECAB1	Rabbit	1:2000	Sigma	HPA023629	AB1848014
NeuN	Mouse	1:250	Millipore	MAB377	AB_2298772
Somatostatin	Rabbit	1:2000	Peninsula Labs	T-4103	AB_518614
VGlut3	Rabbit	1:2000	Synaptic Systems	135-203	AB_887886
VIP	Rabbit	1:1000	ImmunoStar	20077	AB_572270

Microscopy

Imaging was performed on a Zeiss Axio Imager M2 fluorescence upright microscope equipped with an Apotome.2 module for structured illumination microscopy. The microscope uses a metal halide light source (HXP 200 C), Axiocam 506 mono camera, and 10X/0.3 NA EC Plan-Neofluar, 20X/0.8 NA Plan-Apochromat objectives. Z-stack images were acquired and processed as maximum projection images using Zeiss Zen Imaging software, and analyzed offline in ImageJ/FIJI (Schindelin et al., 2012). Images used for quantification between genotypes were acquired using the same exposure

times. Brightness and contrast were adjusted in FIJI to improve visibility of images for publication. Figures were composed in Adobe Illustrator CS6 (Adobe Systems).

Quantification

Quantification of CB_1R terminals in the hippocampus was performed on 5 μm z-stacks acquired using a 20X objective. 10-12 images of the CA1 were acquired from 5-6 sections per animal (technical replicates), and at least three animals per genotype (biological replicates) were used for analysis. Sections were taken from equivalent rostro-caudal positions including the dorsal hippocampus (Bregma between -1.48 to -1.94 mm) using coordinates from the mouse brain atlas (Franklin and Paxinos, 1997). All images used for quantification were processed identically. Briefly, background subtraction (Rolling ball radius = 50) and mean filtering (Smooth function in FIJI) were applied to each image to enhance the detection of CB_1R terminals by thresholding. To measure CB_1R signal, a threshold was manually set and applied equally across images to detect only CB_1R signal. Hoechst signal in the SP (CA regions) were used to align the placement of the ROI in the SO, SP, and SR. Raw integrated density values were averaged across all images for each animal and hippocampal layer, and normalized to the mean intensity of the control group (set to 100% for each ROI).

Statistical analysis

All phenotypic analyses were conducted using tissue collected from at least three mice per genotype from at least two independent litters. The number of mice used for each analysis ("n") are indicated in the text and figure legends. No specific power analyses

were performed, but sample sizes were similar to our previous work and other published literature (Wright et al., 2012; Clements et al., 2017; Lindenmaier et al., 2019). Phenotypes were indistinguishable between male and female mice and were analyzed together. In many cases, highly penetrant phenotypes revealed the genotypes of the mice and no blinding could be performed. For comparisons between two groups, significance was determined using a two-tailed Students t test. Statistical significance was set at alpha = 0.05 ($P < 0.05$) and data presented as means ± s.e.m. All statistical analyses were performed in Prism Graphpad (San Diego, CA).

3.7 ACKNOWLEDGMENTS

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3.8 FIGURES

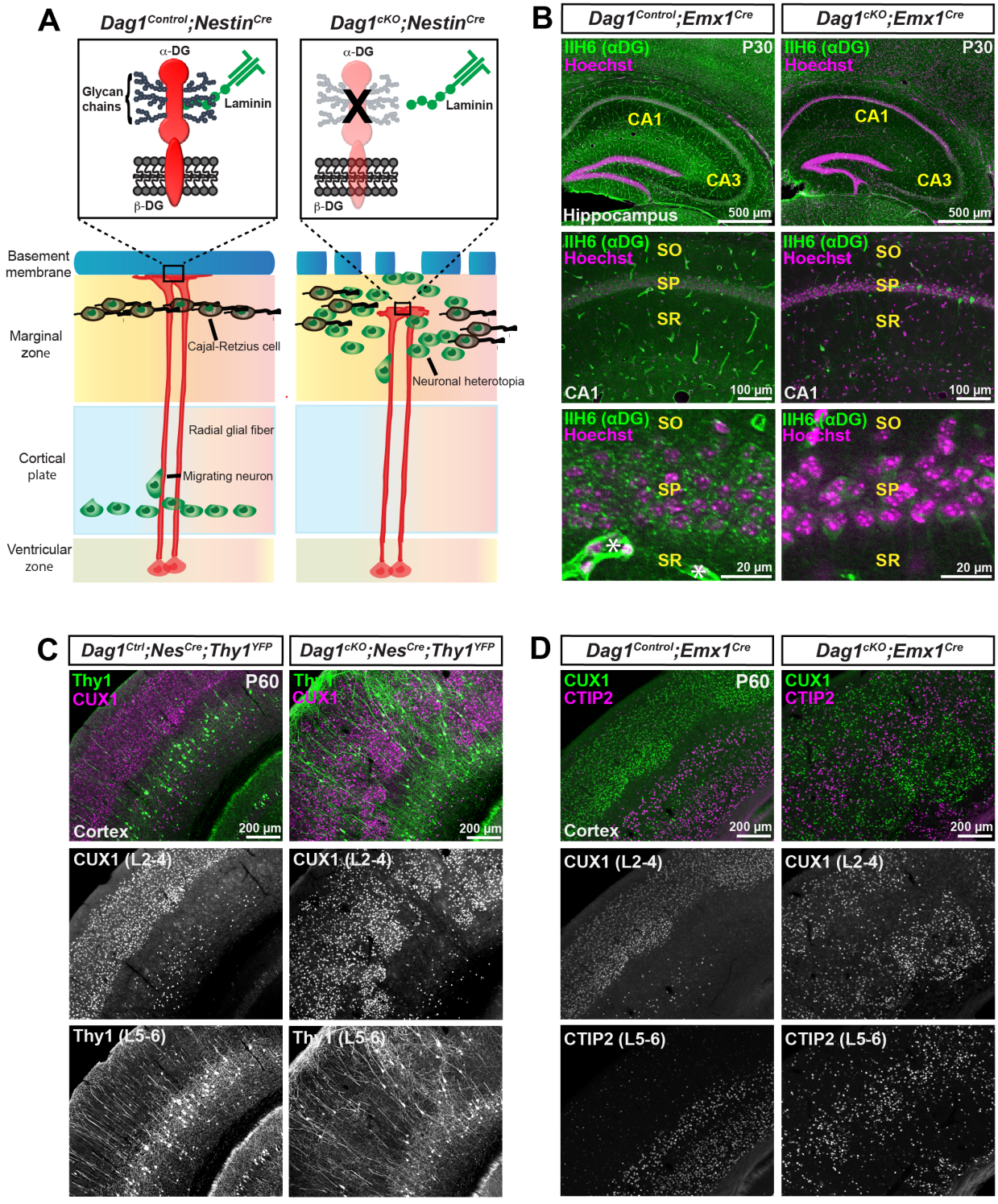

Figure 1. Central nervous system deletion of *Dystroglycan* leads to cortical migration defects resembling cobblestone lissencephaly. (A) Schematic of dystroglycan showing glycan chains on the extracellular alpha subunit (α-DG), and the

transmembrane beta subunit (β-DG). Dystroglycan is localized to radial glial endfeet where it binds laminin at the cortical surface. Deletion of *Dystroglycan* from the neuroepithelium (*Dag1*^{cKO}*;Nestin*^{Cre}) leads to breaches in the basement membrane and neuronal heterotopia (ectopic cortical neurons). **(B)** Coronal sections from P30 *Dag1*^{Control}*;Emx1*^{Cre} mice (left) immunostained for dystroglycan (IIH6, green) shows abundant staining of blood vessels in the cortex and hippocampus (white asterisks, bottom panel). In *Dag1*^{Control}*;Emx1*^{Cre} mice (bottom left panel) dystroglycan puncta (green) are found on cell bodies (Hoechst, magenta) in the pyramidal cell body layer (SP) of CA1. In *Dag1*^{cKO}*;Emx1*^{Cre} mice (right), vascular and synaptic dystroglycan staining are absent from the cortex and hippocampus. CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*. **(C)** Sections from *Dag1*^{Control}*;Nestin*^{Cre} (left) and *Dag1*^{cKO}*;Nestin*^{Cre} mice (right) crossed with a *Thy1*^{YFP} reporter mouse to sparsely label layer 5-6 pyramidal neurons (green). CUX1 (magenta) labels layer 2-4 pyramidal neurons. **(D)** Coronal sections from *Dag1*^{Control}*;Emx1*^{Cre} (left) and *Dag1*^{cKO}*;Emx1*^{Cre} mice (right) immunostained for cortical layer markers CUX1 (green, L2-4) and CTIP2 (magenta, L5-6). Single channel images (gray) are shown below.

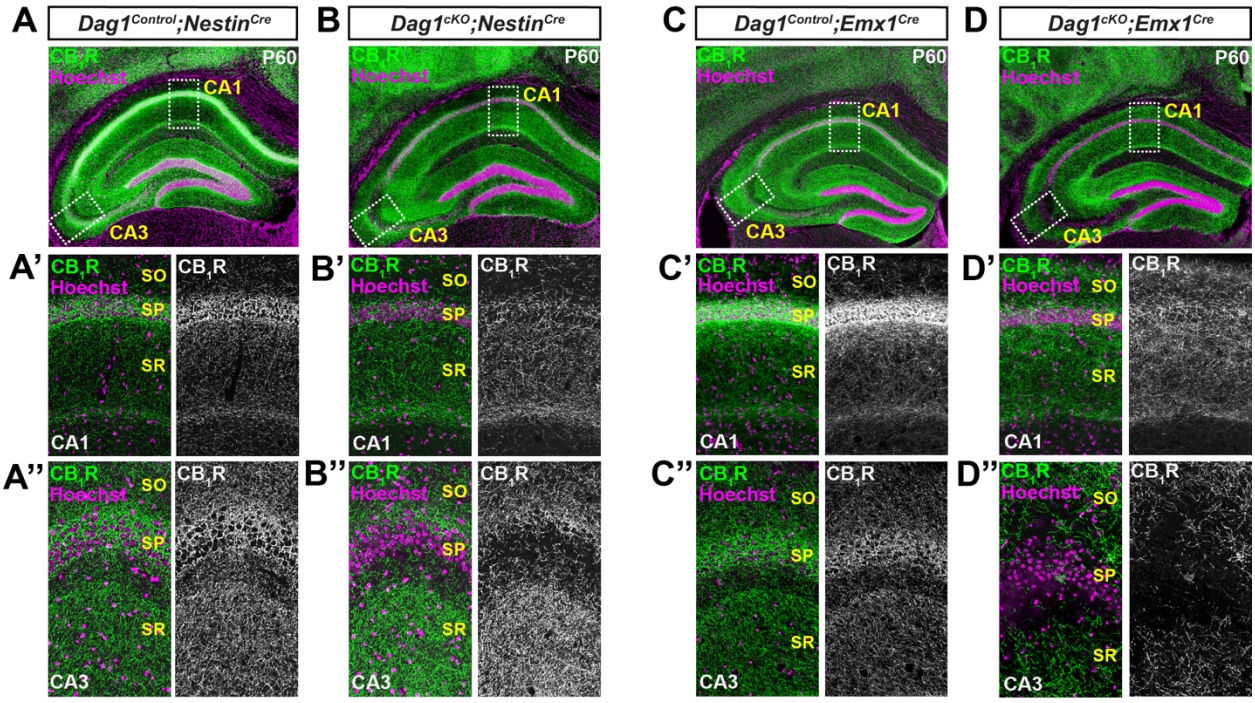

Figure 2. *Dystroglycan* is required non-cell autonomously for CCK+ interneuron axon targeting. (A, B) Immunostaining for CB_1R (green) labels CCK interneuron axon terminals in the hippocampus of P60 *Dag1*^{Control};*Nestin*^{Cre} **(A)** and *Dag1*^{cKO};*Nestin*^{Cre} mice **(B)**. In *Dag1*^{Control};*Nestin*^{Cre} mice **(A)**, CB_1R+ axon terminals are concentrated in the pyramidal cell body layer (SP) of CA1 **(A')** and CA3 **(A'')**. In *Dag1*^{cKO};*Nestin*^{Cre} mice **(B)**, CB_1R+ axon terminal density was reduced in the pyramidal cell body layer (SP) of CA1 **(B')** and CA3 **(B'')**. **(C, D)** Immunostaining for CB_1R (green) in the hippocampus of P60 *Dag1*^{Control};*Emx1*^{Cre} **(C)** and *Dag1*^{cKO};*Emx1*^{Cre} mice **(D)** shows reduced CB_1R+ axon terminal density in the SP of CA1 **(D')** and CA3 **(D'')** compared with *Dag1*^{Control};*Emx1*^{Cre} mice **(C-C'')**. Hoechst (magenta) shows the location of the SP. Single channel CB_1R images (gray) are shown to the right. CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

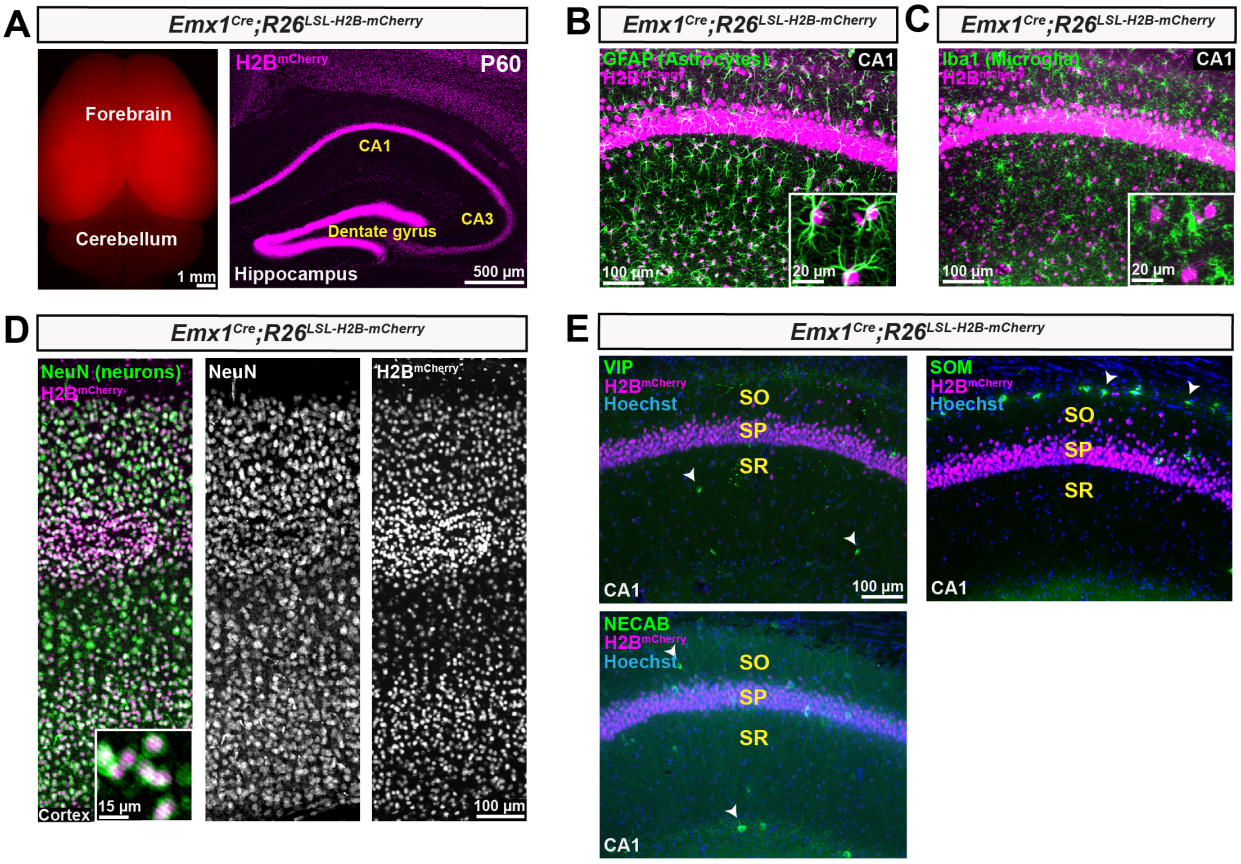

Figure S1. *Emx1*^{Cre} drives recombination in forebrain excitatory neurons and glia, but not interneurons. (A) Left; endogenous red fluorescence in the forebrain of P60 *Emx1*^{Cre};*R26*$^{LSL\text{-}H2B\text{-}mCherry}$ reporter mice. Right; coronal section of the forebrain from *Emx1*^{Cre};*R26*$^{LSL\text{-}H2B\text{-}mCherry}$ mice showing robust nuclear mCherry signal (magenta) in the cortex and hippocampus. **(B-D)** mCherry+ nuclei overlap with markers of multiple cell types in the brain including astrocytes (GFAP, green) **(B)**, microglia (Iba1, green) **(C),** and neurons (NeuN, green) **(D).** Insets show enlarged images of mCherry+ nuclei overlapping with cell type markers. **(E)** mCherry+ nuclei did not overlap with markers for interneuron cell bodies (white arrowheads). CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

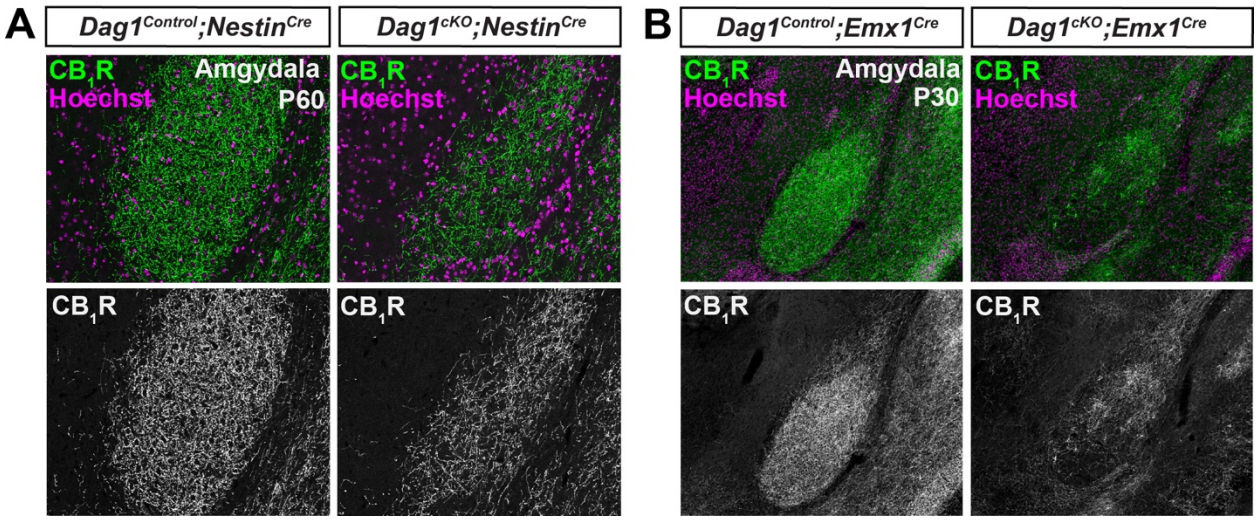

Figure S2. CCK+ interneuron axon targeting is impaired in the amygdala of *Dystroglycan*-cKO mice. (A, B) Immunostaining for CB_1R (green) and Hoechst (magenta) shows abnormal CCK+ axon targeting in the amygdala of **(A)** *Dag1*^{cKO};*Nestin*^{Cre} and **(B)** *Dag1*^{cKO};*Emx1*^{Cre} mice (right panels). Single channel CB_1R images (gray) are shown below.

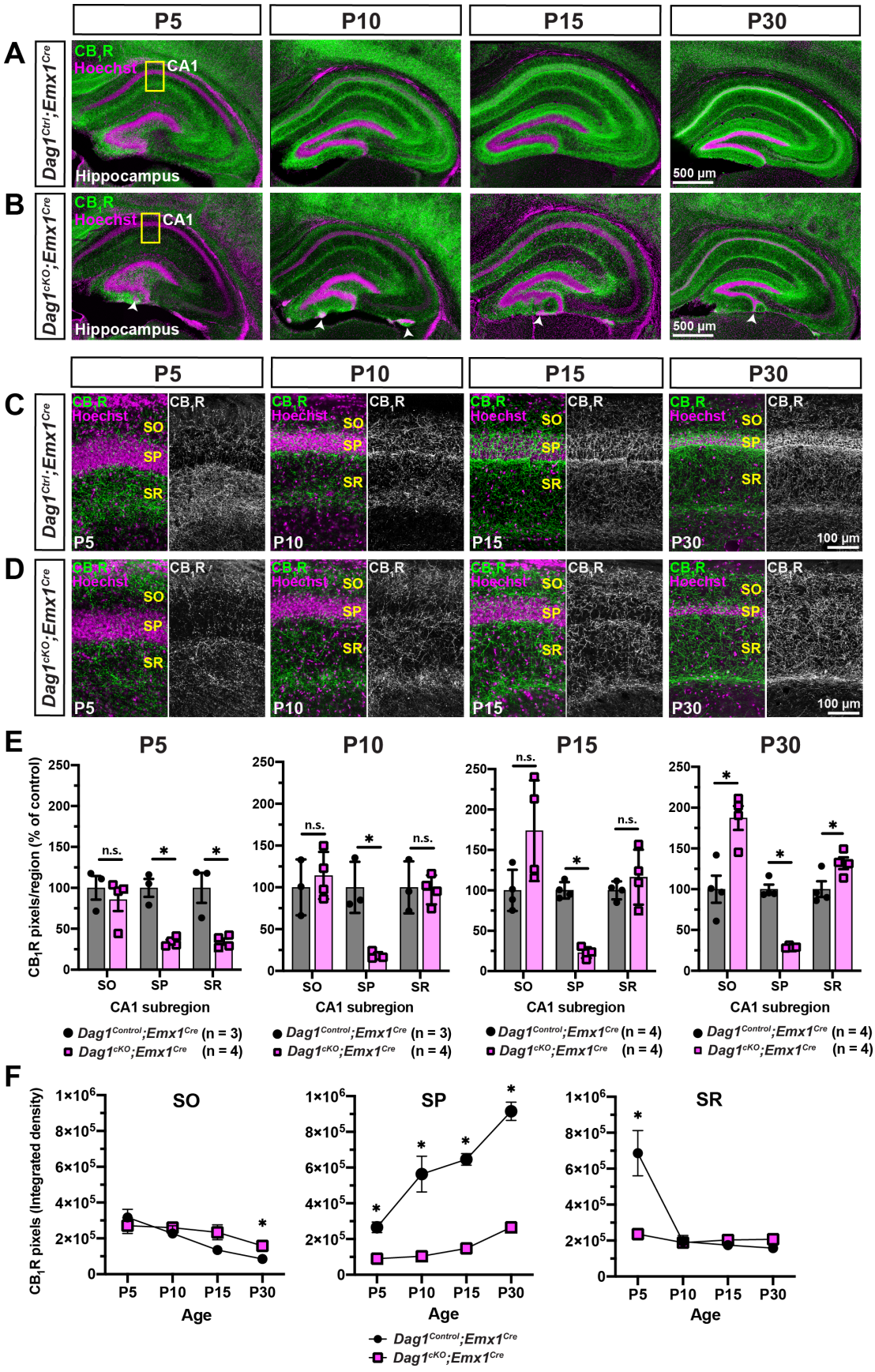
A
B
C
D
E
F
P5
P10
P15
P30
CB1R
Hoechst
CA1
Hippocampus
500 µm
Dag1Ctrl;Emx1Cre
Dag1cKO;Emx1Cre
SO
SP
SR
100 µm
CB1R pixels/region (% of control)
CA1 subregion
n.s.
Dag1Control;Emx1Cre (n = 3)
Dag1cKO;Emx1Cre (n = 4)
Dag1Control;Emx1Cre (n = 4)
CB1R pixels (Integrated density)
Age

Figure 3. *Dystroglycan* is required for CCK+ IN axon targeting during early postnatal development. (A-D) Immunostaining for CB_1R+ axon terminals (green) in the hippocampus **(A, B)** and CA1 **(C, D)** of $Dag1^{Control};Emx1^{Cre}$ mice (top rows) and $Dag1^{cKO};Emx1^{Cre}$ mice (bottom rows) from postnatal day 5 to 30 (P5-30). White arrowheads indicate neuronal migration errors in $Dag1^{cKO};Emx1^{Cre}$ mice. Yellow boxes indicate approximate locations of high magnification images in C and D. **(C-D)** High magnification (20X) images of CB_1R+ axon terminals (green) in the CA1 of $Dag1^{Control};Emx1^{Cre}$ **(C)**, and $Dag1^{cKO};Emx1^{Cre}$ mice **(D)** from P5-P30. Hoechst (magenta) labels the pyramidal cell body layer (SP). Single channel images of CB_1R signal (gray) are shown to the right. **(E)** Quantification of CB_1R pixels in hippocampal CA1 layers SO, SP, and SR of $Dag1^{Control};Emx1^{Cre}$ (gray) and $Dag1^{cKO};Emx1^{Cre}$ (pink) mice (*$P < 0.05$, unpaired two-tailed Student's t-test; n = 3-4 mice/genotype). Data points represent individual animals. Data are presented as mean values ± s.e.m, and normalized to $Dag1^{Control};Emx1^{Cre}$ signal in each CA layer. **(F)** Quantification of non-normalized CB_1R signal in the SO, SP, and SR of $Dag1^{Control};Emx1^{Cre}$ (black) and $Dag1^{cKO};Emx1^{Cre}$ (pink) mice from P5-P30. (*$P < 0.05$, unpaired two-tailed Student's t-test; n = 3-4 mice/genotype). Data are presented as mean values ± s.e.m. CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

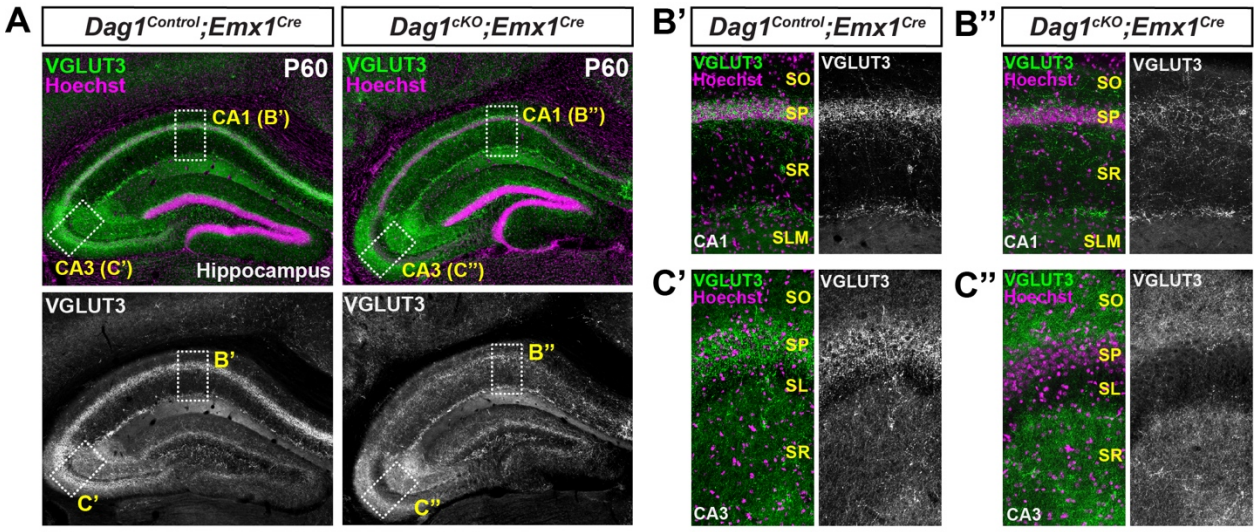

Figure S3. Abnormal distribution of VGLUT3+ terminals in *Dag1^{cKO};Emx1^{Cre}* mice. **(A)** Immunostaining for VGLUT3 (green) in the hippocampus of P60 *Dag1^{Control};Emx1^{Cre}* (left panels) and *Dag1^{cKO};Emx1^{Cre}* mice (right panels). Hoechst (magenta) labels the dentate gyrus and pyramidal cell body layer (SP) of CA1 and CA3. High magnification (20X) images of VGLUT3+ axon terminals (green) in the CA1 **(B', B")**, and CA3 **(C', C")** of *Dag1^{Control};Emx1^{Cre}* and *Dag1^{cKO};Emx1^{Cre}* mice. Single channel images of VGLUT3 signal (gray) are shown below (hippocampus) and to the right (CA1) of merged images. Dotted white boxes indicate locations of high magnification images. SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*; SLM, *stratum lacunosum-moleculare*; SL, *stratum lucidum*.

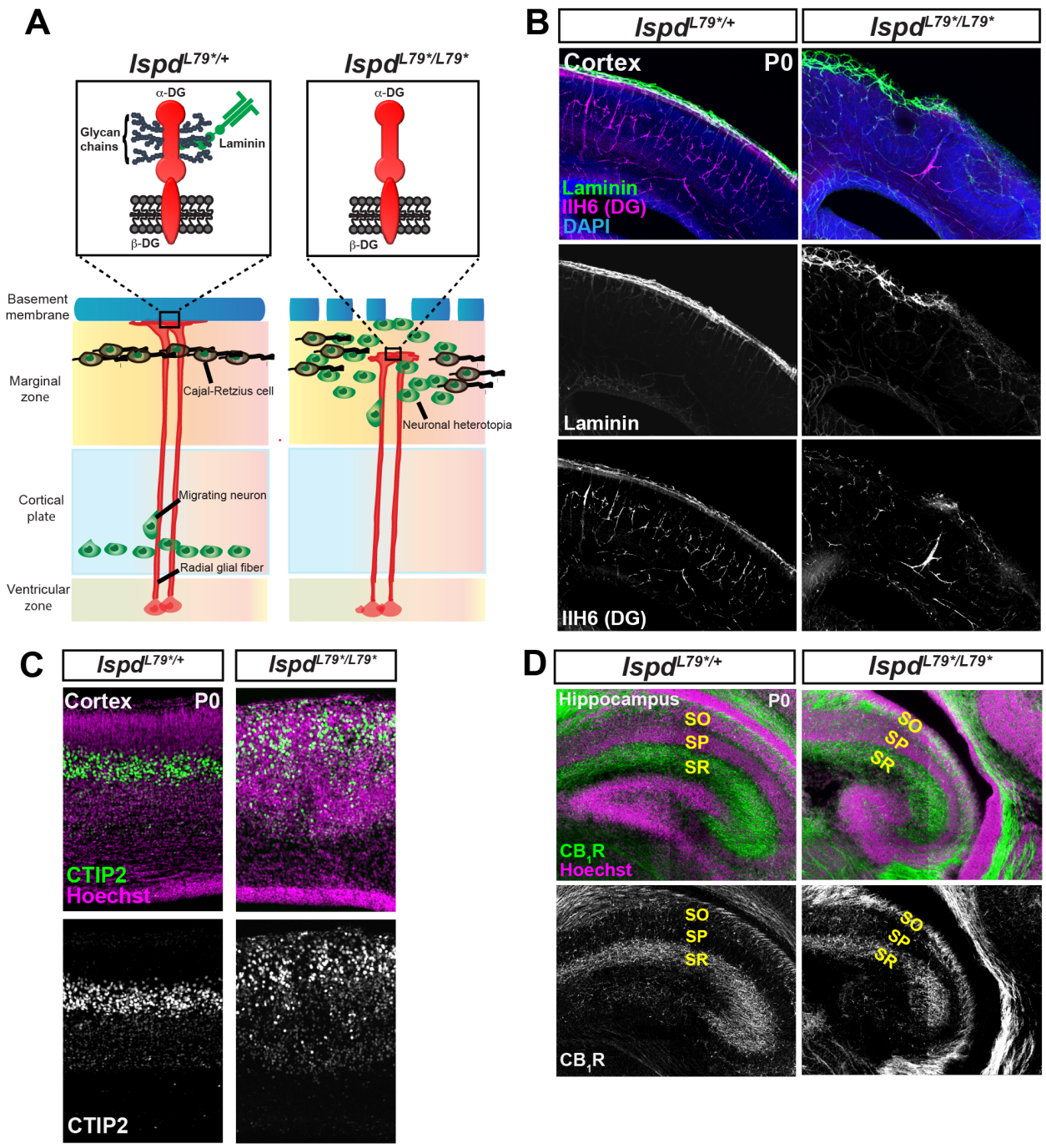

Figure 4. CCK+ interneuron terminals are present at birth in *Ispd*^{L79*} mutant mice lacking dystroglycan glycosylation. (A) Schematic showing lack of sugar chains on alpha dystroglycan (α-DG) and impaired laminin binding due to a point mutation in *Ispd* (right). **(B)** Sections from P0 mice immunostained for the basement membrane marker laminin (green) and dystroglycan (IIH6, magenta). In *Ispd*$^{L79*/+}$ control mice (left), laminin and dystroglycan co-localize at the cortical surface, whereas *Ispd*$^{L79*/L79*}$ mutant mice display loss of dystroglycan staining and disruption of basement membrane architecture. **(C)** Sections immunostained for CTIP2 (L5, green) shows disrupted cortical

layers and ectopic neurons in heterotopia of *Ispd*$^{L79*/L79*}$ mutant mice (right). **(D)** Hippocampal sections from P0 *Ispd*$^{L79*/+}$ control (left) and *Ispd*$^{L79*/L79*}$ mutant mice (right) were immunostained for CB_1R to label nascent CB_1R+ axon terminals. CB_1R+ axon terminals populate the SR of both control and mutant mice. Single channel images (gray) are shown below merged images. CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

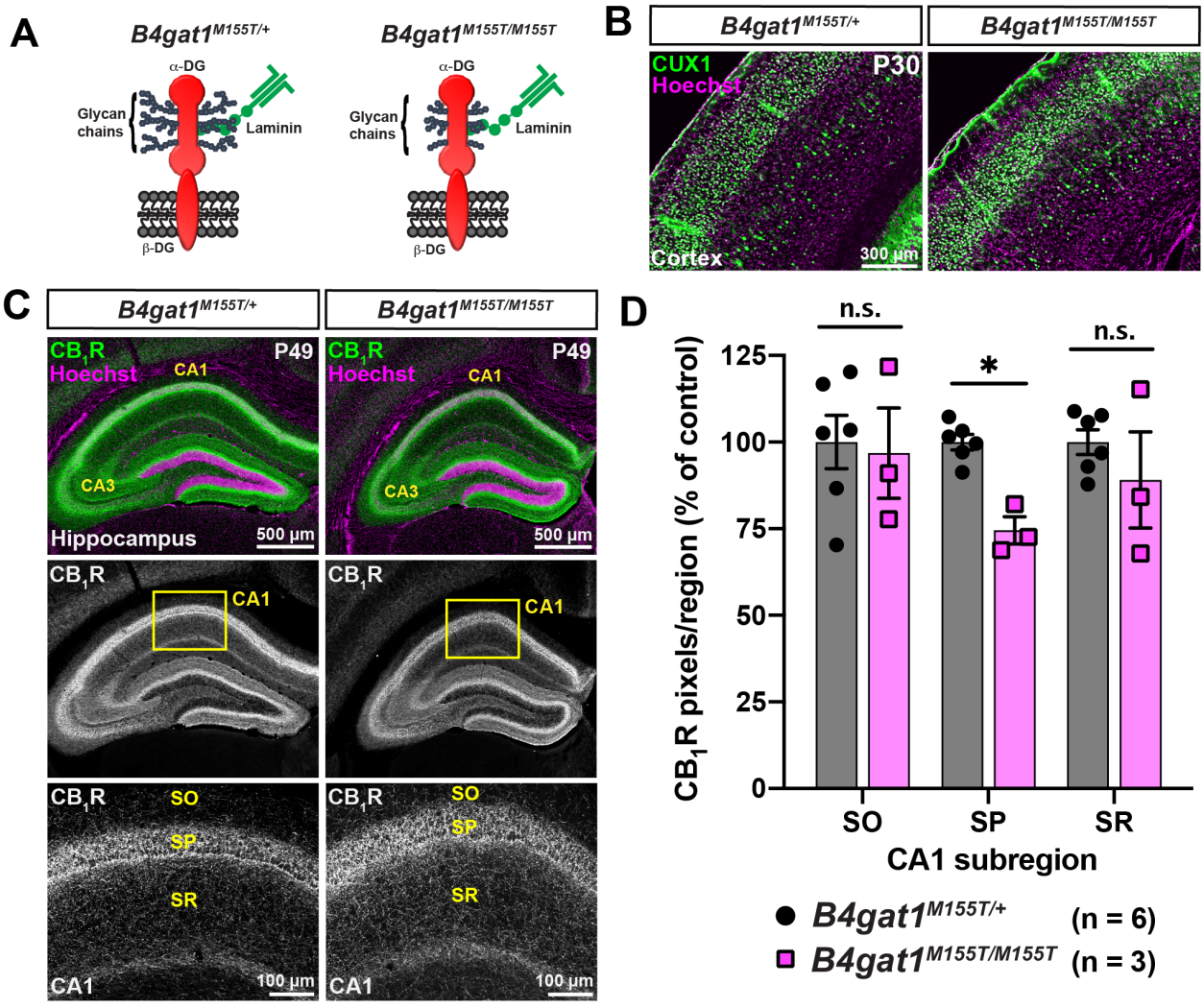

Figure 5. CCK+ interneuron axon targeting is modestly impaired in *B4gat1*^{M155T} mutant mice with reduced dystroglycan glycosylation. (A) Schematic showing fewer sugar chains on alpha dystroglycan (α-DG) due to a point mutation in glycosyltransferase gene *B4gat1* (right). **(B)** Sections immunostained for layer marker CUX1 (L2-3, green) shows normal migration of cortical neurons in *B4gat1*$^{M155T/M155T}$ mutant mice (right). **(C)** Immunostaining for CB_1R shows axon terminals (green) in the hippocampus of P49 *B4gat1*$^{M155T/+}$ control (left panels) and *B4gat1*$^{M155T/M155T}$ mutant mice (right panels). High magnification images (20X) of the CA1 (yellow boxed regions) and single channel CB_1R images (gray) are shown below. **(D)** Quantification of CB_1R pixels in CA1 layers SO, SP, and SR from *B4gat1*$^{M155T/+}$ control (gray) and *B4gat1*$^{M155T/M155T}$ mutant mice (pink) (*$P < 0.05$, unpaired two-tailed Student's t-test; n.s., not significant). Data are presented as mean values ± s.e.m. Data are normalized to the signal measured in control mice for each CA layer. CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

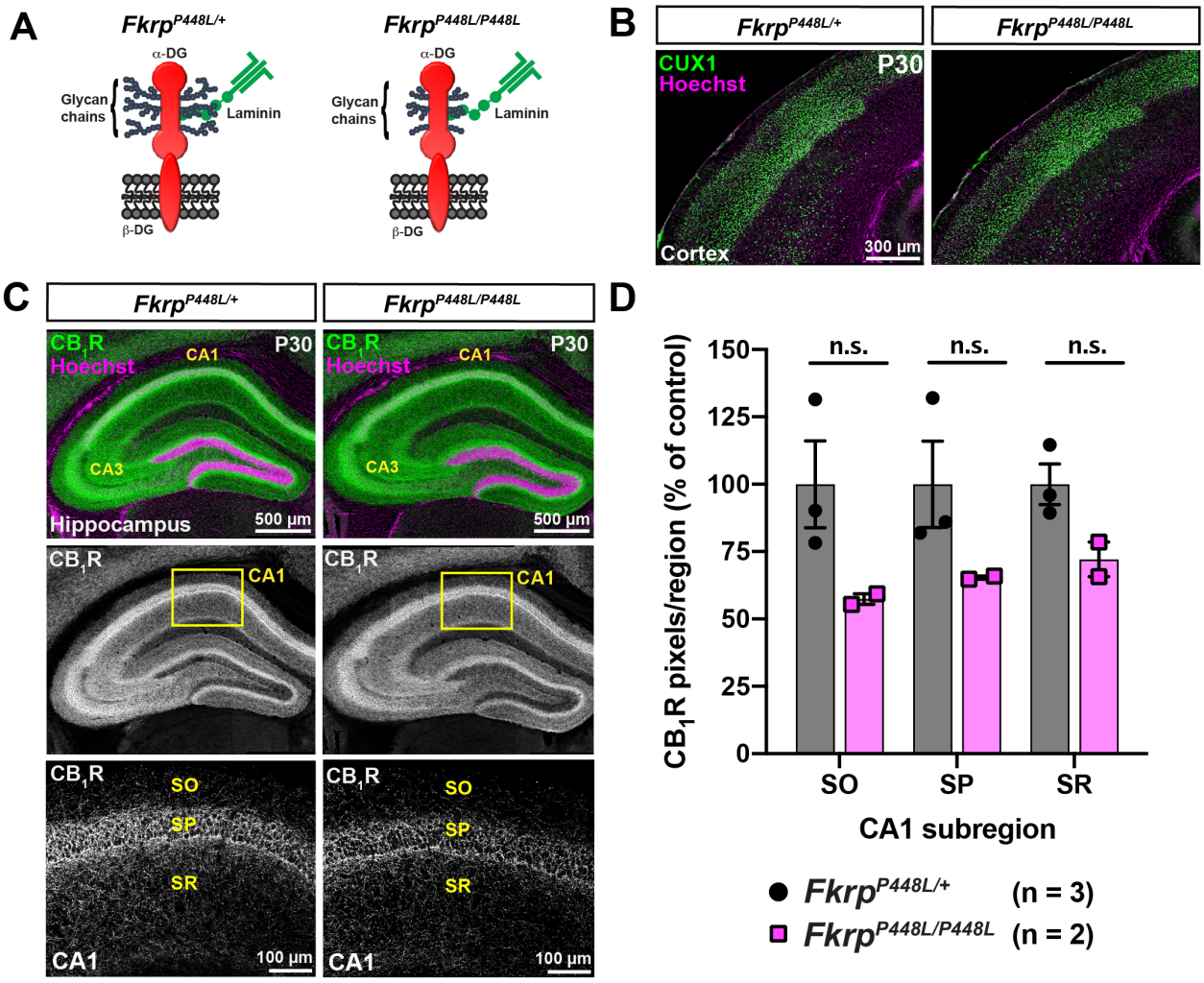

Figure 6. CCK+ interneuron axon targeting is unaltered in *Fkrp*^{P448L} mutant mice with reduced dystroglycan glycosylation. (A) Schematic showing fewer sugar chains on alpha dystroglycan (α-DG) due to a point mutation in the gene *Fkrp* (right). **(B)** Sections immunostained for layer marker CUX1 (L2-3, green) shows normal migration of cortical neurons in *Fkrp*$^{P448L/P448L}$ mutant mice (right). **(C)** Immunostaining for CB_1R shows axon terminals (green) in the hippocampus of *Fkrp*$^{P448L/+}$ control (left panels) and *Fkrp*$^{P448L/P448L}$ mutant mice (right panels). High magnification images (20X) of the CA1 (yellow boxed regions) and single channel CB_1R images (gray) are shown below. **(D)** Quantification of CB_1R pixels in CA1 layers SO, SP, and SR from *Fkrp*$^{P448L/+}$ control (gray) and *Fkrp*$^{P448L/P448L}$ mutant mice (pink). (n.s, not significant; unpaired two-tailed Student's t-test). Data are presented as mean values ± s.e.m. Data are normalized to the signal measured in control mice for each CA layer. CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

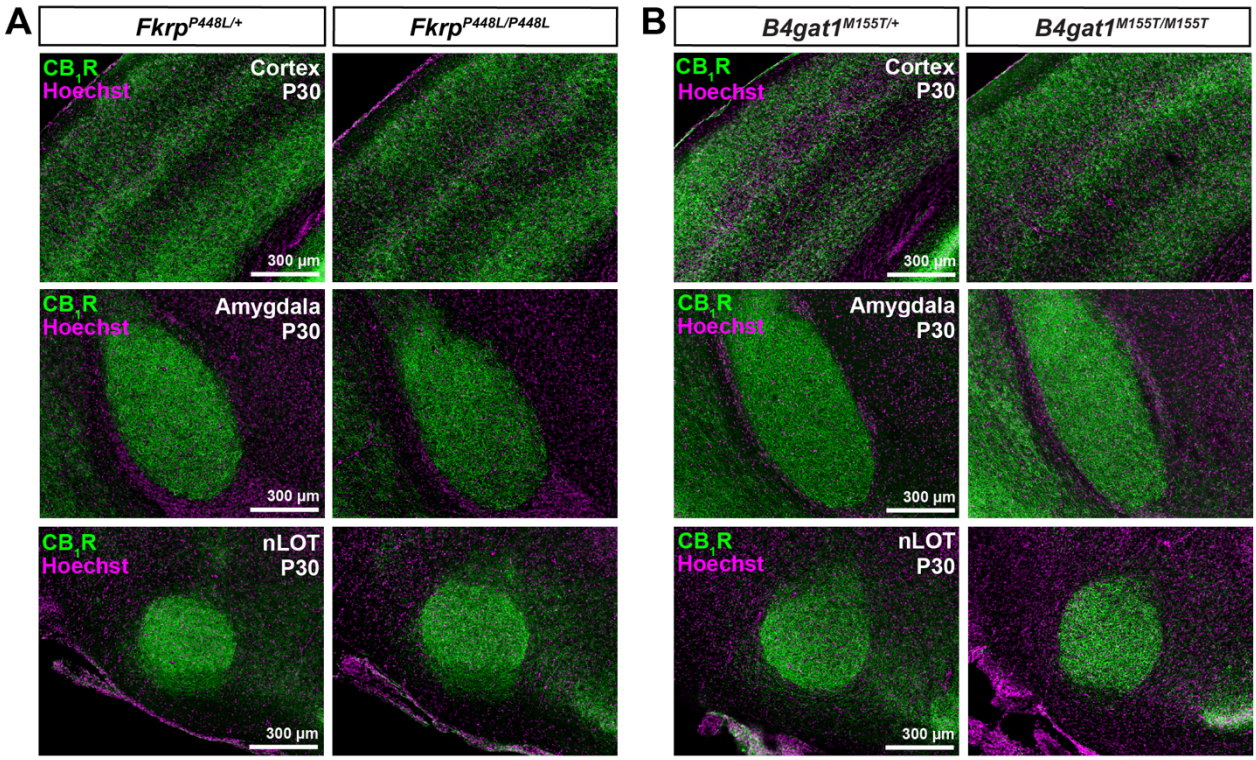

Figure S4. CCK+ interneuron axon targeting in the forebrain is grossly unaffected in dystroglycan glycosylation mutant mice. (A) Immunostaining for CB_1R+ axon terminals (green) in the cortex (top), amygdala (middle) and nucleus of the lateral olfactory tract (nLOT, bottom) of P30 *Fkrp*$^{P448L/+}$ control (left panels) and *Fkrp*$^{P448L/P448L}$ mutant mice (right panels). **(B)** Immunostaining for CB_1R+ axon terminals (green) in the cortex (top), amygdala (middle) and nucleus of the lateral olfactory tract (nLOT, bottom) of P30 *B4gat1*$^{M155T/+}$ control (left panels) and *B4gat1*$^{M155T/M155T}$ mutant mice (right panels).

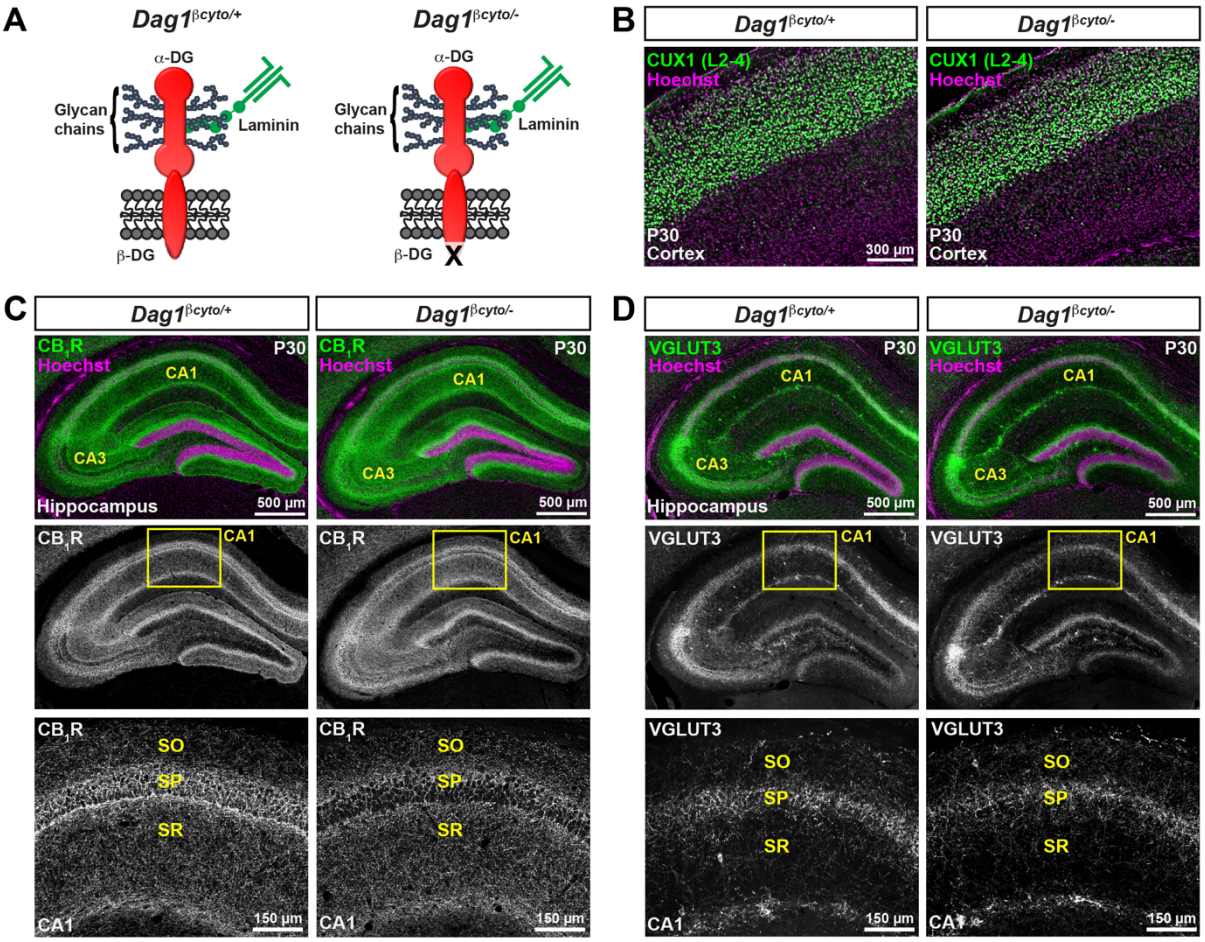

Figure 7. Deletion of the cytoplasmic domain of β-dystroglycan leads to minor alterations in CCK+ interneuron axon targeting. (A) Schematic showing transmembrane β-dystroglycan subunit. *Dag1*$\beta^{cyto/-}$ mutant mice (right) lack the intracellular domain of β-dystroglycan. **(B)** Sections immunostained for layer marker CUX1 (L2-3, green) shows normal migration of cortical neurons in P30 *Dag1*$\beta^{cyto/-}$ mutant mice. **(C)** Sections from *Dag1*$\beta^{cyto/+}$ control (left panels) and *Dag1*$\beta^{cyto/-}$ mutant mice (right panels) immunostained for CB_1R+ axon terminals (green). Single channel images of CB_1R (gray) in the hippocampus and CA1 are shown below the merged images. Yellow boxes indicate approximate locations of CA1 high magnification (20X) images (bottom panels). **(D)** Hippocampal sections from *Dag1*$\beta^{cyto/+}$ control (left) and *Dag1*$\beta^{cyto/-}$ mutant mice (right) immunostained for VGLUT3+ axon terminals (green). Single channel images of VGLUT3 (gray) in the hippocampus and CA1 are shown below the merged images. Yellow boxes indicate approximate locations of CA1 high magnification images (bottom panels)**.** CA1 layers: SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*.

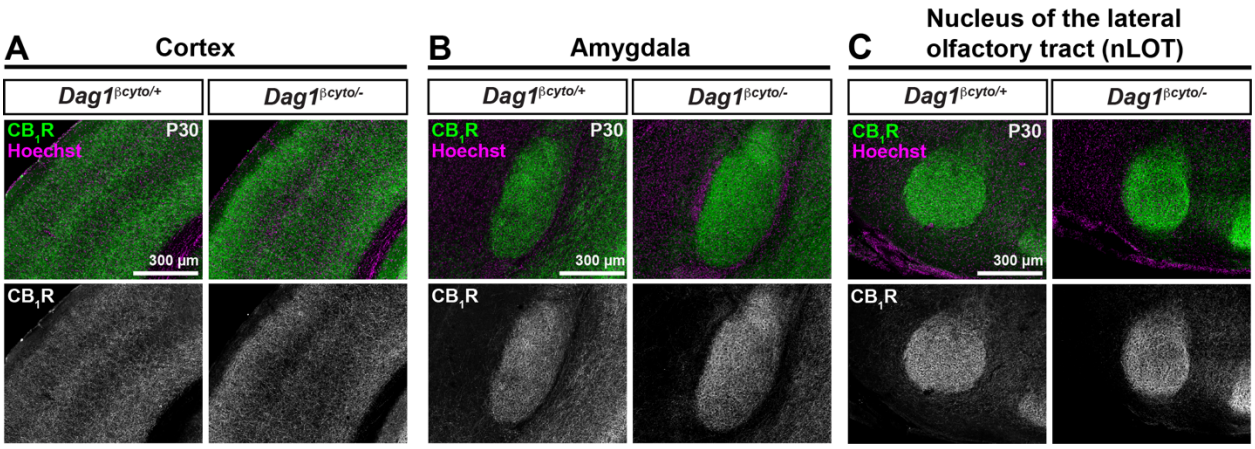

Figure S5. CCK+ interneuron axon targeting is grossly unaffected in multiple forebrain regions of β-dystroglycan mutant mice. (A-C) Immunostaining for CB_1R+ axon terminals (green) and Hoechst (magenta) in the cortex **(A)**, amygdala **(B)**, and nucleus of the lateral olfactory tract **(C)** of P30 *Dag1*$\beta^{cyto/+}$ control (left panels) and *Dag1*$\beta^{cyto/-}$ mutant mice (right panels). Single channel images of CB_1R (gray) are shown below.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 SUMMARY OF RESULTS

Despite decades of research into mechanisms of neural circuit formation, we lack an understanding of how the specificity of synaptic connections or “wiring” in the brain is achieved (Yogev and Shen, 2014). In this dissertation I explored how dystroglycan, a cell adhesion molecule concentrated at CNS inhibitory synapses, regulates the postnatal development of CCK+ INs using mouse genetic tools and mouse dystroglycanopathy models (see **Table 1** for summary of mouse phenotypes).

In Chapter 2 I focused on determining the specific role of dystroglycan in the development of CCK+ IN synapses. Using a conditional mouse genetic approach, I showed that presynaptic CCK+ INs are largely absent from the forebrain at early postnatal ages following deletion of *Dystroglycan* from excitatory neurons. The loss of CCK+ INs before the peak of inhibitory synapse formation suggests that dystroglycan may function in earlier steps of synaptogenesis such as synaptic targeting. The few remaining INs in *Dag1*^{cKO} mice misproject their axons into the nearby striatum where *Dystroglycan* expression is retained. Genetic deletion of the pro-apoptotic gene *Bax*, the main cell death pathway used by INs during development, was not sufficient to rescue CCK+ INs. This indicates that CCK+ INs may undergo cell death in a *Bax*-independent manner when their primary synaptic targets are not capable of being innervated. Furthermore, deletion of *Dystroglycan* from excitatory neurons after synapse formation is complete did not reduce CCK+ IN axon density, suggesting that dystroglycan may not be required for the maintenance of synapses in the adult brain.

In Chapter 3 I explored how CCK+ IN development is affected in mice lacking *Dystroglycan* throughout the central nervous system, as well as in mouse models of dystroglycanopathy. Surprisingly, CCK+ IN axon terminals were established in the brains of mice lacking *Dystroglycan* (CNS-*Dag1*^{cKO}), but the laminar organization of axon terminals in the hippocampus was perturbed. CCK+ IN innervation of the pyramidal cell body layers was reduced, whereas innervation of the dendritic layers was slightly increased. The inability of CCK+ axon terminals to concentrate in cell body layers began shortly after birth when dynamic changes in laminar innervation by CCK+ axons normally occurs. Genetic structure-function analyses revealed modest CCK+ axon targeting impairments in mutant mice with partially reduced glycosylation of α-DG or mice lacking the intracellular domain of β-DG. These results suggest that residual dystroglycan function may be sufficient for most aspects of brain development and CCK+ axon targeting to proceed normally.

The findings from this work demonstrate that dystroglycan on PyNs is important for the development of a specific type of presynaptic CB1R IN. Outside of the retina, few molecules have been identified in the mammalian brain that regulate axon targeting of PyNs by a specific inhibitory IN subtype, especially at the subcellular level (distal dendrite, proximal dendrite, soma targeting) (Favuzzi et al., 2019; Sanes and Zipursky, 2020). A single class of neurochemically defined IN (for instance CB1R or PV) is often comprised of multiple subtypes with remarkable diversity in their preference for targeting specific domains of PyNs. For instance, CB1R INs include basket INs that form perisomatic synapses, but also include dendrite targeting subtypes. In addition, we do not yet have a list of molecules that instruct parvalbumin basket INs to form synapses

onto the cell body of PyNs or somatostatin INs to form dendrite targeted synapses. The cell adhesion molecule L1CAM appears to be important for innervation of the axon initial segment by GABAergic INs in the cortex and cerebellum (Ango et al., 2014; Tai et al., 2019). Dystroglycan is similar in that it is found postsynaptically, and appears to be important for the axon targeting of INs onto a specific subcellular domain. This suggests that other postsynaptic molecules may exist that instruct the proper targeting of specific IN subtypes onto different PyN domains.

Table 1. Summary of phenotypes

Mouse line	Dystroglycan status	Cells/brain regions affected	Axon targeting defect	CB1R axons present	*Phenotype severity
Nex^{Cre}-Dag1^{cKO}	No DG	Excitatory neurons	Y	N	+++++
Emx1^{Cre}-Dag1^{cKO}	No DG	Forebrain excitatory neurons, glia	Y	Y	++++
Nestin^{Cre}-Dag1^{cKO}	No DG	CNS	Y	Y	+++
Dag1^{icKO};Camk2a$_{CreERT2}$	No DG in adult excitatory neurons	Excitatory neurons (adult)	N	Y	-
Dag1$\beta^{cyto/-}$	Deletion of β-DG intracellular domain	All	Y	Y	+
Ispd^{L79*} (P0)	Absence of α-DG glycosylation	All	N	Y	-
Fkrp^{P448L}	α-DG hypoglycosylation	All	Y	Y	+
B4gat1^{M155T}	α-DG hypoglycosylation	All	Y	Y	+

Table 1. *Phenotypic severity scale ranges from mild (+) to severe (+++++). No phenotype is denoted by (-).

4.2 BASIC SCIENCE IMPLICATIONS

What is the function of dystroglycan in CCK+ interneuron development?

What is the precise role of dystroglycan in the development of CCK+ interneurons and their synapses? Synaptogenesis, the formation of synapses, requires multiple distinct steps: (1) synaptic partner recognition (2) recruitment and assembly of core inhibitory/excitatory synaptic machinery (3) differentiation and maturation of synaptic identity, and (4) synaptic maintenance (Sudhof, 2018). Based on data from Chapter 2 and Chapter 3, mice lacking *Dystroglycan* exhibit defects in the development of CCK+ INs at the earliest time point they (or their CB1R+ axons) can be reliably identified (P0-P5). This period precedes the peak of inhibitory synapse formation (P9), suggesting that dystroglycan functions at early stages of synaptogenesis such as synaptic partner recognition (Favuzzi et al., 2019). Early studies characterizing the development of CCK+ INs in the rat brain using light and electron microscopy demonstrated that CCK+ INs are capable of forming synaptic connections at early postnatal ages (P3-4) (Morozov and Freund, 2003). Later work showed that putative CCK+ INs were capable of firing action potentials at late embryonic ages (Calvigioni et al., 2016). Although more research is needed to establish when CCK+ INs form physical contacts onto PyNs, evidence from dystroglycan mutant mice suggest that CCK+ INs may begin contacting immature pyramidal neurons (PyNs) around birth or earlier. Although the peak of inhibitory synapse formation occurs around P9 using general inhibitory synapse markers (VGAT), individual IN subtypes may establish synapses onto their targets at different times. For instance, chandelier cells (a PV+ subtype) begin innervating PyN axon initial segments around P12 and complete synaptogenesis by P28 (Tai et al., 2019). Determining the onset of synapse targeting and formation for

most IN subtypes, including CCK+ INs, is limited by a lack of genetic tools for visualizing and manipulating IN subtypes (discussed below).

Differences in phenotypes between *Dystroglycan* conditional knockout mice

One finding of this work is that loss of *Dystroglycan* results in a spectrum of CCK+ IN phenotypes depending on the Cre line used. Deletion of *Dystroglycan* from excitatory neurons using Nex^{Cre} results in a 95% reduction of CCK+ IN axon terminals in the forebrain, whereas *Dystroglycan* deletion from the central nervous system or forebrain ($Nestin^{Cre}$, $Emx1^{Cre}$) permits CCK+ IN axon terminals to populate the brain, but impairs targeting of pyramidal neuron cell bodies. One explanation for the phenotypic differences is that the timing and robustness of Cre-mediated recombination and gene deletion varies between *Nestin*, *Emx1*, and *Nex* Cre lines. A study directly comparing the timing and efficiency of recombination in $Nestin^{Cre}$ and $Emx1^{Cre}$ lines (both used in this dissertation) showed that despite similar onset of recombination in the embryonic forebrain, $Emx1^{Cre}$ leads to more robust and earlier completion of recombination than $Nestin^{Cre}$ (Liang, Hippenmeyer and Ghashghaei, 2012). The CCK+ IN phenotype observed in $Emx1^{Cre}$-$Dag1^{cKO}$ mice is more severe than in $Nestin^{Cre}$-$Dag1^{cKO}$ mice, supporting the conclusion that early loss of *Dystroglycan* leads to more severe synaptic targeting defects. In line with this interpretation, one study found relatively mild cortical migration abnormalities when using $Nestin^{Cre}$ to delete the *fukutin*, a dystroglycan glycosyltrnasferease, compared with using $Emx1^{Cre}$ (Sudo et al., 2018).

A separate study investigating the role of *Dicer* in the nervous system found more severe hippocampal defects when *Dicer* was deleted using $Emx1^{Cre}$ compared

with *Nestin*^{Cre}. The authors discuss the possibility that the difference in severity of phenotypes observed may be due to *Emx1*^{Cre} deletion genes earlier and more robustly (Kawase-Koga, Otaegi and Sun 2009; Li et al., 2011). It is unclear why CCK+ INs in *Nex*^{Cre}*-Dag1*^{cKO} mice exhibit a severe loss of CB_1R+ axon terminals compared with *Nestin*^{Cre} or *Emx1*^{Cre}. Collectively, these studies highlight the importance of characterizing the timing and patterns of recombination for each Cre line used to dissect the roles of dystroglycan in the CNS, as they can influence the developmental onset and magnitude of the phenotype observed.

Role of the dystrophin-glycoprotein complex (DGC) in CCK+ IN development

In brain and muscle tissue, dystroglycan forms a complex (or complexes) with dystrophin and other proteins, collectively known as the dystrophin glycoprotein complex (DGC). Like dystroglycan, dystrophin is also expressed on PyNs and has been associated with inhibitory synapses in multiple brain regions (Knuesel et al., 1999). Similar to patients with dystroglycanopathy, patients with Duchenne muscular dystrophy, caused by *Dystrophin* mutations, frequently exhibit cognitive impairments in the absence of brain malformations, suggesting a general role for the DGC in synapse development or function (Jagadha and Becker, 1988; Moizard et al., 2000; Naidoo and Anthony, 2020). Interestingly, mice lacking all dystrophin isoforms (*mdx*), a model of Duchenne muscular dystrophy, exhibit defects in CCK+ IN synapse development and CB_1R density in the hippocampus (Krasowska et al., 2014). Currently, no conditional mouse is available to determine the role of *Dystrophin* in specific cell types in the brain. Whether loss of *Dystrophin* specifically from PyNs leads to early postnatal defects in

CCK+ axon targeting is unknown. Future work will be necessary to elucidate the unique contributions of dystrophin, utrophin, and other DGC components to the development of CCK+ INs and inhibitory synaptogenesis. Gaining a better understanding of the various roles of dystroglycan, dystrophin, and other DGC proteins in specific cell types will be necessary to understand their function during normal brain development, and their contribution to neurological symptoms in muscular dystrophy patients.

Progress and challenges for identifying novel regulators of inhibitory synapse development

In the brain, IN subtypes are subdivided based on their molecular identity, physiological properties, and targeting of different subcellular domains of PyNs (Kepecs and Fishell, 2014). Even within a molecularly defined class (CCK+ or PV+ for example), INs target different domains of PyNs (distal/proximal dendrites, soma, axon initial segment), suggesting that unique IN subtypes employ different cell surface molecules during synapse wiring (Pelkey et al., 2017). Loss of dystroglycan selectively impairs the development of perisomatic targeting CCK+ INs (Fruh et al., 2016; Miller and Wright, 2021). In addition to perisomatic targeting CCK+ INs, PV+ IN populations also include a subtype whose axons primarily target the cell body of PyNs (so called perisomatic “basket” INs). Presumably, CCK+ and PV+ INs subtypes that primarily target the dendrites of PyNs would require different sets of cell surface molecules, although this has not been experimentally tested. Using single-cell mRNA profiling or genetic approaches to label and sort different IN populations, recent work has identified cell-type specific profiles of cell surface molecules that may be required for regulating

synapse targeting (Fucillo et al., 2015; Favuzzi et al., 2019). Although some ligand-receptor pairs at excitatory and inhibitory synapses have been identified, these have largely been determined using heterologous cell co-culture systems, and their involvement in the development of IN subtype-specific synaptic wiring *in vivo* is largely unexplored (Scheiffele et al., 2000).

A significant barrier to identifying novel regulators of inhibitory synapse targeting and synaptogenesis in the CNS is the lack genetic tools for isolating and profiling GABAergic INs at early postnatal ages. The majority of cell-type specific IN markers (VGLUT3, PV, VIP, CB1R) are not significantly expressed until the first postnatal week (Carlen et al., 2012; del Rio et al., 1994; Freund and Buzsáki, 1996; Hill et al., 1994; Takeda et al., 1989). For example, the onset of PV expression occurs around ~P12, but inhibitory synapse formation is well underway by this time (peak at P9) Favuzzi et al., 2019). For this reason, much of our knowledge of synapse targeting and synapse formation have been studied *in vitro* or in adult nervous systems.

The specific impairment in CCK+ IN development suggests a trans-synaptic role for dystroglycan. One of the main dystroglycan ligands in the brain is the presynaptic cell adhesion molecule neurexin (Sugita et al., 2001). The neurexin (*NRXN1-3*) family of synaptic cell-adhesion molecules are highly alternatively spliced, and specific isoforms are differentially expressed by CCK+ and PV+ interneurons (Ullrich et al., 1995; Fucillo et al., 2015). α-DG has also been shown to interact biochemically with particular splice isoforms of neurexins present in CCK+ INs (Sugita et al., 2001; Boucard et al., 2005; Reissner et al., 2014; Fucillo, et al., 2015). These findings suggest that specific splice isoforms of neurexin that can bind dystroglycan are differentially expressed by CCK but

not PV interneurons. A recent study knocked out all neurexin genes in PV and SOM interneurons and found only a modest decrease of inhibitory synapses in the cortex, indicating that neurexins may play a more important role in synapse function than synapse formation and maintenance (Chen et al., 2017). Whether neurexins are required for CCK+ IN development has not been directly examined, leaving open the possibility that neurexins participate in CCK+ IN development or synapse formation.

Another challenge for understanding IN development and discovering molecules involved in synaptic specificity is the fact that many molecular markers used for Cre-dependent genetic targeting and manipulation of INs (VIP, PV, CCK) label multiple subtypes of INs within each class, and are often expressed in non-neuronal cell types. For instance, in addition to CCK+ INs, forebrain excitatory neurons also express low levels of CCK (*Cck*) and CB1R (*Cnr1*), limiting the usefulness of CCK^{Cre} and $CB1R^{Cre}$ mice for selective targeting of CCK/CB1R INs. One way to specifically label CCK+ interneurons to identify candidate presynaptic cell-adhesion molecules would be to use the $VGLUT3^{Cre}$ mouse, restricted to perisomatic targeting CCK+ interneurons (Schaffer et al., 2002; Fremeau et al., 2002; Grimes et al., 2011). Although VGLUT3 expression allows targeting of nearly half of CCK+ INs, the onset of its expression during the first postnatal week may limit the usefulness of $VGLUT3^{Cre}$ mice for early developmental studies (Gras et al., 2002; Gras et al., 2005; Pelkey et al, 2020; Somogyi et al., 2004).

Does dystroglycan function in interneurons?

A detailed immunohistochemical study performed on the adult mouse brain found dystroglycan localization highly concentrated on the soma and proximal dendrite of

pyramidal neurons, but also on non-pyramidal neurons attributed to interneurons (Zaccaria et al., 2001; Fig 3B asterisks). Data from Chapter 3 suggest that dystroglycan may be expressed in a subtype of IN. Interestingly, a recent study using Cre lines to isolate genetically defined populations of INs found that *Dystroglycan* was expressed in the vasoactive intestinal peptide/Calretinin subtype (VIP;CR), whose main synaptic targets are other INs. Whether *Dystroglycan* is expressed by or required for synaptic connectivity between VIP/CR+ INs themselves is unknown. To determine whether *Dystroglycan* functions cell-autonomously in CCK+ interneurons, one would need to use a Cre line whose expression begins during embryonic development (*Gad2*^{Cre}) to delete *Dystroglycan* and targets CGE-derived INs that include CCK+ INs. Unfortunately, no Cre lines are available to delete genes specifically from CCK+ INs beginning early in embryonic development. The *5HT3AR*^{Cre} line targets most CGE-derived interneurons, of which CCK+ INs comprise ~10% of the total number labeled, limiting the usefulness of this line for tracking CCK+ IN development (Chittajallu et al., 2013; Lee et al., 2010). Future studies will be necessary to identify unique CCK+ IN markers at early postnatal timepoints, thereby allowing the development of genetic tools to label and profile CCK+ INs for cell adhesion molecules capable of mediating synaptic targeting.

4.3 CLINICAL IMPLICATIONS

CB1R development in humans/non-human primates

Dystroglycan is a critical regulator of forebrain CCK+ IN development in mice (Fruh et al., 2016; Miller and Wright, 2021). Whether dystroglycan is also important for CCK+ IN development and axon targeting in humans and non-human primates is

unknown. In human fetal brains, CB1Rs from putative CCK+ INs can be detected as early as gestational week 19 and are highly concentrated in limbic regions of the brain including the cortex, hippocampus, and amygdala (Glass et al., 1997; Mato et al., 2003; Wang et al., 2003). Similar to rodents, CCK+ IN axon terminals undergo dramatic laminar redistribution during development, ultimately forming characteristic perisomatic "basket" synapses onto excitatory neuron cell bodies (Eggan et al., 2007; Eggan et al., 2010a; Eggan et al., 2010b).

Currently, it is unknown whether specific populations of interneurons such as CCK+ INs develop properly, or whether CB1R+ axon targeting is impaired in the brains of patients with dystroglycanopathy. It would be important to know whether CCK+ INs and their axons are present in the brains of dystroglycanopathy patients with a range disease severity from mild to severe. It is possible that dystroglycan may not play a role in the development of CCK+ INs in humans or in dystroglycanopathy. Although some functions of dystroglycan appear to be species-specific, these are largely limited to invertebrates such as *c. elegans* (Johnson et al., 2006). Many key anatomical defects and pathological mechanisms in human dystroglycanopathy have been successfully modeled and studied using mouse genetic tools, making it likely that model organisms will continue to yield valuable insights into the etiology of human disease.

What are the physiological consequences of altered CCK-IN development?

Cognitive impairments are frequently observed in human patients with dystroglycanopathy and Duchenne muscular dystrophy (Clement et al., 2008). Whether impairments in CCK+ IN development or function contribute to neurological symptoms

in dystroglycanopathy is unknown. Recently, CCK+ INs have been implicated in a wide variety of important neural circuit functions that support diverse forms of cognition including spatial coding, fear extinction, and working memory (Busquets-Garcia et al., 2018; Del Pino et al., 2017; Nguyen et al., 2020; Rovira-Esteban et al., 2019; Veres et al., 2017; Whissell et al., 2019). Future studies are needed to determine whether the loss of CCK+ INs in PyN-*Dag1*^{cKO} mice results in cognitive impairments or seizures. PyN-*Dag1*^{cKO} and CNS-*Dag1*^{cKO} mice do not exhibit obvious behavioral signs of seizures (personal observations), although this has yet to be formally tested. Genetic deletion of CB1R (*Cnr1*) from PyNs increased seizure susceptibility, but deletion from CCK+ interneurons (*Dlx5/6-Cre;CB$_1$R*$^{F/F}$) had no effect suggesting that cannabinoid signaling on PyNs but not CCK+ INs is important for regulating network excitability (Monory et al., 2006). Recent research suggests that CCK+ INs play a role in the expression of long-term potentiation (LTP) in the hippocampus (Jensen et al., 2021). Interestingly, mice lacking dystroglycan from PyNs (*Nex*^{Cre}) exhibit defects in (LTP) (Satz et al., 2010). These mice also lack most CB1R axon terminals, raising the possibility that reduced CB1R function contributes to LTP defects (Miller and Wright, 2021). Moreover, conditional deletion of *Cnr1* from CCK+ INs also leads to defects in LTP (Monory et al., 2015). Collectively, these results suggest that CCK+ INs and their CB1R+ axons are important for multiple forms of memory, and future work will help determine whether CCK+ IN dysfunction contributes to cognitive symptoms in dystroglycanopathy.

Implications for gene therapy development

Defining when and how dystroglycan functions during development of the nervous system is critical for determining therapeutic strategies and time windows for treating symptoms of human dystroglycanopathy. Mouse genetic tools such as the Cre/Lox system have revealed that dystroglycan has critical functions in neurons, astrocytes, oligodendrocytes. People with severe forms of dystroglycanopathy (such as Walker-Warburg syndrome, WWS) frequently have multiple structural brain malformations at birth due to loss of dystroglycan. One open question is whether gene therapy to restore dystroglycan function (via AAV delivery of genes encoding enzymes important for dystroglycan glycosylation) can rescue synaptic connections or synapse function in the context of structural brain abnormalities. It is unlikely that structural brain defects will be amenable to correction with gene therapy due to the role of dystroglycan at the earliest stages of brain development. However, synaptogenesis is one of the final stages in the development of functional neural circuits, raising the possibility that synaptic defects may be amenable to correction in humans (Liu et al., 2012). Encouragingly, in a mouse model of dystroglycanopathy with brain malformations (*POMT2*), postnatal gene therapy restored dystroglycan glycosylation and rescued behavioral impairments (Hu et al., 2016). The ability to provide effective therapies to treat genetic diseases relies on the ability to make accurate and early diagnoses. Walker-Warburg Syndrome can be diagnosed prenatally (first trimester) in humans via ultrasound, raising the possibility that gene therapy or dietary supplementation of sugar precursors could be delivered before major periods of synapse formation begin in the fetal brain (Blin et al., 2005; Huttenlocher and Dabholkar, 1997). Furthermore, mouse models of dystroglycanopathy have revealed that residual levels of dystroglycan

glycosylation may be sufficient to prevent the development of muscular dystrophy symptoms (Kanagawa et al., 2009). These findings also seem to extend to the nervous system, as hypoglycosylation mutant mice have structurally normal brains and relatively mild synaptic alterations (Chapter 3: *FKRP*, *B4gat1*, T190M). In sum, understanding dystroglycan's roles in brain development will allow for more effective gene therapies aimed at restoring cognitive function in human patients with dystroglycanopathy.

4.4 CONCLUSION

In this dissertation, I provide evidence that dystroglycan plays a central role in the postnatal development of forebrain CCK+ interneurons in mice. Using conditional gene inactivation of *Dystroglycan* and mouse models of dystroglycanopathy, I demonstrate that loss of dystroglycan function impairs the ability of CCK+ interneurons to populate the brain and make synaptic connections with excitatory neurons. Given the emerging roles of CCK+ interneurons in supporting learning and memory, CCK+ interneuron dysfunction may contribute to cognitive impairments in human patients with dystroglycanopathy.

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