MOLECULAR MECHANISMS OF DENDRITIC SPINE DEVELOPMENT AND SYNAPTOGENESIS: THE ROLE OF AXON GUIDANCE MOLECULES AND INTRACELLULAR SCAFFOLDING PROTEINS

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Abstract

A major interest in developmental neuroscience is to understand the cellular and molecular mechanisms of neural circuitry formation. The central goal of this project is to approach this complex problem by studying one key process in the establishment of neuronal connectivity—the development of dendritic spines. Dendritic spines are tiny membranous protrusions where the majority of excitatory synapses in the mammalian brain form. The development of dendritic spines and excitatory synapses is a complicated, multi-step process that involves the establishment of axo-dendritic contact, stabilization of trans-synaptic interactions, recruitment of synaptic organelles and proteins, and elimination of exuberant synapses. A comprehensive understanding of the cellular and molecular mechanisms that regulate dendritic spine and synapse development is fundamental to the understanding of neural mechanisms underlying complex behavior and pathogenesis of neuropsychiatric diseases. In this study, we show that the transmembrane semaphorin, semaphorin 5A, signals through plexin A2 to restrict dendritic spine density in both prenatal and adult-born dentate granule neurons. Importantly, Sema5A−/− mice exhibit social behavioral defects reminiscent of patients with autism spectrum disorders. Dendritic spines are specialized subcellular compartments where specific cell-surface receptors and cell-adhesion molecules are concentrated. In the present study, we also address molecular mechanisms that regulate subcellular localization of cell-adhesion molecules during synaptogenesis. Through a non-baised forward genetic screen, we find that the intracellular scaffolding protein, Discs large homologue 5, regulates dendritic spine formation by controlling the
neuronal surface localization of N-cadherin. The results of this study lend further insight into the molecular mechanisms of dendritic spine formation and synaptogenesis and will likely provide a conceptual framework for in vivo approaches to study synapse development and function in the normal and diseased brain.
Preface and Acknowledgements

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Chapter 1

General Introduction

A major interest in developmental neuroscience is to understand the cellular and molecular mechanisms of neural circuitry formation. The central goal of this project is to approach this complex problem by studying one key process in the establishment of neuronal connectivity—the regulation of dendritic spine density. Dendritic spines are tiny membranous protrusions that typically consist of a round head with a long narrow neck connected to the dendritic shaft. The spine head is the main site of excitatory synaptic contact in the mammalian brain, and dendritic spines are widely used as a morphological marker for excitatory synapses. The dendritic spine is thought to function as an isolated subcellular compartment that spatially restricts the signaling events triggered by presynaptic input to a specific synapse (Nimchinsky et al., 2002). Individual spines vary widely in size and shape, and the morphology of dendritic spines is strongly correlated with synaptic strength. For instance, studies have shown that dendritic spines in hippocampal pyramidal neurons increase and decrease their volume during long-term potentiation (LTP) and long-term depression (LTD), respectively (Matsuzaki et al., 2004; Zhou et al., 2004). Accordingly, spine density, distribution, and morphology exert a profound impact on synaptic transmission, integration, and plasticity of neural networks (Ballesteros-Yanez et al., 2006; Bhatt et al., 2009; Elston and DeFelipe, 2002; Hayashi and Majewska,
alterations in spine density and morphology are associated with numerous neurological and psychiatric disorders, including fragile X syndrome, Down syndrome, schizophrenia, and Alzheimer’s disease (Irwin et al., 2000; Kaufmann and Moser, 2000; Knobloch and Mansuy, 2008; Penzes et al., 2011). A comprehensive understanding of the molecular and cellular processes that regulate dendritic spine and synapse development is fundamental to the understanding of neural mechanisms underlying complex behavior and pathogenesis of neuropsychiatric diseases (Lippman and Dunaevsky, 2005; Saneyoshi et al., 2010; Tada and Sheng, 2006; Yoshihara et al., 2009).

Synaptogenesis begins with the formation of transient axon-dendritic contacts. It is generally believed that this initial contact is mediated by dendritic filopodia, which are long, thin, highly motile structures protruding from the dendritic shaft of developing neurons. Stabilization of this transient axon-dendritic contact transforms dendritic filopodia into mature dendritic spines through a multi-step process. (Fiala et al., 1998; Ziv and Smith, 1996). Recent studies using time-lapse imaging have demonstrated that dendritic filopodial motility is crucial for synaptogenesis. For instance, BDNF promotes dendritic spine formation and excitatory synaptogenesis in hippocampal neurons, in part by increasing dendritic filopodial motility. The effect of BDNF on filopodial motility is dependent on TrkB kinase activity and PI3 kinase signaling (Luikart et al., 2005; Luikart et al., 2008). Likewise, EphBs are required for normal dendritic filopodial motility and dendritic spine formation in cortical neurons (Kayser et al.,
The effect of EphB signaling on dendritic spine formation is mediated by the Rac1 guanine exchange factors (GEF) tiam1 and kalirin, which activate rac1 and its effector PAK (Penzes et al., 2003; Tolias et al., 2007). However, EphBs regulate multiple developmental events during synaptogenesis (see below), and the precise role of the Rho family of small GTPases in EphB signaling has not been directly addressed. Despite these recent advances, our understanding of dendritic filopodia is far from complete. For instance, it is unknown whether dendritic filopodia emerge randomly along the dendritic shaft, or whether filopodia formation is induced by extracellular cues or potential presynaptic partners. It is also unclear whether the movement of dendritic filopodia is completely random or whether they are directed by extracellular cues in the environment.

Although the role of dendritic filopodia in synaptogenesis is widely accepted, there are alternative models of synaptogenesis involving filopodia-independent mechanisms (Yuste and Bonhoeffer, 2004). It is highly probable that different types of synapses in the mammalian brain develop by different mechanisms. Alternatively, due to their transient nature, dendritic filopodia may be particularly difficult to observe in certain synapses.

Although most studies on the initial events of synaptogenesis have focused on the role of dendritic filopodia, there is also in vitro evidence that axons may be prepatterned to contain preferred sites of synapse formation. It was observed that synaptic protein transport vesicles (STV) repeatedly pause at predefined sites along the axons of cultured cortical neurons. Remarkably,
formation of stable contacts with dendritic filopodia also occurs at these STV pause sites (Sabo et al., 2006). The molecular nature of these STV pause sites is unknown. Why axon-dendrite contacts preferentially occur at these sites is also elusive. Perhaps these pause sites represent areas where attractive cues and trans-synaptic adhesion molecules are locally concentrated.

Once transient axo-dendritic contacts are formed, cell surface proteins are required to establish stable trans-synaptic interactions. Numerous molecules involved in this process have been identified, including neurexins, neuroligins, ephrins, Ephs, leucine-rich-repeat proteins, and cadherins (Arikkath and Reichardt, 2008; Craig and Kang, 2007; de Wit et al., 2009; Henkemeyer et al., 2003; Kayser et al., 2008; Ko et al., 2009; Siddiqui and Craig, 2011; Suzuki and Takeichi, 2008). Previous studies have shown that these proteins not only mediate cell adhesion, but also interact with intracellular scaffolding proteins to recruit other components of the synaptic machinery (see below). However, the precise role of these cell surface proteins in synaptogenesis is not fully understood. One potential caveat is that many studies on trans-synaptic cell adhesion molecules are performed using in vitro neuronal cultures, and the results may not truly reflect their in vivo functions. For instance, overexpression of neuroligin in non-neuronal cells induces synaptic vesicle clustering and formation of functional release sites in axons that contact these cells (Scheiffele et al., 2000). Similarly, overexpression of neurexins in non-neuronal cells induces postsynaptic differentiation in neighboring neurons (Graf et al., 2004). However, neuroligin 1; neuroligin 2; neuroligin 3 triple knockout mice show
deficits in synaptic transmission but normal synapse density and synapse morphology (Varoqueaux et al., 2006). Therefore, neuroligin-neurexin interactions are crucial for synapse maturation \textit{in vivo}, possibly by recruitment of other synaptic proteins to immature synapses, but not for initial synapse formation. In co-culture assays, overexpression of neuroligin and neurexin \textit{in vitro} may lead to local clustering of other cell-adhesion molecules that are sufficient to induce synapse differentiation. Indeed, a major challenge in the field is to elucidate which of these molecules are required primarily for the initial stabilization of trans-synaptic contacts, and which are involved primarily in reinforcement and maturation of these contacts. A better understanding of trans-synaptic adhesion molecule function will likely come from phenotypic analysis of mutant mice \textit{in vivo}.

Recent studies suggest that cell adhesion molecules regulate synaptogenesis at specific subsets of synapses. For instance, cadherin 9, was shown to regulate the formation of select hippocampal synapses (Williams et al., 2011). Different splice forms of neuroligin and neurexin determine binding selectivity among these proteins and whether they function primarily at glutamatergic or GABAergic synapses (Craig and Kang, 2007). A better understanding of the neuronal specific expression of these cell-adhesion molecules will provide further insight into how synapse specificity is established.

Once axon-dendritic contacts become stabilized, organelles and additional proteins are recruited and assembled to form the synaptic machinery at the nascent synapse. These include neurotransmitter receptors, ion channels,
scaffolding proteins, and signaling proteins on the postsynaptic spine; they also include synaptic proteins, vesicle docking proteins, and ion channels on the presynaptic terminal. Trans-synaptic cell adhesion molecules play an important role in this process. In some cases, cell-adhesion molecules bind directly to neurotransmitter receptors and regulate their localization and function. For instance, EphBs interact with NMDA receptors (Dalva et al., 2000), and neurexins interact with GABA_A receptors (Zhang et al., 2010). Intriguingly, N-cadherin interacts directly with the extracellular domain of GluR2, and this interaction accounts for the increase in spine density when Glur2 is overexpressed in hippocampal neurons (Passafaro et al., 2003; Saglietti et al., 2007). However, the in vivo significance of this interaction has yet to be determined. In other cases, cell-adhesion molecules regulate synaptic protein clustering indirectly by interacting with other intracellular scaffolding proteins. For example, EphB2 regulates AMPA receptor localization through interaction with PDZ domain-containing proteins (Kayser et al., 2006). Interfering with trans-synaptic adhesion could also affect presynaptic function. N-cadherin knockdown in the postsynaptic neuron reduced the basal release probability of presynaptic terminals that form synapses onto this neuron (Vitureira et al., 2012). The molecular mechanisms that couple trans-synaptic adhesion to presynaptic vesicle release are unknown.

Lastly, exuberant synapses are eliminated in response to extracellular cues or by activity-dependent mechanisms. Semaphorin 3F (Sema3F) is a repulsive axon guidance molecule that also plays a role in synapse elimination in
the infrapyramidal tract of the hippocampus (Bagri et al., 2003), collateral branches of visual cortex corticofugal projections (Low et al., 2008) and apical dendrites of layer V cortical neurons (Tran et al., 2009). Intriguingly, the Rac-GAP β2-chimaerin is required for the function of Sema 3F on infrapyramidal tract pruning, but not axon repulsion or dendritic spine pruning in the cortex (Riccomagno et al., 2012). This suggests that axon repulsion, elimination of presynaptic terminals, and elimination of postsynaptic spines are fundamentally distinct processes that may require different signaling pathways. Mossy fibers of the cerebellum are another example of synaptic pruning mediated by extracellular cues. During early development, mossy fibers form elaborate synaptic contacts with Purkinje cells. These synapses are eventually eliminated by Purkinje cell-derived BMP4 (Kalinovsky et al., 2011). Other synapses are pruned by activity-dependent mechanisms. In the developing cerebellum, a Purkinje cell is initially innervated by multiple climbing fibers. Later on, the climbing fiber with the highest synaptic efficacy takes over the whole synaptic field to establish a one climbing fiber-one Purkinje cell innervation pattern (Hashimoto et al., 2009). Similar mechanisms exist for the establishment of the neuromuscular junction (Buffelli et al., 2003). Interestingly, the formation of excessive synapses in climbing fibers and neuromuscular junctions is thought to be important to ensure that all postsynaptic targets are innervated. However, mossy fiber-Purkinje cell synapses and corticofugal collateral branches of the visual cortex represent innervation patterns that are non-existent in the adult. Why these types of synapses form transiently in the juvenile brain remains unknown.
The major aim of my thesis is to understand how synapse density is tightly controlled in the mammalian CNS. Several recent studies have demonstrated that axon guidance molecules play a crucial role in regulating synaptogenesis and synaptic plasticity (Shen and Cowan, 2010). Indeed, axon guidance and synaptogenesis share several common features. On a cellular level, growth cones and dendritic filopodia are both highly motile structures that actively sample their microenvironment in search of their targets or synaptic partners. On a molecular level, the process of growth cone navigation, dendritic filopodial motility, and spine remodeling all rely on signaling events that ultimately lead to remodeling of the actin cytoskeleton. Previous work has shown that the secreted semaphorin Sema3F plays a crucial role in eliminating excessive spines in layer V cortical neurons and dentate gyrus (DG) granule neurons (Tran et al., 2009). Here, we show that the transmembrane semaphorin Sema5A plays a similar role in restricting dendritic spine density in DG granule neurons. We provide evidence that Sema5A signals through the PlexinA2 (PlexA2) receptor to reduce dendritic spine density in hippocampal neurons. Interestingly, a recent genome-wide association study (GWAS) found a significant association between the SEMA5A locus and autism. Moreover, expression of SEMA5A is reduced in the brain of autism patients (Weiss et al., 2009). We find that Sema5A−/− mice show abnormal behavior in the three-chamber social interaction test, mirroring some of the key clinical symptoms of human autism patients.

Like growth cones, dendritic spines are specialized subcellular compartments where specific cell surface receptors, cell-adhesion molecules,
and signaling machinery are concentrated. The correct targeting and maintenance of guidance receptors and cell-adhesion molecules at the neuronal surface is crucial for the function of growth cones and developing dendritic spines. How is the subcellular localization of these proteins controlled? We address this question by investigating the mechanisms that regulate cell surface localization of N-cadherin, the most abundant and widely distributed classical cadherin in the mammalian brain (Togashi et al., 2002; Yagi and Takeichi, 2000). We find that neuronal surface localization of N-cadherin is dependent on Discs large homologue 5 (DLG5), a member of the membrane associated guanylate kinase (MAGUK) family of scaffolding proteins. Dlg5 loss-of-function leads to a dramatic reduction of neuronal surface N-cadherin without affecting total N-cadherin protein levels. This results in a significant reduction in dendritic spine density and synaptic transmission in cortical neurons derived from Dlg5 mutant mice. We provide evidence that N-cadherin and DLG5 genetically interact in vitro and in vivo, suggesting that they function in the same molecular pathway to regulate dendritic spine density.

In the present study, we expand our current knowledge of how axon guidance molecules function to restrict dendritic spine density. We also address molecular mechanisms by which subcellular localization of cell surface proteins is regulated during synaptogenesis. Importantly, these studies are based on analysis of dendritic spine phenotypes in vivo, and functional relevance is confirmed using electrophysiology and behavioral analyses. The results of this study lend further insight into molecular mechanisms of synaptogenesis and will
likely provide a conceptual framework for in vivo approaches to study synapse
development and function in the normal and diseased brain.

References:


Ballesteros-Yanez, I., Benavides-Piccione, R., Elston, G.N., Yuste, R., and
DeFelipe, J. (2006). Density and morphology of dendritic spines in mouse

review of physiology 71, 261-282.

Buffelli, M., Burgess, R.W., Feng, G., Lobe, C.G., Lichtman, J.W., and Sanes,
J.R. (2003). Genetic evidence that relative synaptic efficacy biases the outcome

development. Current opinion in neurobiology 17, 43-52.

Dalva, M.B., Takasu, M.A., Lin, M.Z., Shamah, S.M., Hu, L., Gale, N.W., and
Greenberg, M.E. (2000). EphB receptors interact with NMDA receptors and

de Wit, J., Sylwestrak, E., O'Sullivan, M.L., Otto, S., Tiglio, K., Savas, J.N.,
interacts with Neurexin1 and regulates excitatory synapse formation. Neuron 64, 799-806.


Semaphorin 5A Inhibits Synaptogenesis in Early Postnatal and Adult-Born Hippocampal Dentate Granule Neurons

2.1 Introduction

Several recent studies have shown that molecules involved in axonal growth and guidance play crucial roles at various stages of dendritic spine formation and synaptogenesis (Shen and Cowan, 2010). For instance, BDNF-TrkB signaling promotes dendritic spine formation and excitatory synaptogenesis by increasing dendritic filopodia motility (Luikart et al., 2005; Luikart et al., 2008). Ephrin Bs and EphBs regulate multiple processes of synaptogenesis, including dendritic filopodial motility (Kayser et al., 2008), stabilization of initial axon-dendritic contacts (Henkemeyer et al., 2003), and recruitment of AMPA and NMDA receptors (Dalva et al., 2000; Kayser et al., 2006). More recently, Sema3F-neuropilin2/plexinA3 signaling was shown to eliminate excessive excitatory synapses in the apical dendrites of cortical pyramidal neurons (Tran et al., 2009).

The class 5 semaphorins Sema5A and Sema5B, two closely related and evolutionarily conserved type-1 transmembrane proteins, are comprised of a 500 amino acid sema domain followed by seven type-1 thrombospondin repeats (TSRs). The cytoplasmic domain of Sema5s is ~ 90 amino acid residues in length and harbors no obvious signaling motifs (Adams et al., 1996; Tran et al., 2007). Previous work has shown that Sema5A functions as a bidirectional cue
for medial habenular axons that form the fasciculus retroflexus (FR). Sema5A is an attractive cue in the presence of heparan sulfate proteoglycans (HSPGs), but is converted into a repulsive cue in the presence of chondroitin sulfate proteoglycans (CSPGs). This dual function of Sema5A ensures that the FR projects to the midbrain as a tight fascicle between prosomere 1 and prosomere 2 of the developing diencephalon (Kantor et al., 2004). Furthermore, Sema5A and Sema5B are required \textit{in vivo} for the correct laminar targeting of retinal ganglion cell, amacrine cell, and bipolar cell neurites in the developing inner plexiform layer. Functional studies have demonstrated that Sema5A and Sema5B inhibit retinal neurite outgrowth through PlexinA1 and PlexinA3 receptors (Matsuoka et al., 2011). Interestingly, Sema5A and Sema5B continue to be expressed at high levels in the postnatal hippocampus, suggesting a role for these cues beyond axonal growth and guidance (Adams et al., 1996).

Here we show that Sema5A, but not Sema5B, negatively regulates synaptogenesis in dentate gyrus (DG) granule neurons and CA1 pyramidal neurons. Sema5A signals through PlexinA2, and possibly PlexinA3, to restrict dendritic spine and excitatory synapse density. Furthermore, Sema5A serves a similar function in adult-born DG granule neurons to restrict dendritic spine density. Interestingly, a recent genome-wide association study showed a significant association between a single nucleotide polymorphism (SNP) near the \textit{SEMA5A} gene and autism. Analysis of brain tissue from autistic patients showed that the expression of \textit{Sema5A} mRNA is significantly decreased compared to control brains (Weiss et al., 2009). We find that \textit{Sema5A}^{-/-} mice show
abnormalities in social interaction behavior mirroring core clinical manifestations of autistic patients. Taken together, these observations lend further insight into the role of guidance molecules in synaptogenesis and could provide an informative model for the study of autism spectrum disorders.

2.2 Results

**Sema5A and Sema5B Are Expressed in the Postnatal Hippocampus and Enriched in Postsynaptic Density Fractions**

Earlier studies have shown that Sema5A and Sema5B are expressed at high levels in the postnatal hippocampus, suggesting a role beyond axonal growth and guidance (Adams et al., 1996). To better define the expression pattern of class 5 semaphorins during postnatal brain development, we first examined Sema5A expression using the \textit{Sema5A}^{tm1Dgen/J} reporter mouse generated by Deltagen, where a gene trap insertion of LacZ is present in the last coding exon of Sema5A. In this mouse, \(\beta\)-galactosidase (\(\beta\)-gal) is under transcriptional control of the \textit{Sema5A} promoter. Therefore, \(\beta\)-gal signal can be used to indicate the expression pattern of \textit{Sema5A} transcripts. Whether the gene trap insertion causes a loss-of-function allele of \textit{Sema5A} has not been fully characterized (Gunn et al., 2011; see below). We observe robust \(\beta\)-gal signal in the granule cell layer of DG and weaker signal in a subset of cells in the hilus, CA3, CA1, and entorhinal cortex (EC) at P18 and P30 (Figures 2-1A). In an independent approach, we monitored mRNA levels of \textit{Sema5A} and \textit{Sema5B} by \textit{in situ}
hybridization. We observed robust expression of Sema5A mRNA in the DG and weaker expression in the CA3 and CA1 at P18 and P30 (Figure 2-1B), consistent with the β-gal staining. Sema5B mRNA is expressed in a similar pattern but at lower levels (Figure 2-1B).

To determine the subcellular localization of Sema5A and Sema5B protein, we performed subcellular fractionation of the P18 hippocampus to enrich for postsynaptic densities (PSD), monitoring the quality of our isolation by immunoblotting with the pre- and postsynaptic markers, synaptophysin and PSD95, respectively. Sema5A is highly enriched in the PSD fractions (Figure 2-2), suggesting that Sema5A is a postsynaptic protein. Sema5B is also enriched in PSD fractions (Figure 2-2); however, the protein levels are significantly lower.

**Sema5A Regulates Dendritic Spine Density in DG**

We observe strong expression of Sema5A and Sema5B in the hippocampus at a period of active synaptogenesis and synaptic remodeling, and we also find synaptic localization of Sema5A and Sema5B protein. These observations raise the possibility that class 5 semaphorins may be involved in dendritic spine development and synaptogenesis. To examine whether class 5 semaphorins indeed regulate dendritic spine development in vivo, we used the Golgi labeling technique to analyze dendritic spine morphology in wild type, Sema5A−/−, Sema5B−/−, and Sema5A−/−; Sema5B−/− brains (Matsuoka et al., 2011) at P60. We observed a 30% increase in dendritic spine density in DG granule neurons (Figures 2-3A, B, and C) of Sema5A−/− mice. There was no defect in spine
density in Sema5B<sup>−/−</sup> neurons, and Sema5A<sup>−/−</sup>; Sema5B<sup>−/−</sup> neurons show the same degree of dendritic spine density increase as Sema5A<sup>−/−</sup> neurons (Figure 2-3C). Therefore, we conclude that Sema5A, but not Sema5B, restricts dendritic spine density in the postnatal hippocampus.

We also analyzed dendritic spine density in wild type and Sema5A<sup>−/−</sup> mice at P16, at a point when active synaptogenesis is occurring. At this earlier age, we observe a 20% increase in dendritic spine density in DG granule neurons and CA1 pyramidal neurons of Sema5A<sup>−/−</sup> mice (Figure 2-3F). This suggests that Sema5A functions early during synaptogenesis to restrict the formation of exuberant synapses. Sema5A may have additional roles in synapse elimination at later stages, since the severity of dendritic spine abnormality in Sema5A<sup>−/−</sup> mice increases with age. This is in contrast to Sema3F, which functions at later stages of synapse elimination (Tran et al., 2009).

In an independent approach, Thy1-GFP (m line) mice were crossed to a Sema5A<sup>−/−</sup> and Sema5B<sup>−/−</sup> background to label hippocampal neurons. Dendritic spine morphology of DG granule neurons and CA1 pyramidal neurons were analyzed at P33. Using this method, we observe a 30% increase in dendritic spine density in DG granule neurons of Sema5A<sup>−/−</sup> mice (Figures 2-3D and E). There is no change in dendritic spine density in Sema5B<sup>−/−</sup> DG granule neurons, and Sema5A<sup>−/−</sup>; Sema5B<sup>−/−</sup> neurons show the same degree of dendritic spine density increase as Sema5A<sup>−/−</sup> neurons (Figures 2-3D and E). These findings are consistent with our Golgi analysis, and they suggest that Sema5A, but not Sema5B, regulates dendritic spine density in the postnatal hippocampus. We do
not observe any difference in dendritic spine density in either primary or secondary branches of apical dendrites of CA1 pyramidal neurons by GFP labeling across all genotypes examined (Figures 2-3D and E).

The effect of Sema5A is not secondary to hippocampal developmental defects since neurogenesis and neuronal migration is apparently normal in \textit{Sema5A}^{−/−}; \textit{Sema5B}^{−/−} mice, as shown by immunostaining with the mature DG granule neuron marker calbindin, and the immature DG granule neuron markers calretinin and doublecortin (Figure 2-4). The function of Sema5A is apparently confined to select populations of neurons, since we do not observe any change in spine density in Layer V (Figures 2-5A, B, and E) or layer II/III (Figures 2-5C, D, and E) cortical pyramidal neurons.

**Increased Postsynaptic Density of Excitatory Synapses in \textit{Sema5A}^{−/−} Hippocampal Neurons**

Dendritic spines are sites of excitatory synaptic contact (Nimchinsky et al., 2002), and they are widely used as a morphological marker for excitatory synapses. To investigate whether there is a concomitant increase in excitatory synapses in \textit{Sema5A}^{−/−} mice, we analyzed synapse density in cultured hippocampal neurons by immunostaining with synaptic markers. Hippocampal neurons from E16.5 embryos were cultured for 21 DIV and stained for the presynaptic marker vGlut1 and postsynaptic marker PSD95. Co-localization of vGlut1 and PSD95 was then used to assess excitatory synapse density. Consistent with our dendritic spine analysis, we observe a 25% increase in PSD95 puncta density in cultured
Sema5A\(^{-}\) hippocampal neurons (Figures 2-6A and B). In contrast, vGlut1 puncta density and vGlut1/PSD95 double-positive puncta density were not significantly increased (Figures 2-6A and B). These results suggest that Sema5A functions to restrict the number of postsynaptic components of excitatory synapses, with only a negligible effect on presynaptic terminals.

Furthermore, our \textit{in vitro} observations predict that excitatory synapses in the hippocampus of Sema5A\(^{-}\) mice should exhibit morphological abnormalities, with either an increased number of postsynaptic spines with no presynaptic partner or multiple postsynaptic spines making contact with a single presynaptic terminal. To access synaptic morphology \textit{in vivo}, we used transmission electron microscopy (TEM) to examine synaptic morphology in the DG of Sema5A\(^{-}\) mice in greater detail. Consistent with our \textit{in vitro} results, we observe abnormal ultrastructure of excitatory synapses in the DG molecular layer of Sema5A\(^{-}\) brains by TEM analysis, with a significant increase in dendritic spine density (Figures 2-7A and B), presynaptic terminals that contact multiple post-synaptic partners (Figure 2-7E), and increased number of split PSDs (Figure 2-7F). Furthermore, Sema5A\(^{-}\) DG excitatory synapses show a significant decrease in PSD length (Figure 2-7G).

We also stained cultured hippocampal neurons with the inhibitory presynaptic marker vGAT and the inhibitory postsynaptic marker gephyrin. We observe no change in vGAT puncta, gephyrin puncta, or vGAT/gephyrin double-positive puncta density in cultured Sema5A\(^{-}\) hippocampal neurons (Figures 2-8A and B), suggesting that Sema5A does not regulate inhibitory synaptogenesis.
Sema5A Regulates Dendritic Spine Density in Adult-born Granule Neurons

The DG is one of the two well-studied neurogenic regions in the adult mammalian brain (Ming and Song, 2005). Adult-born granule neurons constitute a minor population of neurons in the DG with distinct functional properties, including enhanced synaptic plasticity (Ge et al., 2007). Recent studies demonstrate that adult neurogenesis is crucial for the formation of new episodic memories by transforming similar experiences into discrete representations, a process known as “pattern separation” (Nakashiba et al., 2012; Sahay et al., 2011). Impaired adult neurogenesis has been associated with neuropsychiatric diseases such as depression (Santarelli et al., 2003) and schizophrenia (Kim et al., 2012). The integration of adult-born granule neurons into pre-existing hippocampal circuitry represents a remarkable but poorly understood form of neural plasticity. Whether the same molecular programs regulate maturation and synaptic integration of both early and adult-born granule neurons is unknown.

To assess whether Sema5A regulates dendritic spine density in adult-born granule neurons, we injected a self-inactivating retrovirus that includes a GFP reporter into the DG of Sema5A−/− mice and their wild type littermates at P35-P42 to birth-date and label a sparse population of adult-born granule neurons (Ge et al., 2006). Dendritic spine density of the labeled granule neurons was examined at 19-21 days post-injection by immunostaining for GFP. We observe a 50% increase in dendritic spine density in adult-born granule neurons of Sema5A−/− mice (Figures 2-9A, B, and C). These results demonstrate that Sema5A restricts
dendritic spine density in adult-born granule neurons, in addition to its role in at early postnatal times in newly born GC neurons. The molecular mechanisms that regulate synaptogenesis and synaptic integration in adult-born granule neurons are poorly understood. Our results provide an example of adult-born granule neurons employing the same molecular cues as early-born granule neurons to control key aspects of synaptic development.

**Sema5A−/− Mice Exhibit Altered Synaptic Properties**

Since there is continuous adult neurogenesis in the DG, whole cell patch-clamp recording of randomly sampled DG granule neurons results in recordings from granule neurons at different stages of maturation with distinct electrophysiological properties. To circumvent this problem, we performed our recordings on adult-born granule neurons birth-dated by retrovirus injection. The DG of Sema5A−/− mice and their wild type littermates were injected with a retrovirus carrying a GFP reporter at P35-P42, and whole cell patch-clamp recordings were performed on GFP positive cells at 19-21 days post-injection. This guarantees that the recordings are performed on DG granule neurons of the same developmental stage. Analysis of miniature excitatory postsynaptic currents (mEPSC) revealed a significant increase in mEPSC amplitude in Sema5A−/− neurons (Figures 2-10A, C, and D). The increase in mEPSC frequency did not reach statistical significance (Figures 2-10A and B). These results demonstrate an increase in excitatory synaptic strength in Sema5A−/− neurons, consistent with the increase in dendritic spine density.
Sema5A Can Function Cell-autonomously and as a Ligand to Reduce Dendritic Spine Density in vitro

To investigate whether Sema5A functions cell-autonomously or non-cell autonomously, dissociated hippocampal neurons derived from P1-P2 wildtype and Sema5A/− embryos were transfected with Sema5A or eGFP expression constructs at DIV 4 and dendritic spine density was analyzed at DIV21. Prox1 immunostaining was used to label DG granule neurons. Consistent with in vivo results, a significant increase in spine density is observed in Sema5A/− (2.23 ± 0.08 /µm) compared to wildtype controls (1.67 ± 0.04 /µm) (Figures 2-11D, F, and K). Importantly, overexpression of exogenous Sema5A leads to a significant (p<0.0001) decrease in GC spine density in Sema5A/− (1.10 ± 0.04 /µm) and Sema5A+/+ (1.18 ± 0.04 /µm) neurons (Figures 2-11E, G, and K). Because only a small fraction of neurons (<1%) is transfected in the hippocampal cultures, these experiments demonstrate that in GCs Sema5A can inhibit the formation of supernumerary spines in a cell-autonomous manner.

In addition to their conventional role as ligands, previous studies have shown that transmembrane Semaphorins can function as receptors to mediate reverse signaling (Cafferty et al., 2006; Toyofuku et al., 2004). To assess whether Sema5A functions as a ligand or receptor in GCs, we expressed a deletion mutant lacking the cytoplasmic domain (S5AΔcyto) and asked whether this construct is sufficient to rescue the Sema5A/− dendritic spine phenotype. Similar to wildtype Sema5A (1.10 ± 0.04 /µm), S5AΔcyto (1.09 ± 0.03 /µm) rescues
the Sema5A\(^{-}\) spine phenotype (Figures 2-11H and K), suggesting that the intracellular portion of Sema5A is dispensable for the regulation of spine density.

To determine which extracellular domain of Sema5A mediates inhibition of spine density, additional deletion constructs were generated. A mutant that lacks the Sema domain S5A\(^{\Delta}\text{Sema}\) (1.94 ± 0.05 /µm) fails to rescue the supernumerary spine phenotype of Sema5A\(^{-}\) GCs, while a deletion construct that lacks the seven TSRs S5A\(^{\Delta}\text{TSR}\) (1.11 ± 0.05 /µm) is sufficient for rescue (Figures 2-11I, J and K). Together these studies show that Sema5A functions as a ligand and that the Sema domain of Sema5A is necessary and sufficient to control dendritic spine density in GCs in a cell-autonomous manner.

**PlexinA Family Members Are Expressed in the Postnatal Hippocampus**

A recent study showed that class 5 semaphorins signal through plexinA1 and plexinA3 to regulate laminar targeting of retinal ganglion cells, amacrine cells, and bipolar cells in the inner plexiform layer (Matsuoka et al., 2011).

Furthermore, in vitro cell binding assays demonstrate that Sema5A binds to plexinA1 and plexinA2, but not plexinA3, through the Sema domain (Figure 2-12). To determine whether the same receptors might mediate Sema5A signaling to restrict spine formation in the hippocampus, we analyzed the expression pattern of PlexinA1, PlexinA2, and PlexinA3 mRNA at P7 and P30 by in situ hybridization. We observe robust expression of PlexinA1 in the CA regions, and weak expression in the DG (Figures 2-13A and D). PlexinA2 is expressed strongly in all areas of the hippocampus (Figures 2-13B and E). PlexinA3 shows robust
expression in the DG, and weak expression in the CA regions (Figures 2-13C and F). These results suggest that plexinA family members are present at the right time and right place to affect dendritic spine development in the postnatal hippocampus.

**Sema5A Signals Through PlexinA2 to reduce dendritic Spine Density in vitro and in vivo**

Similar to the dendritic spine phenotype observed in cultured Sema5A−/− GCs (2.23 ± 0.08 /µm) at DIV21, cultured PlexinA2−/− GCs (1.94 ± 0.04 /µm) exhibit a significant (p< 0.0001) increase in dendritic spine density compared to wildtype controls (1.47 ± 0.05 /µm) (Figure 2-14). As shown in figure 2-11, transfection of wildtype GCs with a full-length Sema5A expression construct leads to a significant (p< 0.001) decrease in spine density (1.13 ± 0.05 /µm). However, in PlexinA2−/− cultures, overexpression of Sema5A does not lead to a decrease in spine density (1.88 ± 0.05 /µm), demonstrating that plexinA2 is required for Sema5A mediated regulation of GC spine density in vitro. To investigate whether plexinA2 functions as a receptor to inhibit GC spine density, cultured PlexinA2−/− hippocampal neurons were transfected with full-length plexinA2 and plexinA2Δcyto, a deletion construct lacking the cytoplasmic domain (Figure 2-14). While expression of plexinA2 in PlexinA2−/− cultures is sufficient to reduce GC spine density (1.38 ± 0.10/µm), no significant reduction was observed following transfection of plexinA2Δcyto (2.03 ± 0.09/µm), indicating that plexinA2 cytoplasmic domain is necessary to reduce spine density.
Based on the interaction between Sema5A with plexinA1 and plexinA2 in heterologous cell binding assays, we next investigated the role of plexinA1 and plexinA2 \textit{in vivo} by examining GC dendritic spine density in \textit{PlexinA1;Thy1-eGFP} and \textit{PlexinA2;Thy1-eGFP} mice at P30. Compared to wildtype GCs (1.70 ± 0.04/µm), there is a significant increase in dendritic spine density in \textit{PlexinA2}^{-/-} (2.24 ± 0.07/µm), but not in \textit{PlexinA1}^{-/-} (1.79± 0.05/µm) GCs (Figures 2-15A and B). Interestingly, the increase in GC spine density observed in \textit{PlexinA2}^{-/-} (2.24 ± 0.07/µm) is comparable to \textit{Sema5A}^{-/-} mice (2.05 ± 0.05/µm, p= 0.165). To investigate whether there is a genetic interaction between \textit{Sema5A} and \textit{PlexinA2}, we assessed GC dendritic spine density in P33 single heterozygous \textit{Sema5A}^{+/+}, \textit{PlexinA2}^{+/+} and compound heterozygous \textit{Sema5A}^{+/+}; \textit{PlexinA2}^{+/+} mice. \textit{Sema5A}^{+/+} (1.65 ± 0.06/µm) and \textit{PlexinA2}^{+/+} (1.86 ± 0.05/µm) mice exhibit GC dendritic spine density similar to wildtype mice (1.71± 0.05/µm). In marked contrast, \textit{Sema5A}^{+/+}; \textit{PlexinA2}^{+/+} mice (2.39± 0.05/µm) show a significant (p< 0.0001) increase in GC dendritic spine density (Figures 2-15C and D), comparable to \textit{Sema5A}^{-/-} and \textit{PlexinA2}^{-/-} single mutants. These experiments demonstrate that \textit{Sema5A} and \textit{PlexinA2} interact genetically, suggesting that they function in the same signaling pathway.

\textbf{Sema5A}^{-/-} Mice Exhibit Altered Social Behavior

Recently, a genome-wide association study in a large sample of autism families revealed a significant association between the SEMA5A locus and autism. Moreover, expression of SEMA5A is reduced in the brain and other tissues of
To investigate whether Sema5A−/− mice recapitulate clinical symptoms of human autism patients, we bred these mice into congenic C57Bl/6 background and performed a panel of behavioral assays. In a three-chamber social interaction assay (Moy et al., 2004), wild type mice spent more time interacting with another mice over an inanimate object, whereas Sema5A−/− mice show no preference (Figure 2-16A). When given a choice between a familiar mouse and stranger mouse, wild type mice spent more time interacting with the stranger over the familiar mouse; however, Sema5A−/− mice show no clear preference (Figure 2-16B).

The behavior of Sema5A−/− mice was similar to that of wild type mice in all other respects. Sema5A−/− mice show normal locomotor behavior in the open field (Figure 2-17A), as shown by the total number of beam breaks. They also exhibit normal levels of anxiety, as shown by the comparable percentage of time spent in the center and periphery of the open field (Figure 2-17B), similar rearing behavior (Figure 2-17C), and similar percentage of time spent in open and closed arms of the elevated plus maze (Figure 2-18).

A recent study examining Sema5ALacZ/LacZ mice, where a gene trap insertion of LacZ is present in the last coding exon of Sema5A, found no significant behavioral defects, and therefore concluded that Sema5A mutant mice do not meet the behavioral criteria for a mouse model of autism (Gunn et al., 2011). However, there is no evidence that the Sema5ALacZ is indeed a loss-of-function allele. There are no data showing that LacZ insertion causes a decrease in Sema5A protein, and the anatomical phenotypes of Sema5ALacZ/LacZ
mice have not been characterized. Therefore, we examined hippocampal neurons of Sema5ALacZ/+; Sema5B−/− and Sema5ALacZ/LacZ; Sema5B−/− mice by Golgi staining. We observe no change in dendritic spine density in DG granule neurons (Figures 2-19A, B, and E) and CA1 pyramidal neurons (Figures 2-19C, D, and E) of Sema5ALacZ/LacZ; Sema5B−/− mice. This demonstrates that the Sema5ALacZ allele does not lead to loss of Sema5A function with regard to regulation of dendritic spine density. Moreover, these findings reinforce the correlation between the anatomical changes in the hippocampus and the behavioral abnormality we observe in Sema5A−/− mice.

2.3 Discussion

Semaphorin Signaling and Dendritic Spine Morphogenesis

In the present study, we identified Sema5A-plexinA2 forward signaling as a key mechanism for restricting dendritic spine and excitatory synapse density in hippocampal DG granule neurons during development and adult neurogenesis. We provide several lines of evidence to show that plexinA2 is a functional Sema5A receptor. First, plexinA2 binds to Sema5A in a heterologous cell-based binding assay. Secondly, cultured PlexinA2−/− hippocampal neurons are resistant to the effects of Sema5A overexpression. Moreover, PlexinA2−/− mice phenocopy the dendritic spine defects of Sema5A−/− mice and Sema5A and plexinA2 show genetic interaction by trans-heterozygous analysis. Experiments are underway to examine the effect of Sema5A overexpression in PlexinA2−/−.
dentate gyrus neurons \textit{in vivo} by stereotactic injection of Sema5A-expressing lentivirus.

Interestingly, Sema5A signals through both plexinA1 and plexinA3 to regulate laminar targeting of retinal ganglion cells, amacrine cells, and bipolar cells in the developing retina (Matsuoka et al., 2011). Sema5A apparently employs different receptors, and possibly distinct signaling pathways, in different cell types to regulate a diverse range of developmental processes.

An outstanding question in this study relates to how Sema5A can act cell-autonomously as a ligand to restrict dendritic spine density \textit{in vivo}. Previous studies have shown that the closely related Sema5B protein can undergo ADAM17-mediated proteolytic cleavage to release a secreted ectodomain fragment (O.Conner et al., 2009) \textit{in vitro}. However, the ADAM17 cleavage site is not conserved in Sema5A, and we do not observe ectodomain shedding of Sema5A. One plausible model is that Sema5A interacts with plexinA2 \textit{in cis} on the same dendritic segment to prevent formation of dendritic spines at inappropriate sites. This model is supported by a recent study in \textit{C. elegans} where it was found that in developing DA9 motoneurons, membrane bound semaphorin \textit{Smp-1} and \textit{Plexin-1} function cell-autonomously to restrict synaptic bouton formation at the appropriate subaxonal segment (Mizumoto and Shen, 2013). Nevertheless, other modes of signaling cannot be formally ruled out. For instance, when two closely-spaced dendritic spines occupy the same presynaptic space, Sema5A may signal cell-autonomously and \textit{in trans} through plexinA2 expressed on the neighboring spine to induce spine retraction.
The closely related Sema5B functions redundantly with Sema5A to regulate IPL laminar targeting in the retina (Matsuoka et al., 2011). A previous paper showed that Sema5B is proteolytically processed to release a secreted ectodomain fragment, which induces the elimination of excitatory synapses in cultured hippocampal neurons (O’Connor et al., 2009). We do not find any dendritic spine defects in Sema5B−/− mice in vivo. This discrepancy could be related to the age and genetic background of these mice or the exact hippocampal neuronal subtype examined. Nevertheless, given the synaptic localization of Sema5B, it is possible that Sema5B could regulate other aspects of excitatory synapse development.

**Sema5A and Autism Spectrum Disorders**

Autism is a neurodevelopmental disorder with a strong genetic basis. The genetics of autism spectrum disorders is complex, and it is not known whether autism is mostly caused by rare, highly penetrant, mutations with different subsets of patient harboring different mutations, or by a combination of common mutations, with each mutation conferring a certain degree of risk to develop disease. Although mutations in SEMA5A have not yet been identified in autism patients, a significant decrease of Sema5A mRNA level in the brains of autism patients has been described (Weiss et al., 2009). Our behavioral studies indicate that Sema5A−/− mice show abnormalities in social interaction, recapitulating a key clinical feature of human autism patients. Previous studies using Sema5ALacZ/LacZ mice failed to show a significant alteration in behavior. However, we have shown
that $\text{Sema5A}^{\text{LacZ/LacZ}}$ mice exhibit normal dendritic spine density and, therefore, $\text{Sema5A}^{\text{LacZ}}$ is not a loss-of-function allele. $\text{Sema5A}^{-/-}$ mice may serve as a valuable model for the study of neural developmental mechanisms of social behavior in autism spectrum disorders.

Recent advances in our understanding of autism pathogenesis have mostly been derived from the study of rare genetic mutations that cause autism in a small population of patients, and also from the study of neuropsychiatric syndromes with autistic features, including fragile X syndrome and Rett syndrome. However, even though mouse models of these different mutations all recapitulate a subset of clinical features of autism, the anatomical and physiological abnormalities observed in each model are different. For instance, $\text{Cntnap2}^{-/-}$ mice show defects in cortical interneuron migration and decreased numbers of GABAergic interneurons in the somatosensory cortex (Penagarikano et al., 2011). Conversely, a $\text{Neuroligin-3 R451C}$ knockin mouse exhibits increased inhibitory synaptic transmission in the somatosensory cortex (Tabuchi et al., 2007). $\text{Shank3}^{-/-}$ mice show increased dendritic arbor complexity and decreased dendritic spine density in medium spiny neurons of the striatum (Peca et al., 2011). $\text{Sema5A}^{-/-}$ mice show abnormal synaptic transmission, increased spine density in CA1 pyramidal neurons, early-born and adult-born granule neurons (this study), and defects in cortical migration (unpublished results). Therefore, the neural mechanism underlying abnormal autism-like behavior in this mutant remains unclear. In addition, the link between these structural and functional phenotypes and behavioral phenotypes is not straightforward. In this
regard, Sema5A<sup>ff</sup> mice and conditional alleles of other genes implicated in autism could be valuable for investigating which anatomical defect directly contributes to specific behavioral abnormalities. More sophisticated methods will be required to examine the functional changes in neural circuitry that arise from these anatomical defects. It is possible that different genetic influences on autism affect different developmental processes and distinct brain structures, but ultimately leads to similar changes in neural circuitry, including perturbed synaptic plasticity (Auerbach et al., 2011; Baudouin et al., 2012) or imbalance of excitation/inhibition in the cortex (Yizhar et al., 2011).

2.4 Materials and Methods

**Mouse breeding, transgenic mice, and genotyping**

Sema5A<sup>−/−</sup>, Sema5B<sup>−/−</sup>, PlexinA1<sup>−/−</sup>, PlexinA2<sup>−/−</sup>, and PlexinA3<sup>−/−</sup> mice were previously described (Cheng et al., 2001; Feng et al., 2000; Matsuoka et al., 2011; Suto et al., 2007; Yoshida et al., 2006). For behavioral studies Sema5A mice were backcrossed to a C57bl/6 background.

**DNA constructs**

Sema5A constructs was obtained by PCR amplification of full length Sema5A coding sequence from rat Sema5A cDNA (Accession: NM_001107659), and subsequently cloned in frame into pCAG vector with 6xMyc N-terminal tag and Ig Kappa signal sequence with EcoR1/MfeI and Ascl.
**Primary neuronal culture**

The day of plug was designated E0.5 for all timed-pregnancies. E16.5 hippocampi were dissected in ice cold L-15 Leibovitz Medium (Sigma) and incubated in trypsin-EDTA (0.05%; Gibco) at 37 °C for 15 minutes. The tissues were washed once at room temperature in Ca²⁺- and Mg²⁺- free Hank’s balanced salt solution (HBSS; Gibco) and dissociated with a fire-polished glass Pasteur pipette in HBSS containing 0.025% DNAse I. Dissociated cells were plated on poly-D-lysine (Sigma) coated 12mm coverslips at a density of 2X10⁵ cells per coverslip and grown in 24-well plates in Neurobasal medium (Gibco) containing 2% B27 supplement (Gibco), 50 U/ml penicillin, 50 mg/ml streptomycin (Gibco), and 2 mM glutaMAX (Gibco) at 37 °C, with medium changes every other day. Transfection was performed on DIV 7, with 1 µg DNA and 2 µl Lipofectamine (Invitrogen) as described in manufacturer’s manual (Invitrogen). Following transfection, culture medium was switched to the aforementioned medium plus 2.5% FBS (Gibco), and cultured for another 10-13 DIV, with medium changes every other day.

**X-Gal staining**

P18 and P30 Sema5A<sup>Im1<sup>10gen/J</sup></sup> mice were perfused transcardially with 4% paraformaldehyde (PFA), the brains dissected, post-fixed in 4% PFA for another 30 min and cryo-protected in 30% sucrose in PBS. Brains were flash frozen in dry-ice cooled isopentane. Horizontal sections were cut at 30 µm thickness. Sections were post-fixed in 4% PFA for 15 min, rinsed with 1x PBS twice,
incubated with X-Gal solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl2, and 1mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside [X-Gal] in PBS) and the reaction was developed at 37˚C overnight.

In situ hybridization
In Situ hybridization was performed as previously described (Giger et al., 2000). Digoxigenin-labeled antisense riboprobes specific for *Sema5A*, *Sema5B* and *PlexA3* were synthesized as previously described (Matsuoka et al., 2011). *PlexA1* and *PlexA2* specific riboprobes were transcribed from cDNA fragments harboring 552-2730 bp for *PlexA1*, and 2311-3228 bp for *PlexA2*, respectively.

Isolation of synaptic density fractions and Western blot analysis
Freshly dissected mouse brains were homogenized in ice-cold 4 mM HEPES pH 7.4, 0.32 M sucrose, and protease inhibitors (Roche). Brain lysate was centrifuged at 2000 g for 10 min to give S1 (supernatant) and P1 (pellet) fractions. The S1 fraction was centrifuged at 37,000 g for 30 min to give S2 (supernatant) and P2 (pellet) fractions. The P2 pellet was resuspended in 4 mM HEPES and centrifuged at 82,500 g for 2 hours in a sucrose gradient consisting of 4 mM HEPES plus 0.85 M, 1.0 M, and 1.2 M sucrose. The synaptosomal fraction was harvested at the interface between the 1.2 M and 1.0 M part of the gradient, centrifuged at 150,000 g for 30 min, and resuspended in 80 mM Tris-HCl pH 7.8. To isolate PSD fractions, synaptosomes were incubated with 40 mM
Tris-HCl pH 8.0, 0.5% Triton-X for 15 min on ice, then centrifuged at 32,000 g for 20 min. The pellet was resuspended in 40 mM Tris-HCl pH 8.0 to give PSD I. The PSD I fraction was incubated again with 40 mM Tris-HCl pH 8.0, 0.5% Triton-X for 15 min on ice and centrifuged at 201,800 g for 1 hour. The pellet was resuspended in 40 mM Tris-HCl pH 8.0 with 0.3% SDS to give PSD II fraction. To isolate PSD III fraction, PSD I was incubated with 40 mM Tris-HCl pH 8.0, 3% sarkosyl for 15 min and centrifuged at 201,800 g for 1 hour. The pellet was resuspended in 40 mM Tris-HCl pH 8.0 to give PSD III. Protein concentrations were measured by BCA assay (Pierce) and mixed in Laemmli’s sample buffer. 5 µg of each sample fractions were used for Western blot analysis. Primary antibodies used were mouse anti-PSD95 (1:2000; Millipore), rabbit anti-synaptophysin (1:1000; Santa Cruz), rabbit anti-sema5A (1:1000), rabbit anti-sema5B (1:250), rabbit anti-plexinA1 (1:2000; gift from Yutaka Yoshida), rabbit anti-plexinA2 (1:2000; gift from Hajime Fujisawa), rabbit anti-plexinA3 (1:500; Abcam), and mouse anti-neuropilin-2 (1:1000; Cell Signaling). Generation of rabbit anti-sema5A and rabbit anti-sema5B antibody has been described previously (Matsuoka et al., 2011). Species-specific secondary antibody was used at 1:5000 (Jackson).

Immunocytochemistry

Briefly, primary neurons were fixed with 4% PFA at 21 DIV, and blocked with 1% horse serum, 0.1% Triton X-100 in PBS (PHT) for 1hr. Then cells were incubated with indicated primary antibodies in PHT overnight at 4°C, followed by 1hr
incubation with fluorophore-conjugated secondary antibodies at room temperature. Primary antibodies used in this study included: Rabbit anti-Prox1 (1:5000, Millipore), mouse anti-myc (1:1000, Developmental Studies Hybridoma Bank at the University of Iowa), chicken anti-GFP (1:1000; AVES), guinea pig anti-vGlut1 (1:1000; Millipore), mouse anti-PSD95 (1:500; Millipore), rabbit anti-VGAT (1:1000; Synaptic Systems), and mouse anti-gephyrin (1:1000; Synaptic Systems). Species-specific secondary antibodies (Invitrogen) were used at 1:1000.

**Immunohistochemistry**

Coronal brain sections (30-40 µm thick) were cryosectioned and fixed in 4% PFA for 15 min and rinsed twice with 1xPBS for 5 min, then sections were blocked with 5% horse serum, 0.3% Triton X-100 in PBS for 1hr at room temperature, and followed by incubation overnight at 4°C with primary antibodies: Rabbit anti-PSD95 (1:500, Millipore), guinea pig anti-vGlut1 (1:1000, Millipore), Rabbit anti-Calbindin (1:1000, swant), goat anti-Calretinin (1:1000, swant), goat anti-Doublecortin (1:200, Santa Cruz), goat anti-GFP antibody (1:500, Rockland). Sections then washed 5 times with 1xPBS for 5 min each, and incubated with Alexa Fluor conjugated secondary antibodies for 1hr at room temperature to visualize the staining using either fluorescent or confocal microscopy.

**Retrovirus birthdating of adult-born granule neurons**
Engineered self-inactivating murine retroviruses with GFP reporter under the control of ubiquitin promoter were used to label and birth-date the proliferating cells and their progeny as previously described (Ge et al., 2006). High titers of engineered retroviruses were produced by co-transfection of retroviral vectors and VSVG into HEK293gp cells followed by ultracentrifugation of viral supernatant as previously described (Ge et al., 2006).

Young adult (5-6 weeks old) Sema5A⁻/⁻ mice and their littermates housed under standard conditions were anaesthetized with ketamine/xylazine and retroviruses were stereotaxically injected into the dentate gyrus at 4 sites (0.5 μl per site) with the following coordinates as previously described², ³: anterioposterior = - 2 mm from bregma; lateral = ±1.5 mm; ventral = 2.5 mm. All animal procedures were performed in accordance with institutional guidelines.

Three weeks later, brains were isolated, coronal sections cut with 40 μm thickness, and then proceed for standard anti-eGFP immunohistochemistry (Ge et al., 2006). Images were acquired with 40x oil objective plus 5x zoom on a Zeiss LSM 710 META multiphoton confocal system (Carl Zeiss, Thornwood, NY, USA) using a multi-track configuration. Only dorsal molecular layer of DG were imaged and included in the analysis.

Golgi analysis

Freshly dissected P60 mouse brains were incubated in Golgi solution A+B (FD Rapid GolgiStain Kit, FD NeuroTechnologies) for 8 days. After incubation, all brains were washed thoroughly with Solution C for 2-4 days at room temperature,
and embedded in OCT embedding medium (Tissue-Tek). Coronal sections (100 µm) through the somatosensory cortex and hippocampus were cut with a Leica CM3050 cryostat and mounted on 3% gelatin coated slides. Staining procedures were followed as described (FD NeuroTechnologies), and slides were dehydrated in ethanol, cleared in xylene, and mounted for microscopy. Only Layer II-III and V pyramidal neurons from the somatosensory cortex and granule cells from dorso-medial dentate gyrus were included in our analyses.

**Transmission electron microscopy**

P60 mice were fixed by transcardial perfusion with 3.0% formaldehyde/1.5% glutaraldehyde in 0.1 M Na+-cacodylate, 3 mM Ca2+ and 2.5% sucrose, at pH 7.4. Brains were dissected and the hippocampus was trimmed and fixed overnight in the aforementioned fixative. The tissue was washed with 0.1 M Na+-cacodylate/ 2.5% sucrose, reduced with 2% OsO4, then dehydrated in ethanol, infiltrated and flat embedded in EPON. Semi-thin and ultra-thin sections were collected using a Leica Ultracut microtome and analyzed with a Hitachi 7600 TEM. Only identified synapses on dendritic spines of DG granule cells were included in these analyses.

**Electrophysiology**

Mice housed under standard conditions were anaesthetized 19-21 days after retroviral injection and processed for slice preparation as previously described (Ge S, et al, 2007). The brains were quickly removed into the ice-cold solution (in
mM: 110 choline chloride, 2.5 KCl, 1.3 KH₂PO₄, 25.0 NaHCO₃, 0.5 CaCl₂, 7 MgSO₄, 20 dextrose, 1.3 sodium L-ascorbate, 0.6 sodium pyruvate, 5.0 kynurenic acid). Slices (275 µm thick) were cut using a vibrotome (Leica VT1000S) and transferred to a chamber containing the external solution (in mM: 125.0 NaCl, 2.5 KCl, 1.3 KH₂PO₄, 1.3 MgSO₄, 25.0 NaHCO₃, 2 CaCl₂, 1.3 sodium L-ascorbate, 0.6 sodium pyruvate, 10 dextrose, pH 7.4, 320 mOsm), bubbled with 95% O₂/5% CO₂. Electrophysiological recordings were obtained at 32°C - 34°C. GFP⁺ cells within the subgranular zone were visualized by DIC and fluorescence microscopy. The whole-cell patch-clamp configuration was employed in voltage-clamp mode (Vₘ = -65 mV). Microelectrodes (4–6 MΩ) were pulled from borosilicate glass capillaries and filled with the internal solution containing (in mM): 120 potassium gluconate, 15 KCl, 4 MgCl₂, 0.1 EGTA, 10.0 HEPES, 4 ATP (magnesium salt), 0.3 GTP (sodium salt), 7 phosphocreatine (pH 7.4, 300 mOsm). Data were collected using an Axon 200B amplifier and acquired with a DigiData 1322A (Axon Instruments) at 10 kHz. Series and input resistances were monitored, and only those with changes less than 20% during experiments were analyzed. The series resistance ranged from 10–30 MΩ and was uncompensated.

**Behavioral studies**

All behavioral experiments were performed on 3-4 month old male C57Bl/6 congeneric Sema5A⁻/- mice and their WT littermates.
Open field

Open field was performed using the Photobeam Activity System (San Diego Instruments). Mice were placed individually in the chambers and were allowed to move freely for 30 minutes. The number of beam interruptions caused by both horizontal and vertical movement was recorded. Total number of beam breaks was used to assess general locomotor activity. Percentage of time spent in center was used to assess anxiety level. Rearing represents the total number of vertical movements and was used to assess exploratory behavior.

Elevated plus maze

Elevated plus maze was used to assess anxiety level. Mice were placed in the center of the elevated plus maze and were allowed to move freely in the maze for 5 minutes. The behavior of the mice were videotaped and analyzed by Any-maze video tracking system (Stoelting Co.).

Social interaction

Social interaction was examined by the three-chamber social interaction test as described previously (Yang et al., 2011). Briefly, the subject mouse was placed in the center chamber with nothing in the side chambers and allowed to acclimate for 5 minutes. Subsequently, a cage with a WT C57Bl/6 mouse and a cage with an inanimate object were placed in each of the side chambers. The subject mouse was allowed to enter each chamber freely and interact with the mouse or object for 5 minutes. Finally, the inanimate object was replaced with a
new WT C57Bl/6 mouse, and the subject mouse was allowed to enter each chamber freely and interact with either mouse for 5 minutes. The behavior of the mice were videotaped and analyzed by Any-maze video tracking system (Stoelting Co.).

2.5 Acknowledgements

This work was done in close collaboration with Yuntao Duan from Roman Giger’s laboratory and Juan Song from Hongjun Song’s laboratory. Yuntao Duan performed all the dendritic spine analysis with GFP, Sema5A-plexin binding assays, and Sema5A overexpression experiments. Juan Song performed dendritic spine analysis and electrophysiological recordings on retrovirus-labeled adult born GC’s. Kimberly Christian supervised the behavioral assays. Ryota Matsuoka generated \textit{Sema5A}^{-/-} and \textit{Sema5B}^{-/-} mice and other reagents and provided valuable input throughout the study.

2.6 References:


Figure 2-1. *Sema5A* And *Sema5B* are Expressed in The Postnatal Hippocampus

(A) Horizontal section of P18 and P30 *Sema5A*\textsuperscript{Im1Dgen/J} LacZ reporter mouse brain. Strong β-galactosidase signal is observed in the dentate gyrus (DG) granule cells (GCs). Hilus, CA3 pyramidal neurons, a subset of CA1 pyramidal neurons and neurons in deep layers of the entorhinal cortex (EC) show weaker staining.
(B) *In situ* hybridization for *Sema5A* and *Sema5B* transcripts on coronal section of P18 and P30 mouse hippocampus. Robust expression of *Sema5A* is observed in the dentate granule cells. *Sema5B* is also expressed in granule cells, but at a lower level.
Figure 2-2. Sema5A and Sema5B are Enriched in Postsynaptic Density (PSD) Fractions

Subcellular fractionation of P18 mouse hippocampus was performed to generate fractions progressively enriched in synaptic membranes. Western blots show that both Sema5A and Sema5B are enriched postsynaptically. S1, homogenate; S2, cytosolic fraction; P2, membrane fraction; Syn, synaptosomal fraction; PSDI-III, purified postsynaptic fractions, with progressive enrichment of core PSD proteins. Synaptophysin (SYP) and PSD95 were used as pre- and postsynaptic markers, respectively.
Figure 2-3. Sema5A, but not Sema5B, Regulates Dendritic Spine Density in DG Granule Neurons

(A and B) Representative images of DG granule neurons from Sema5A+/- (A) and Sema5A-/- (B) mice examined using Golgi staining at P60. There is a significant increase in dendritic spine density in Sema5A-/- neurons.

(C) Quantification of dendritic spine density in hippocampal DG granule neurons at P60. (Sema5A+/-; Sema5B+/- DG: 0.605±0.023 spines/µm; Sema5A-/-;
Sema5B<sup>+/+</sup> DG: 0.836±0.029 spines/µm; Sema5A<sup>+/+</sup>; Sema5B<sup>−/−</sup> DG: 0.558±0.023 spines/µm; Sema5A<sup>−/−</sup>; Sema5B<sup>−/−</sup> DG: 0.739±0.039 spines/µm). Number of neurons quantified: n=50 neurons from 5 animals for Sema5A<sup>+/+</sup>; Sema5B<sup>+/+</sup>, n=30 neurons from 3 animals for Sema5A<sup>−/−</sup>; Sema5B<sup>+/+</sup>, n=30 neurons from 3 animals for Sema5A<sup>−/−</sup>; Sema5B<sup>+/+</sup>, n=30 neurons from 5 animals for Sema5A<sup>−/−</sup>; Sema5B<sup>−/−</sup>. ***p<0.001. Error bar represents SEM.

(D) Quantification of dendritic spine density in hippocampal DG granule neurons at P16. (Sema5A<sup>+/+</sup> DG: 0.488±0.014 spines/µm; Sema5A<sup>−/−</sup> DG: 0.587±0.021 spines/µm). Number of neurons quantified: n=20 neurons from 2 animals for Sema5A<sup>+/+</sup>, and n=20 neurons from 2 animals for Sema5A<sup>−/−</sup>. ***p<0.001. Error bar represents SEM.

(E) The Thy1-GFP (m line) mouse was crossed to a Sema5A<sup>−/−</sup> and Sema5B<sup>−/−</sup> background to label hippocampal neurons. Representative images of GFP positive dendrites of DG granule neurons and CA1 pyramidal neurons in P33 mice of various genotypes are shown. Scale bar = 1 µm.

(F) Quantification of dendritic spine density revealed a significant increase in GCs of Sema5A<sup>−/−</sup> but not Sema5<sup>−/−</sup> mice. Deletion of both Sema5A and Sema5B does not lead to a further increase in spine density compared to Sema5A<sup>−/−</sup> single mutants. There is no difference in dendritic spine density of primary or secondary branches in CA1 pyramidal neurons across all genotypes examined. Number of neurons quantified: n=50-60 neurons from 3-4 animals for each genotype. **p<0.01 by two-tailed student t test.
Figure 2-4. Normal Neurogenesis and Neuronal Migration in Sema5A\textsuperscript{+/-}; Sema5B\textsuperscript{-/-} mice

(A-D) Coronal sections of hippocampus from P18 and P30 Sema5A\textsuperscript{+/-}; Sema5\textsuperscript{+/-} and Sema5A\textsuperscript{+/-}; Sema5B\textsuperscript{+/-} mice were immunostained with calbindin (green), a marker for mature DG neurons, and calretinin (red), a marker for immature neurons. No difference was observed between Sema5A\textsuperscript{+/-}; Sema5\textsuperscript{+/-} and Sema5A\textsuperscript{+/-}; Sema5B\textsuperscript{-/-} mice. Dash lines indicate the borders of granule neuron layer. n=3.

(E-F) Coronal sections of hippocampus from P30 Sema5A\textsuperscript{+/-}; Sema5\textsuperscript{+/-} and Sema5A\textsuperscript{+/-}; Sema5B\textsuperscript{-/-} mice were immunostained with doublecortin, a marker for new-born granule neurons. No difference in the immature granule cell layer was observed between Sema5A\textsuperscript{+/-}; Sema5\textsuperscript{+/-} and Sema5A\textsuperscript{+/-}; Sema5B\textsuperscript{-/-} mice. n=3. Scale bar in (F) = 100\mu m for (A to F).
Figure 2-5. Sema5A Does Not Regulate Dendritic Spine Density in Cortical Pyramidal Neurons

(A and B) Layer V cortical pyramidal neurons from Sema5A<sup>+/+</sup>; Sema5B<sup>+/+</sup> (A) and Sema5A<sup>−/−</sup>; Sema5B<sup>−/−</sup> (B) mice were examined using Golgi staining at P60. There is no change in dendritic spine density in Sema5A<sup>−/−</sup>; Sema5B<sup>−/−</sup> neurons.
(C and D) Layer II/III cortical pyramidal neurons from Sema5A⁺/⁺; Sema5B⁺/⁺ (C) and Sema5A⁻/-; Sema5B⁻/- (D) mice were examined using Golgi staining at P60. There is no change in dendritic spine density in Sema5A⁻/-; Sema5B⁻/- neurons. Scal bar in (A) = 3µm for (A) to (D).

(E) Quantification of dendritic spine density in layer V and layer II/III cortical neurons at P60. (Sema5A⁺/⁺; Sema5B⁺/⁺ layer V: 0.532±0.031 spines/µm; Sema5A⁻/-; Sema5B⁻/- layer V: 0.559±0.032 spines/µm; Sema5A⁺/⁺; Sema5B⁺/⁺ layer II/III: 0.552±0.033 spines/µm; Sema5A⁻/-; Sema5B⁻/- layer II/III: 0.563±0.036 spines/µm). Number of neurons quantified: n=22 layer V and 22 layer II/III cortical neurons from 3 animals for Sema5A⁺/⁺; Sema5B⁺/⁺, and n=22 layer V and 22 layer II/III cortical neurons from 3 animals for Sema5A⁻/-; Sema5B⁻/-. Error bar represents SEM.
Figure 2-6. Sema5A Regulates Postsynaptic Density of Excitatory Synapses in Cultured Hippocampal Neurons

(A) Hippocampal neurons derived from E16.5 mouse embryos were cultured for 21 DIV and stained for the post- and presynaptic markers PSD95 and VGlut1, respectively. Representative images of double-labeled dendrites from Sema5A+/+ and Sema5A−/− neurons are shown. Scale bar = 10 µm.

(B) Quantification of PSD95, VGlut1 and VGlut1/PSD95 double-labeled puncta. In Sema5A−/− neurons, the density of PSD95 positive puncta is significantly increased. No significant difference in VGlut1 positive puncta and VGlut1/PSD95 double-labeled puncta was observed in Sema5A+/+ cultures. (Sema5A+/+ PSD95: 0.308±0.017 puncta/µm; VGlut1: 0.233±0.018 puncta/µm; VGlut1/PSD95: 0.168±0.013 puncta/µm. Sema5A−/− PSD95: 0.381±0.018 puncta/µm; VGlut1: 0.272±0.021 puncta/µm; VGlut1/PSD95: 0.21±0.019 puncta/µm). Number of neurons quantified: n=26 from 3 independent cultures for Sema5A+/+, and n=26
from 3 independent cultures for *Sema5A*<sup>−/−</sup>. Error bars represent SEM. **p<0.01 by two-tailed Student’s t test.
Figure 2-7. Abnormal HippocampalSynaptic Ultrastructure in Sema5A\(^{-/-}\) Mice

(A and B) Transmission electron microscopic images of the DG molecular layer at P45 show that the number of postsynaptic densities (PSDs, marked by blue arrows) is significantly higher in Sema5A\(^{-/-}\) mice compared to control mice.

Scale bar in (B) = 500 nm for (A) and (B).

(C to F) Higher magnification images show a single presynaptic terminal making contact with multiple postsynaptic spines in Sema5A\(^{-/-}\) mice (E). Moreover, there is increased number of split PSDs in Sema5A\(^{-/-}\) mice (F).

Scale bar in (F) = 500 nm for (C) to (F).

(G) Quantification of PSD length in the DG molecular layer of Sema5A\(^{+/+}\) and Sema5A\(^{-/-}\) mice. Excitatory synapses in Sema5A\(^{-/-}\) DG show significant decrease in PSD length. (Sema5A\(^{+/+}\): 101.34 ± 5.82 nm; Sema5A\(^{-/-}\): 62.86 ± 3.6 nm.)

Number of synapses quantified: n=20 synapses from 2 animals for Sema5A\(^{+/+}\) and Sema5A\(^{-/-}\). Error bars represent SEM. ***p<0.001 by two-tailed student t test.
Figure 2-8. Sema5A Does Not Regulate Inhibitory Synapses

(A) Hippocampal neurons derived from E16.5 mouse embryos were cultured for 21 DIV and stained for the inhibitory post- and presynaptic markers gephyrin and VGAT, respectively. Representative images of double labeled dentrites from Sema5A+/+ and Sema5A−/− neurons are shown. Scale bar = 10 µm.

(B) Quantification of gephyrin, VGAT and VGAT/gephyrin double labeled puncta. In Sema5A−/− neurons, no change in density of gephyrin, VGAT, or VGAT/gehyrin double-labeled puncta is observed. (Sema5A+/+ gephyrin: 0.734±0.073 puncta/µm; VGAT: 0.728±0.056 puncta/µm; VGAT/gephyrin: 0.338±0.038 puncta/µm. Sema5A−/− gephyrin: 0.773±0.078 puncta/µm; VGAT: 0.595±0.065 puncta/µm; VGAT/gephyrin: 0.29±0.033 puncta/µm). Number of neurons quantified: n=10 from 3 independent cultures for both Sema5A+/+ and Sema5A−/−. Error bars represent SEM.
Figure 2-9. Sema5A Regulates Dendritic Spine Density in Adult-Born GC

Granule Neurons

(A and B) Self-inactivating retrovirus with GFP reporter was stereotaxically injected into the DG of P35-P42 Sema5A\(^{+/+}\) and Sema5A\(^{-/-}\) mice to label and birth-date adult-born granule neurons. Adult-born granule neurons of Sema5A\(^{-/-}\) mice show significant increase in dendritic spine density. Scale bar in (A) = 3\(\mu\)m for (A and B).

(C) Quantification dendritic spine density of birth-dated adult-born granule neurons at 19-21dpi. There is a significant increase in dendritic spine density in Sema5A\(^{-/-}\) neurons. (n=19 for Sema5A\(^{+/+}\): 1.009 \(\pm\) 0.081 spines/\(\mu\)m; and n=16 for Sema5A\(^{-/-}\): 1.554 \(\pm\) 0.111 spines/\(\mu\)m ). Number of neurons quantified: n = 19
neurons from 3 animals for Sema5A^{+/+}, and n= 16 neurons from 3 animals for Sema5A^{-/-}. Error bars represent SEM. *** p = 0.0003 by two-tailed unpaired Student’s t-test.
**Figure 2-10. DG Granule Neurons of Sema5A⁻/⁻ Mice Show Increased mEPSC Amplitude**

(A-D) Spontaneous mEPSCs recorded in GFP labeled adult-born granule neurons at 19-21dpi. Shown are sample recording traces from WT (top) and KO (bottom) animals before and after adding CNQX (20 µM) (A); and quantification of mean frequency (B), mean amplitude (C), and distribution plot of mEPSC amplitudes from both groups (D). Recordings were performed in the presence of TTX (1 µM) and bicuculline (10 µM). Values represent mean±SEM (n=10 cells each group; student t-test).
**Figure 2-11. Sema5A Can Function Cell-Autonomously as a Ligand**

(A-C) Representative images of a hippocampal neuron derived from P1-P2 mice and transfected with GFP and 6x-myc-Sema5A at DIV4. At DIV 21 cultured hippocampal neurons were labeled with anti-GFP (A), anti-prox-1 (B), and anti-myc (C). Scale bars in (A) and (C) = 10 μm.

(D and E) Overexpression of Sema5A results in significant reduction in dendritic spine density in cultured wild type hippocampal neurons.

(F-J) Overexpression of full length Sema5A (G), Sema5A lacking the cytoplasmic domain (H), and Sema5A lacking the TSR domain (J) results in significant
reduction in dendritic spine density in cultured Sema5A−/− hippocampal neurons. In contrast, overexpression of Sema5A lacking the Sema domain shows no change in dendritic spine density. Scale bar in (J) = 5 µm for (D) to (J).

(K) Quantification of dendritic spine density in cultured hippocampal neurons at 21 DIV. (Wild type: 1.67±0.04 spines/µm; Wild type + Sema5A: 1.18±0.04 spines/µm. Sema5A−/−: 2.23±0.08 spines/µm; Sema5A−/− + Sema5A: 1.10±0.04 spines/µm, Sema5A−/− + Sema5AΔcyto: 1.09±0.03 spines/µm, Sema5A−/− + Sema5AΔsema: 1.94±0.05 spines/µm, Sema5A−/− + Sema5AΔTSR: 1.11±0.05 spines/µm) Number of neurons quantified are indicated in brackets. Error bar represents SEM from 3-4 independent cultures per condition. ***P < 0.0001 by two-tailed student T test.
Figure 2-12. Sema5A Binds to plexinA1 and plexinA2 Through The Sema Domain

COS7 cells were transfected with Myc-tagged plexinA1, A2, A3 expression constructs. Application of oligomerized Sema5A ligands show that both Sema5A Ecto-domain and Sema5A Sema domain bind to cells expressing plexinA1 and plexinA2, but not plexinA3. Sema5A TSR domain does not bind to plexinA1, A2, or A3.
Figure 2-13. PlexinA’s are Expressed in the Postnatal Hippocampus

(A-C) In situ hybridization for plexinA1, plexinA2, plexinA3 mRNA on coronal sections of P7 and P30 mouse hippocampus. *PlexinA1* shows robust expression in the CA regions and weak expression in the DG. *PlexinA2* is strongly expressed throughout the hippocampus. *PlexinA3* shows strong expression in the DG and weak expression in the CA regions.
Figure 2-14. Sema5A signals through PlexinA2 to reduce dendritic spine density in vitro

(A) Representative images of primary hippocampal neurons derived from P1-P2 mice and transfected with GFP and myc-Sema5A, myc-PlexinA2, or myc-PlexinA2Δcyto. At DIV21 cultures were labeled with anti-GFP, anti-myc and anti-prox1 antibodies. PlexinA2Δcyto GCs show a significant increase in dendritic spine density. Overexpression of Sema5A significantly reduced dendritic spine density in PlexinA2Δcyto but not in PlexinA2Δcyto GCs. Overexpression of PlexinA2, but not PlexinA2Δcyto, significantly reduced dendritic spine density in PlexinA2Δcyto GCs. Scale bar = 5 µm.

(B) Quantification of GC dendritic spine density at DIV21. (Wild type: 1.47 ± 0.05 spines/µm; Wild type + Sema5A: 1.13 ± 0.05 spines/µm. PlexinA2Δcyto: 1.94 ± 0.04 spines/µm; PlexinA2Δcyto + Sema5A: 1.88 ± 0.05 spines/µm; PlexinA2Δcyto + PlexinA2:...
1.38 ± 0.10 spines/µm; *PlexinA2*<sup>−/−</sup> + PlexinA2<sup>Δcyto</sup>: 2.03 ± 0.09 spines/µm.) Error bars represent SEM from 3 animals for each condition. Numbers in brackets indicate the cells analyzed per condition. *** indicates p < 0.0001 by two-tailed unpaired student t-test.
Figure 2-15. PlexinA2 mediates Sema5A signaling in vivo

(A) Representative images of GC dendritic segments of P30 Thy1-eGFPm positive wildtype (wt), PlexinA1<sup>−/−</sup> and PlexinA2<sup>−/−</sup> mice. There is a significant increase in dendritic spine density in PlexinA2<sup>−/−</sup>, but not PlexinA1<sup>−/−</sup> mice. Scale bar = 5µm.

(B) Quantification of GC dendritic spine density at P30. (Wt: 1.70 ± 0.04 spines/µm; PlexinA1<sup>−/−</sup>: 1.79 ± 0.05 spines/µm; PlexinA2<sup>−/−</sup>: 2.24 ± 0.07 spines/µm.) Error bars represent SEM from 3-4 animals for each genotype.
Numbers in brackets indicate number of neurons analyzed for each genotype.

*** p<0.001 by two-tailed unpaired student t-test.

(C) Representative images of GC dendritic segments of P30 Thy1-eGFPm positive wt, Sema5A+/-, PlexinA2+/-, and Sema5A+/--; PlexinA2+/-- mice. There is a significant increase in GC dendritic spine density in Sema5A+/--; PlexinA2+/-- mice, but not Sema5A+/-- or PlexinA2+/-- mice.

(D) Quantification of GC dendritic spine densities at P30. (Wt: 1.71 ± 0.05 spines/µm; Sema5A+/--: 1.65 ± 0.06 spines/µm; PlexinA2+/--: 1.86 ± 0.05 spines/µm; Sema5A+/--; PlexinA2+/--; 2.39 ± 0.05 spines/µm.) Error bars represent SEM from 4 animals for each genotype. Numbers in brackets indicate number of neurons analyzed for each genotype. *** p<0.0001 by two-tailed unpaired student t-test.
Figure 2-16. *Sema5A*^−/−^ Mice Show Abnormal Behavior in the Three-Chamber Social Interaction Test

(A and B) Three-chamber social interaction test. Given the choice, *Sema5A*^+/+^ mice spend significantly more time in the chamber with a mouse compared to the chamber with an inanimate object, whereas *Sema5A*^−/−^ mice show no preference (A). *Sema5A*^+/+^ mice spend significantly more time in the chamber with a stranger mouse compared to the chamber with the familiar mouse, whereas *Sema5A*^−/−^ mice show no preference (B). Number of animals tested: n=9 *Sema5A*^+/+^ mice and 9 *Sema5A*^−/−^ mice for mouse vs. inanimate object, and n=8 *Sema5A*^+/+^ mice and 8 *Sema5A*^−/−^ mice for familiar vs. stranger mouse. Error bar represents SEM. *p<0.05 by two-tailed Student’s t test.
Figure 2-17. *Sema5A*<sup>−/−</sup> Mice Exhibit Normal Behavior in the Open Field Test

(A to C) Open field test. *Sema5A*<sup>−/−</sup> mice show no abnormality in locomotion, as measured by total beam breaks (A), or anxiety level, as measured by percentage of time spent in the center of the field (B) and number of rearing (C). Number of animals tested: n = 7 for *Sema5A*<sup>+/+</sup> mice and n = 9 for *Sema5A*<sup>−/−</sup> mice. Error bar represents SEM.
Figure 2-18. *Sema5A*\(^{-/-}\) Mice Show Normal Behavior in the Elevated Plus Maze Test

Elevated plus maze. *Sema5A*\(^{-/-}\) mice show no abnormality in anxiety level, as measured by time spent in the open arm versus the closed arm. Number of animals tested: \(n=7\) *Sema5A*\(^{+/+}\) mice and 9 *Sema5A*\(^{-/-}\) mice. Data represent mean ± SEM.
Figure 2-19. *Sema5A<sup>LacZ/LacZ</sup>; Sema5B<sup>−/−</sup>* Mice Show Normal Dendritic Spine Density in the DG and CA1

(A and B) DG granule cells from *Sema5A<sup>LacZ/+</sup>; Sema5B<sup>−/−</sup>* (A) and *Sema5A<sup>LacZ/LacZ</sup>; Sema5B<sup>−/−</sup>* (B) mice were examined by Golgi staining at P60. There is no change in dendritic spine density in *Sema5A<sup>LacZ/LacZ</sup>; Sema5B<sup>−/−</sup>* DG granule neurons.
(C and D) CA1 pyramidal neurons from \( \text{Sema5A}^{\text{LacZ/+}}; \text{Sema5B}^{-/-} \) (C) and \( \text{Sema5A}^{\text{LacZ/LacZ}}; \text{Sema5B}^{-/-} \) (D) mice were examined by Golgi staining at P60. There is no change in dendritic spine density in \( \text{Sema5A}^{\text{LacZ/LacZ}}; \text{Sema5B}^{-/-} \) CA1 pyramidal neurons.

Scale bar in (A) = 3\( \mu \)m for (A to D).

(E) Quantification of dendritic spine density in DG granule cells and CA1 pyramidal neurons (apical dendrites) at P60. (\( \text{Sema5A}^{\text{LacZ/+}}; \text{Sema5B}^{-/-} \) DG: 0.825±0.025 spines/\( \mu \)m; \( \text{Sema5A}^{\text{LacZ/LacZ}}; \text{Sema5B}^{-/-} \) DG: 0.854±0.028 spines/\( \mu \)m; \( \text{Sema5A}^{\text{LacZ/+}}; \text{Sema5B}^{-/-} \) CA1: 0.725±0.025 spines/\( \mu \)m; \( \text{Sema5A}^{\text{LacZ/LacZ}}; \text{Sema5B}^{-/-} \) CA1: 0.747±0.022 spines/\( \mu \)m). Number of neurons quantified: n=15 neurons from 2 animals for both DG neurons and CA1 neurons for \( \text{Sema5A}^{\text{LacZ/+}}; \text{Sema5B}^{-/-} \) mice, and n=20 neurons from 2 animals for both DG neurons and CA1 neurons for \( \text{Sema5A}^{\text{LacZ/LacZ}}; \text{Sema5B}^{-/-} \) mice. Error bar represents SEM. N.S. p=0.43 for DG and p=0.51 for CA1 by two-tailed Student’s t test.
Chapter 3

Discs Large Homologue 5 Regulates Dendritic Spine Formation and Synaptogenesis by Controlling Subcellular Localization of N-Cadherin

3.1 Introduction

A critical early event in dendritic spine formation and synaptogenesis is the stabilization of trans-synaptic contacts mediated by cell surface proteins; these proteins include neurexins, neuroligins, ephrins, leucine-rich-repeat proteins, and cadherins (Arikkath and Reichardt, 2008; Craig and Kang, 2007; de Wit et al., 2009; Henkemeyer et al., 2003; Kayser et al., 2008; Ko et al., 2009; Siddiqui and Craig, 2011; Suzuki and Takeichi, 2008). N-cadherin is the most abundant and widely distributed classical cadherin in the mammalian brain (Togashi et al., 2002; Yagi and Takeichi, 2000). In cultured hippocampal neurons, N-cadherin knockdown using shRNA results in a decrease in dendritic spine density (Saglietti et al., 2007). Neuronal N-cadherin becomes anchored to the actin cytoskeleton by binding to α-catenin and β-catenin through its intracellular domain (Okuda et al., 2007; Yagi and Takeichi, 2000). However, the intracellular mechanisms that localize N-cadherin to the cell surface during dendritic spine formation and synaptogenesis are unknown.

MAGUK proteins are intracellular scaffolding proteins with well-established roles in synapse development and function. The best-studied synaptic MAGUK protein, PSD95, regulates synaptic strength and plasticity by controlling the
organization and trafficking of glutamate receptors and associated signaling proteins at the postsynaptic density of excitatory synapses (Feng and Zhang, 2009; Kim and Sheng, 2004). Whether MAGUK proteins are required for the formation of dendritic spines in vivo remains unclear. A previous study on PSD95 mutant mice revealed an increase in spine density localized to specific dendritic segments of hippocampal neurons (Vickers et al., 2006). However, this is in contrast to earlier studies showing an increase in spine density in cultured hippocampal neurons that overexpress PSD95 (El-Husseini et al., 2000). Dlg5 is a member of the discs large (Dlg) family of MAGUK proteins (de Mendoza et al., 2010). However, the size of the DLG5 protein and its domain organization are distinct from other classical Dlg family members. In addition to the PDZ (PDZ-95, Dlg, ZO-1), SH3 (Src Homology 3), and GUK (Guanylate Kinase) domains, Dlg5 also contains an N-terminal CARD (Caspase Activation and Recruitment Domain) domain, a DUF (Domain of Unknown Function) domain, and a coiled-coil domain (Nechiporuk et al., 2007). Previous work shows that DLG5 is important for the delivery and stabilization of cadherin-catenin protein complexes to the plasma membrane of epithelial cells. Loss of Dlg5 in vivo leads to disruption of adherens junctions and epithelial cell polarity in Dlg5−/− mutant mice, resulting in enlarged cortical ventricles and renal cysts (Nechiporuk et al., 2007). In addition, DLG5 facilitates the localization of citron kinase to the ventricular surface in neural progenitor cells of the developing neocortex, and Dlg5−/− mutant mice show decreased number of mitotic neural progenitors in the ventricular
zone (Chang et al., 2010). DLG5 functions in other cell types and organ systems, however, are unknown.

We have identified a novel loss-of-function allele of Dlg5, Dlg5\(^{LP}\), through a forward genetic screen for recessive mutations affecting brain development. Dlg5\(^{LP/LP}\) mice show a reduction of dendritic spine density in cortical neurons. DLG5 promotes dendritic spine formation and synaptogenesis in cortical pyramidal neurons by facilitating N-cadherin localization to the neuronal cell surface. The Dlg5\(^{LP}\) mutant protein abolishes key protein domain interactions required for Dlg5 neuronal functions. Taken together, these observations lend further insight into the neuronal functions of MAGUK proteins and reveal a critical molecular mechanism for regulating the subcellular localization of cell adhesion molecules during synaptogenesis.

3.2 Results

**A Forward Genetic Screen Identifies a Novel Dlg5 Allele**

Using a three-generation forward genetic screen for recessive mutations affecting murine brain development (Figure 3-1A) (Merte et al., 2010a; Merte et al., 2010b), we identified a mutant mouse line (line #3007) that exhibits severely enlarged ventricles (Figures 3-1B and C). The affected G3 mice suffer from progressive enlargement of the cortical ventricles and usually die around weaning. The genetic lesion underlying this brain defect was mapped to a 3.1Mb interval on chromosome 14, between D14Mit207 and rs30102223, which is a region that contains 15 protein-coding genes (Table 3-1; see Experimental
Procedures). We identified *Discs large homolog 5 (Dlg5)* as a candidate gene harboring this genetic lesion based on the phenotypic similarity between this mutant and a previously described *Dlg5*<sup>−/−</sup> mouse mutant (Nechiporuk et al., 2007). We sequenced all 32 exons of *Dlg5* and identified a T→C transition in exon 26 that produces a L1642P missense mutation in the evolutionarily conserved single SH3 domain of the DLG5 protein (Figure 3-2A, B, and C). Western blot analysis reveals that DLG5<sup>LP</sup> protein is present in *Dlg5*<sup>LP</sup> mutant brains; however, protein levels are reduced to ~50% of wild type DLG5 levels (Figures 3-2D and E). We designate this novel *Dlg5* allele *Dlg5*<sup>LP</sup>. The severity of hydrocephalus in *Dlg5*<sup>LP/LP</sup> mice is equivalent to that observed in *Dlg5*<sup>−/−</sup> mice (Nechiporuk et al., 2007). This suggests that the *Dlg5*<sup>LP</sup> mutant is either a null, or strong hypomorphic, allele of *Dlg5*. Although *Dlg5*<sup>LP/LP</sup> mutants exhibit enlarged ventricles, cortical lamination appears normal in *Dlg5*<sup>LP/LP</sup> mice since immunostaining for the cortical layer markers BRN2, CUX1, CTIP2 and TBR1 is apparently normal (Figure 3-3).

**DLG5 Is Expressed in the Postnatal Murine Brain**

Previous work shows that the hydrocephalus phenotype observed in *Dlg5<sup>−/−</sup>* mice is caused by disruption of adherens junctions and loss of cell polarity in ventricular epithelial cells, resulting in abnormal closure of the cerebral aqueduct (Nechiporuk et al., 2007). To investigate whether DLG5 functions during neural development, we first analyzed *Dlg5* expression in the brain using *in situ* hybridization. We observed strong expression of *Dlg5* mRNA in the ventricular
zone and cortical plate at embryonic day 14.5 (E14.5) (Figures 3-4A and B). By postnatal day 0 (P0), Dlg5 mRNA becomes widely expressed in various regions of the brain, including the cortex, striatum (Figures 3-4C and D), dorsal thalamus, and cerebellum (data not shown). Dlg5 mRNA expression levels in the cortex and hippocampus remains high at P10 (Figures 3-4E and F) and gradually decreases after P21 (data not shown).

To better define where DLG5 protein is localized, we performed subcellular fractionation of P21 brains to enrich for postsynaptic densities (PSDs), verifying the fidelity of our isolation by immunoblotting for the pre- and postsynaptic markers synaptophysin and PSD95, respectively. DLG5 is highly enriched in PSD fractions in wild type brains, and the mutant DLG5LP protein, though present at lower levels than wild type, retains its localization in the PSD fraction (Figure 3-5). These results show that DLG5 is a PSD component and that it is included in the PSD core protein-containing detergent-insoluble PSDIII fraction.

**DLG5 Regulates Dendritic Spine Density* in vivo**

Strong expression of Dlg5 in the cerebral cortex during early postnatal development, in combination with the localization of DLG5 in PSD fractions, raise the possibility that DLG5 may function in cortical neurons, perhaps by regulating dendritic spine formation and synaptogenesis. This idea is supported by the structural similarity between DLG5 and other MAGUK proteins that serve well-established roles in synapse formation and function (Kim and Sheng, 2004), and
also by interactions previously described in neuroepithelial cells between DLG5 and N-cadherin, β-catenin and syntaxin 4 (Nechiporuk et al., 2007)–known regulators of spine and synapse formation (Arikkath and Reichardt, 2008; Kennedy et al., 2010; Saglietti et al., 2007; Togashi et al., 2002). To determine if DLG5 regulates dendritic spine development in vivo, we used the Golgi labeling technique to analyze dendritic spine morphology in P21 cortical pyramidal neurons. Layer V pyramidal neurons in Dlg5<sup>LP/LP</sup> mice exhibit a 24.5%, 25% and 23% reduction in dendritic spine density along apical dendrites, side branches off of apical dendrites, and basal dendrites, respectively (Figures 3-6A, B, and C). This reduction in spine density is not restricted to specific dendritic regions, nor is there any obvious abnormality in spine morphology (data not shown). This decrease in dendritic spine density is already apparent at P14 (27.5%, 26.8% and 27.3% for apical dendrites, side branches and basal dendrites, respectively; Figures 3-7A, B, and C), suggesting that DLG5 is involved in the initial stages of dendritic spine formation rather than later elimination of excessive spines (Tran et al., 2009; Yuste and Bonhoeffer, 2004). Layer II/III pyramidal neurons in Dlg5<sup>LP/LP</sup> mice also show reductions in dendritic spine density of 20% and 15% in the apical dendrites and basal dendrites, respectively, but no significant change in spine density on the side branches of apical dendrites was observed at P21 (Figures 3-8A, B, and C). However, DLG5 apparently affects select neuronal populations since Dlg5<sup>LP/LP</sup> hippocampal dentate gyrus (DG) granule cells and CA1 pyramidal neurons show no change in spine density (Figure 3-9A, B, C, D, and E).
To address whether the \textit{Dlg5}^{LP} point mutation is indeed the genetic lesion underlying the dendritic spine density phenotype we observe in our \textit{Dlg5}^{LP/LP} mutant, we performed a complementation test by breeding \textit{Dlg5}^{LP/LP} mice to \textit{Dlg5}^{null/null} knockout mice (Nechiporuk et al., 2007). Layer V pyramidal neurons in \textit{Dlg5}^{LP/null} mice show a 30% decrease in dendritic spine density (Figures 3-10A, B, and C). The lack of complementation between \textit{Dlg5}^{LP} and \textit{Dlg5}^{null} alleles shows that the decrease in dendritic spine density is caused by the \textit{Dlg5}^{LP} mutation, and also supports \textit{Dlg5}^{LP/LP} being a null, or strong hypomorphic, allele of \textit{Dlg5}.

**DLG5 Functions Cell-autonomously to Regulate Dendritic Spine Development**

To investigate whether the reduction in dendritic spine density we observe in \textit{Dlg5}^{LP/LP} neurons is a secondary consequence of the enlarged cortical ventricles observed in this mutant, or other as yet uncharacterized defects associated with the \textit{Dlg5}^{LP/LP} mutant, we next examined dendritic spine density in cultured cortical pyramidal neurons. Cortical neurons were cultured from E13.5 embryos, when layer V pyramidal neurons are born, transfected with a DNA construct expressing GFP at 8 days \textit{in vitro} (DIV) to visualize dendritic spines, and then cultured for a total of 18 DIV. At E13.5, when cortical neurons are dissected from the mouse embryo and placed in culture, enlargement of the lateral ventricles is not observed in \textit{Dlg5} mutants (data not shown). Consistent with our \textit{in vivo} observations, \textit{Dlg5}^{LP/LP} pyramidal neurons \textit{in vitro} show a 25% reduction in spine
density (Figures 3-11A, B, and C). There is, however, no significant difference in spine morphology, length, or area (Figures 3-11D and E). These results strongly suggest that the $Dlg5^{LP/LP}$ dendritic spine phenotype we observe in vivo does not result from the progressive enlargement of the lateral ventricles that occurs in these mutants, or any other defects associated with $Dlg5^{LP}$. Furthermore, $Dlg5^{-/-}$ pyramidal neurons in vitro also show a 25% reduction in spine density (Figures 3-11F, G, and H), equivalent to that observed in $Dlg5^{LP/LP}$ neurons. This confirms that the $Dlg5^{LP}$ mutant is a null, or very strong loss-of-function, $Dlg5$ allele.

The enrichment of the DLG5 protein in PSD fractions raises the possibility that DLG5 functions cell-autonomously in postsynaptic neurons to regulate dendritic spine formation and synaptogenesis. To test this idea, we designed shRNA constructs that reduce DLG5 protein expression to undetectable levels in HEK293 cells by western blot (Figure 3-12A) and very low levels in cortical neurons by immunostaining (Figures 3-12B and C) that were transfected to produce robust levels of DLG5 protein. Wild type cortical neurons derived from E13.5 embryos were transfected with $Dlg5$ shRNA at 8 DIV and cultured for a total of 18–21 DIV. This construct also expresses GFP to allow for monitoring transfected neurons. shRNA knockdown of $Dlg5$ causes a 39% reduction in dendritic spine density (Figures 3-13A, B, and D). To eliminate the possibility of $Dlg5$ shRNA off-target effects, we designed a construct that expresses an shRNA silencing-resistant $Dlg5$ together with the $Dlg5$ shRNA (Figure 3-12A). Cortical neurons transfected with this rescue construct show wild type dendritic spine
densities (Figure 3-13C and D), confirming that the shRNA phenotype we observe is indeed caused by DLG5 loss-of-function (LOF).

To assess cell autonomous DLG5 requirements in vivo, we performed in utero electroporation of wild type E13.5 brains using our Dlg5 shRNA construct and a GFP-expressing control construct. At P21, Layer V cortical neurons electroporated with Dlg5 shRNA show a 25% reduction in dendritic spine density compared to neurons electroporated with the GFP control. (Figures 3-13E, F, and G). In cortical neuron cultures in vitro, and also in the electroporated brains in vivo, only a very small number of neurons receive the Dlg5 shRNA construct (fewer than 1% of neurons were transfected in culture, and ~100 neurons per cortical hemisphere were transfected by in utero electroporation), resulting in single, isolated, DLG5-negative neurons surrounded by wild type neurons and glia. These results demonstrate that DLG5 functions cell-autonomously in postsynaptic neurons to regulate dendritic spine development. Given that our shRNA acutely down-regulates DLG5, these results further suggest that the reduction in dendritic spine density observed in Dlg5LP/LP mutants is due to a lack of neuronal DLG5 function and does not result from progressive enlargement of lateral ventricles.

**Dlg5 Mutants Exhibit Decreased Excitatory Synapse Density and Altered Synaptic Properties**

Dendritic spines are sites of excitatory synaptic contact (Nimchinsky et al., 2002). To investigate whether there is a corresponding change in excitatory synapse
number in $\text{Dlg}5^{LP/LP}$ mice, we first analyzed synapse density in cultured cortical neurons by immunostaining with synaptic markers. Cortical neurons from E13.5 embryos were cultured for 21 DIV and then stained for the excitatory presynaptic marker vGlut1 and the postsynaptic marker PSD95. Co-localization of vGlut1 and PSD95 puncta was then determined to assess excitatory synapse number. Consistent with our dendritic spine analysis, we observe a 27% decrease in vGlut1/PSD95 double-positive puncta in dendrites of cultured $\text{Dlg}5^{LP/LP}$ neurons (Figures 3-14A, B, and C). In addition, we stained cultured cortical neurons for the inhibitory presynaptic marker VGAT and postsynaptic marker gephyrin. We observe no change in VGAT/gephyrin double-positive puncta in cultured $\text{Dlg}5^{LP/LP}$ cortical neurons (Figures 3-14D, E, and F), suggesting that DLG5 does not influence inhibitory synaptogenesis. To investigate the effects of the $\text{Dlg}5^{LP}$ mutation on spine morphology and synapse formation in vivo, we used transmission electron microscopy (TEM) to analyze $\text{Dlg}5^{LP/LP}$ cortical neurons at high resolution (Figures 3-15A, B, C, and D). In agreement with our in vitro observations, we observe a 25% decrease in synapse density in layer V $\text{Dlg}5^{LP/LP}$ cortical neurons (Figure 3-15E); however, excitatory synapse morphology is apparently normal (Figures 3-15F, G, and H). Quantification of spine area, PSD length and synaptic vesicle number per terminal shows that synapses in the $\text{Dlg}5^{LP/LP}$ cortex are indistinguishable from those observed in wild type. This is consistent with the normal appearance of dendritic spines in $\text{Dlg}5^{LP/LP}$ neurons that we observe using Golgi analysis in vivo, and also our GFP immunostaining of transfected mutant neurons in vitro. These results suggest that DLG5 is
involved primarily in initial synapse formation but not subsequent synaptic maturation.

To investigate the functional consequences of decreased excitatory synapse density in Dlg5 mutants, we performed whole cell patch-clamp recordings of Layer V cortical neurons in cortical slices at P21–P28. mEPSC analysis reveals a 36% reduction in mEPSC frequency in Dlg5LP/LP neurons (Figures 3-16A, B, C, and D). The altered cortical morphology at P21 and later in postnatal development due to enlarged cortical ventricles precluded paired-pulse recordings from Layer V cortical neurons. Nevertheless, we have shown that DLG5 functions cell-autonomously in postsynaptic neurons to regulate dendritic spine density (Figures 3-13), and Dlg5LP/LP neurons have normal presynaptic terminal morphology by TEM analysis (Figures 3-15F, G, and H). Therefore, the reduction of mEPSC frequency in Dlg5LP/LP neurons is most likely a result of decreased numbers of excitatory synapses, rather than a decrease in presynaptic release probability. There is a slight increase in mEPSC amplitude of ~10% in Dlg5LP/LP neurons, possibly due to homeostatic scaling (Figures 3-16E and F). These results reveal a significant reduction in excitatory synaptic transmission onto layer V cortical neurons in Dlg5LP/LP mice, consistent with a reduction in the number of excitatory synapses.

THE DLG5L1642P SH3 Domain Mutation Disrupts Interactions with the DLG5 GUK Domain and also Affects DLG5–β-catenin Binding
Although DLG5 protein levels in the Dlg5<sup>L/P</sup>LP mutant brain are reduced by ~50%, the reduction in Dlg5<sup>L/P</sup>LP dendritic spine density is comparable to that observed in Dlg5<sup>null/null</sup> mice (Figures 3-12F, G, and H) and Dlg5<sup>+/LP</sup> mice show normal dendritic spine density, suggesting that the L1642P mutation affects the normal function of DLG5. The L1642P point mutation lies within the DLG5 SH3 domain, which is also present in other MAGUK proteins, including PSD95 (Kim and Sheng, 2004). The PSD95 SH3 domain structure differs greatly from canonical SH3 domains, lacking the characteristic secondary structure required for interactions with proline-rich motifs (McGee et al., 2001). Instead, the PSD95 SH3 domain binds to its own GUK domain in an intramolecular fashion, and this interaction may be important for the regulated assembly of PSD95 oligomers (McGee and Bredt, 1999; McGee et al., 2001; Tavares et al., 2001), or the formation of protein conformations essential for interactions with other cell surface proteins and signaling molecules (Bhattacharyya et al., 2009; Colledge et al., 2000; Shin et al., 2000). In the crystal structure of a related MAGUK protein, human ZO-1 (Nomme et al., 2010), isoleucine562 in the ZO-1 SH3 domain, which is homologous to leucine1642 in DLG5, is positioned ~4.3A apart from tryptophan799 located in the GUK domain, and these two residues make contact within the hydrophobic core (Figure 3-17A). The close proximity of this amino acid pair suggests that leucine1642 in DLG5 SH3 domain is important for the interaction between SH3 and GUK domains of DLG5. The conformational rigidity introduced by the proline residue that replaces leucine in DLG5<sup>L/P</sup> mutant could destabilize this interaction, given the position of leucine1642 at the binding
interface between SH3 and GUK domains. In addition, the association between the PDZ-ligand JAM-A and PDZ3 domain of ZO-1 is dependent upon the presence of the ZO-1 SH3 domain (Nomme et al., 2010). Therefore, amino acid changes within the hydrophobic β-sheets of the SH3 domain, such as DLG5LP, have the potential to disrupt interactions with the adjacent PDZ3 domain and thereby alter its function. To begin to address how DLG5LP affects DLG5 domain interactions, we asked whether the DLG5 SH3 domain binds to the DLG5 GUK domain, and whether the L1642P mutation disrupts this interaction. We co-transfected HEK293T cells with a construct expressing HA-tagged WT, or L1642P, SH3 domains, and a construct expressing MYC-tagged GUK. Immunoprecipitation of cell lysates derived from these HEK293T cells with anti-HA, and detection of MYC-GUK in the anti-HA immunoprecipitates, reveals that the DLG5 SH3 domain indeed does bind to the DLG5 GUK domain. Interestingly, the binding of the DLG5L1642P SH3 domain to the DLG5 GUK domain is greatly reduced by the L1642P amino acid substitution (Figure 3-17B).

How might the disruption of the SH3–GUK domain interaction affect DLG5 function? A known DLG5 binding partner is β-catenin, and in epithelial cells, DLG5 regulates targeting and stabilization of β-catenin–N-cadherin complexes at the plasma membrane (Nechiporuk et al., 2007). Since the Dlg5LP point mutant exhibits enlarged ventricles identical to those observed in the Dlg5 null mutant mouse (Nechiporuk et al., 2007), DLG5LP may affect β-catenin binding to DLG5. To address this possibility, forebrain lysates from Dlg5+/+ and Dlg5LP/LP mice were immunoprecipitated with an antibody directed against β-catenin. The amount of
co-immunoprecipitated DLG5 protein was quantified and normalized to the amount of input DLG5. We observe a 45% decrease in the fraction of DLG5 protein that co-immunoprecipitated with β-catenin in *Dlg5*<sup>LP/LP</sup> forebrains, as compared to wild type (Figures 3-18A and B). Interestingly, the association of DLG5 with β-catenin in a heterologous cell system is mediated by the binding of β-catenin to the DLG5 N-terminal CARD, DUF, coiled-coil domains and the 3rd and 4th DLG5 PDZ domains, not the DLG5 SH3 or GUK domains (Nechiporuk et al., 2007). Several studies suggest that the interaction between MAGUK proteins and their binding partners is modulated by intramolecular interactions between different MAGUK protein domains (Brenman et al., 1998; Nomme et al., 2011). Therefore, it is possible that disruption of the DLG5 SH3-GUK interaction affects the association of adjacent PDZ domains to their binding partners (Nomme et al., 2010). Taken together, these observations suggest that DLG5 SH3-GUK interactions are important to create a binding platform for β-catenin and perhaps other synaptic proteins.

**Neuronal Subcellular Localization of β-catenin and Cell Surface Localization of N-cadherin are Compromised in *Dlg5*<sup>LP/LP</sup> Mice**

To determine how decreased β-catenin association with DLG5 affects cortical neurons, we cultured cortical neurons from E13.5 *Dlg5<sup>+/+</sup>* and *Dlg5*<sup>LP/LP</sup> mice for 21 DIV and then immunostained for β-catenin. We observed a punctate distribution of β-catenin along the dendrites in both *Dlg5<sup>+/+</sup>* and *Dlg5*<sup>LP/LP</sup> neurons; however, we found a ~30% reduction in the number of β-catenin puncta in
*Dlg5*<sup>LP/LP</sup> dendrites (Figures 3-19A and B). Cultured cortical neurons were also immunostained for vGlut1 to illuminate sites of presynaptic contact. Assessment of vGlut1/β-catenin double-positive puncta was used to define synaptically localized β-catenin, and we observed a ~30% reduction of vGlut1/β-catenin double-labeled puncta in *Dlg5*<sup>LP/LP</sup> neurons (Figures 3-19A and B). Therefore, reduced protein-protein interactions resulting from the DLG5<sup>LP/LP</sup> amino acid substitution affect the targeting, or stabilization, of β-catenin at sites of excitatory synaptic contact in cortical neurons. These results strongly suggest that the mis-localization of β-catenin is the underlying cause of the dendritic spine and synaptogenesis defects we observe in *Dlg5*<sup>LP/LP</sup> neurons.

Catenin-cadherin protein complexes are essential components of adherens junctions and synapses (Arikkath and Reichardt, 2008). β-catenin is driven into dendritic spines upon depolarization (Murase et al., 2002), and it is required for regulating synaptic strength and spine morphology in hippocampal neurons *in vitro* (Okuda et al., 2007). N-cadherin regulates dendritic spine density (Saglietti et al., 2007), morphology (Togashi et al., 2002), and stabilization following long-term potentiation *in vitro* in hippocampal slice cultures (Bozdagi et al., 2010; Mendez et al., 2010). In epithelial cells, DLG5 serves to deliver β-catenin–N-cadherin complexes to the plasma membrane (Nechiporuk et al., 2007). Given that the *Dlg5*<sup>LP/LP</sup> mutation affects binding of DLG5 to β-catenin, and also that β-catenin is localized to synapses in cortical neurons, this interaction could serve to direct the surface localization of N-cadherin in cortical neurons. Therefore, we used a surface biotinylation assay to assess N-cadherin
cell surface levels in cultured cortical neurons. Though the total amount of N-cadherin protein remains unchanged in \textit{Dlg5}^{LP/LP} cortical neurons, we observe a dramatic decrease in N-cadherin cell surface levels (Figure 3-20A). Quantification of surface N-cadherin, normalized to total N-cadherin, reveals an ~80\% decrease in the fraction of N-cadherin protein targeted to the cell surface in \textit{Dlg5}^{LP/LP} cortical neurons (Figure 3-20B). DLG5 does not affect the surface localization of synaptic proteins non-specifically, since surface levels of AMPA receptor GluR1 subunit and neuropilin-2 (Nrp2) remain unchanged in \textit{Dlg5}^{LP/LP} cortical neurons (Figure 3-20C). Taken together, these results show that the DLG5^{L1642P} mutation markedly disrupts cell surface localization of N-cadherin, presumably resulting from the compromised interaction between DLG5 and β-catenin that, in turn, leads to a decrease in the delivery, or stability, of β-catenin–N-cadherin complexes at the cortical neuron cell surface. Interestingly, surface N-cadherin levels remain unchanged in \textit{Dlg5}^{LP/LP} hippocampal neurons (Figure 3-20D). Therefore, regulation of N-cadherin localization by DLG5 is specific to select neuronal populations. Importantly, we do not observe any change in dendritic spine density in DG granule cells or CA1 pyramidal neurons in \textit{Dlg5}^{LP/LP} mice. These results suggest that DLG5 regulates dendritic spine density in select neuronal populations by facilitating the surface localization of N-cadherin in these neurons.

\textbf{DLG5 and N-Cadherin Functionally Interact \textit{in vitro} and \textit{in vivo}}
We find that DLG5 is required for dendritic spine formation and cell surface localization of N-cadherin. To ask if decreased cell surface N-cadherin is responsible for the reduction in dendritic spine density in Dlg5 mutants, we performed a series of experiments to investigate functional interactions between N-cadherin and DLG5. Previous studies on the role of N-cadherin in spine development and synaptic function have been performed in vitro using hippocampal neurons (Bozdagi et al., 2010; Mendez et al., 2010; Saglietti et al., 2007; Togashi et al., 2002). In contrast, little is known about how N-cadherin affects these developmental processes in cortical neurons. To address this issue, we used a previously characterized shRNA (Saglietti et al., 2007) to knockdown N-cadherin in cortical neurons in vitro. Cultured cortical neurons were transfected with N-cadherin shRNA at DIV 8 and cultured for a total of 21 DIV. We observed a 45% decrease in dendritic spine density following N-cadherin knockdown (Figures 3-21A, B, C, and D), demonstrating that N-cadherin is indeed required for spine formation in cortical neurons. Interestingly, the degree of spine density reduction is comparable to what we observe in Dlg5 mutants.

If the decrease in spine density in Dlg5<sup>LP/LP</sup> neurons is caused by reduction of cell surface N-cadherin, this phenotype should be suppressed by N-cadherin gain of function. Therefore, we performed Dlg5 spine phenotype rescue experiments in cultured Dlg5<sup>LP/LP</sup> neurons by overexpressing N-cadherin. Cortical neurons cultured from E13.5 Dlg5<sup>+/+</sup> and Dlg5<sup>LP/LP</sup> mice were transfected with a construct expressing GFP-tagged N-cadherin at 8 DIV and then cultured
for a total of 21 DIV. While overexpression of N-cadherin had no effect on spine density in $Dlg5^{+/+}$ neurons, it restored spine density in $Dlg5^{LP/LP}$ neurons to wild type levels (Figures 3-22A, B, C, and D). These results demonstrate that the reduction in spine density in $Dlg5^{LP/LP}$ neurons is ameliorated by restoring surface N-cadherin levels through N-cadherin overexpression.

Finally, we asked whether $Dlg5$ and $N$-cadherin genetically interact in vivo. $N$-cad$^{-/-}$ mice die at E10 due to multiple embryonic abnormalities, including severe cardiovascular defects (Radice et al., 1997) that preclude assessment of spine development in $N$-cad$^{-/-}$ mice in vivo. Therefore, we addressed this question by performing transheterozygous genetic analysis. To obtain $N$-cad$^{+/+}$ mice we first bred $N$-cad$^{floxflo}$ mice (Kostetskii et al., 2005) to Sox2-Cre mice, inducing germline deletion of $N$-cad. $N$-cad$^{+/+}$ mice were then bred to $Dlg5^{LP/+}$ mice to obtain $Dlg5^{LP/+}; N$-cad$^{+/+}$ mice. Layer V cortical pyramidal neurons in P21 mice were then examined by Golgi labeling. Though dendritic spine density of $Dlg5^{LP/+}$ or $N$-cad$^{+/+}$ cortical pyramidal neurons does not differ from wild type, there is a 20% reduction in dendritic spine density in $Dlg5^{LP/+}; N$-cad$^{+/+}$ transheterozygous neurons (Figures 3-23A, B, C, D, and E). Taken together, these results strongly suggest that decreased cell-surface N-cadherin is the underlying cause of the spine density reduction observed in $Dlg5^{LP/LP}$ neurons.

3.3 Discussion

$DLG5$ Regulates Dendritic Spine Development
The DLG family of MAGUK proteins includes intracellular scaffolding components composed of multiple protein interaction domains that facilitate the assembly of large molecular complexes at synapses. One of the best-studied DLG family members, PSD95, controls the localization and trafficking of glutamate receptors through direct interactions with NMDA receptors and also indirect interactions with AMPA receptors that are mediated by transmembrane AMPA receptor regulatory proteins (TARPs). PSD95 also interacts with a myriad of signaling proteins to regulate synaptic properties and plasticity (Feng and Zhang, 2009; Kim and Sheng, 2004). Consistent with a role in regulating glutamate receptor trafficking and synaptic plasticity, PSD95 mutant mice show facilitated LTP induction, impaired LTD, and impaired spatial learning (Carlisle et al., 2008; Cuthbert et al., 2007; Elias et al., 2006; Migaud et al., 1998). In contrast, the role of PSD95 in regulating dendritic spine and synapse densities in vivo remains enigmatic (Beique et al., 2006; El-Husseini et al., 2000; Migaud et al., 1998; Vickers et al., 2006). It is also not clear whether other members of the DLG family, including PSD93, SAP97, and SAP102 regulate spine formation or synaptogenesis in vivo.

DLG5 is unique among DLG family members owing to its large N-terminus, which contains additional protein domains. DLG5 binds to β-catenin through its N-terminal CARD, DUF, coiled-coil domains, and 3rd and 4th PDZ domains, and it regulates the localization of catenin-cadherin complexes at the adherens junction of epithelial cells (Nechiporuk et al., 2007). Here, we show that DLG5 is important for the localization of β-catenin–N-cadherin complexes to
the cell surface at sites of excitatory synaptic contact. We demonstrate that DLG5 is an important regulator of dendritic spine and excitatory synapse density and function in cortical pyramidal neurons. These findings, therefore, identify a unique mechanism by which a MAGUK protein affects spine formation and synaptogenesis, involving the regulation of trans-synaptic adhesion molecule subcellular localization.

**N-cadherin Regulation of Dendritic Spine Formation**

Previous studies in cultured hippocampal neurons have identified β-catenin and N-cadherin as important regulators of spine morphogenesis and synaptogenesis (Okuda et al., 2007; Saglietti et al., 2007; Togashi et al., 2002). However, the mechanisms by which N-cadherin promotes spine formation are not well understood. Here, we show that DLG5 plays a critical role in promoting the cell surface localization of N-cadherin in neurons. A key early event in dendritic spine formation and synaptogenesis is the stabilization of trans-synaptic contacts mediated by cell surface proteins. The availability of cell adhesion molecules at the neuronal cell-surface could therefore determine whether trans-synaptic contacts can successfully develop into synapses. In addition to providing trans-synaptic adhesion, N-cadherin may also regulate cytoskeletal dynamics in dendritic spines through multiple signaling pathways, including signaling by members of the Rho GTPase family and PI3 kinase (Arikkath and Reichardt, 2008; Stepniak et al., 2009). Future work will determine whether these, or other, signaling pathways are affected by loss of DLG5.
Recently, the classical cadherin Cad-9, was shown to regulate the formation of select hippocampal synapses (Williams et al., 2011). In addition, cadherin-6 mediates retinal ganglion cell targeting of certain retinorecipient targets in the mouse brain (Osterhout et al., 2011). These findings suggest different cadherins mediate axon targeting and synaptogenesis in distinct subsets of neurons. N-cadherin is widely expressed in the CNS. However, lack of neuronal N-cadherin does not result in a complete loss of dendritic spines; there is a 40% decrease in spine density in both hippocampal (Saglietti et al., 2007) and cortical neurons (this study) *in vitro*. These findings may indicate that certain synapses are more dependent on N-cadherin function than others, a difference that could depend upon distinct presynaptic input.

**Polarity in Epithelial and Neural Cells**

DLG5 is required for correct cell polarity in epithelial cells (Nechiporuk et al., 2007). The precise mechanism by which DLG5 regulates cell polarity remains unknown, but the targeted delivery of N-cadherin–catenin complexes to correct plasma membrane domains is crucial for establishing apical-basal polarity in epithelial cells. In both *Dlg5<sup>LP/LP</sup>* and *Dlg5<sup>−/−</sup>* mice, however, neuronal polarity is apparently normal, suggesting that cell polarity in mature neurons is determined by distinct mechanisms. Previous studies demonstrate that TGF-beta signaling (Yi et al., 2010) and the LKB1-SAD kinase pathway (Barnes et al., 2007; Shelly et al., 2007) promotes axon specification *in vivo*, whereas localized activation of cAMP and cGMP in undifferentiated neurites can promote axon and dendrite
formation, respectively, in vitro (Shelly et al., 2010). We show here that DLG5 regulation of N-cadherin localization in mature neurons is important for dendritic spine formation and synaptogenesis, but apparently not for the establishment of neuronal polarity.

**Role of DLG5 SH3-GUK Domain Interactions**

Using a heterologous in vitro system, we found that the Dlg5LP mutant protein disrupts the interaction between DLG5 SH3 and GUK domains. Previous work on PSD95 indicates that the SH3 domain in MAGUK proteins lacks the characteristic secondary structure required for binding to proline-rich motifs (McGee et al., 2001). Instead, the PSD95 SH3 domain binds to its own GUK domain. It has been proposed that SH3-GUK domain interaction is critical for the formation of PSD95 oligomers important for clustering and stabilization of channels, cell-adhesion molecules, and other cell surface proteins. (Bhattacharyya et al., 2009; Colledge et al., 2000; Kim and Sheng, 2004; McGee and Bredt, 1999; Shin et al., 2000; Tavares et al., 2001). Previously, *Drosophila Dlg* mutations that disrupt SH3-GUK interactions were shown to affect septate junction formation and cell polarity (Woods et al., 1996). Here we provide additional observations that suggest SH3-GUK domain interactions are important for neuronal MAGUK protein function in vivo. We show that a single amino acid substitution in the DLG5 SH3 domain disrupts DLG5 protein functions that are required for dendritic spine formation, both in vitro and in vivo. At the molecular level, the $DLG5^{LP}$ mutant shows decreased binding to $\beta$-catenin and results in
dramatically reduced neuronal cell surface localization of N-cadherin. We propose that the DLG5 SH3-GUK domain interaction modulates binding of DLG5 to β-catenin, and possibly other as yet unidentified binding partners, thereby affecting DLG5 protein function during dendritic spine formation and synaptogenesis.

Taken together, our observations demonstrate that DLG5 is critical for dendritic spine formation, synaptogenesis, and synaptic transmission in cortical neurons. At the molecular level, DLG5 affects synaptogenesis by regulating the subcellular localization of β-catenin and N-cadherin. We provide in vivo evidence for the importance of MAGUK proteins in dendritic spine formation, and we have identified a critical molecular mechanism that mediates N-cadherin cell surface localization during synaptogenesis. The availability of cell adhesion molecules such as N-cadherin at the cell surface is a critical determinant of whether trans-synaptic contacts are stabilized and subsequently develop into synapses. The identification of molecules that regulate the subcellular localization of other cell surface proteins during synaptogenesis will further advance our understanding of the mechanisms that orchestrate the establishment of neural connectivity.

3.4 Materials and Methods

Mutagenesis and screening

ENU mutagenesis was performed as described previously (Merte et al., 2010a; Merte et al., 2010b). Briefly, C57BL/6 (B6) male mice were injected with three doses of 100mg/kg body weight ENU to induce random mutations throughout the
genome. Using a three-generation breeding scheme (Figure S1A), we outbred the mutations in C3H/Hej (C3H) mice and analyzed G3 mice for developmental abnormalities. Freshly dissected P5 mouse brains were fixed in 4% paraformaldehyde (Sigma) overnight and equilibrated in 30% sucrose. Sagittal sections (150 µm) were cut on a Leica sliding microtome. Sections were stained with anti-neurofilament antibody as previously described (Giger et al., 2000).

Mapping

Genomic DNA from mutant lines was analyzed for a panel of 768 single nucleotide polymorphisms (SNP), performed by the Partners Healthcare Center for Genetics and Genomics. The genetic lesion responsible for hydrocephalus was mapped to a 7.3Mb region on chromosome 14 between rs6175633 and rs13482117 using standard linkage analysis of SNPs between C57Bl/6 and C3H/Hej mice. Additional polymorphisms within this region, D14Mit207, rs3666933, and rs30102223 were analyzed, and this allowed us to map the genetic lesion to a 3.1Mb region on chromosome 14 between D14Mit207 and rs30102223, containing 15 protein-coding genes. Dlg5 was identified as a candidate gene based on phenotypic similarity of our mutant to previously described Dlg5−/− mice (Nechiporuk et al., 2007). We sequenced all 32 exons of Dlg5 and identified a T→C transition in exon26 as the genetic mutation responsible for hydrocephalus in our mutant line. D14Mit207 was analyzed by genotyping with primers TCCAACTAGTCCCCCTCTACTT and CTGTGACTATCTGTACAAGACCTGC, which produces a PCR product of 126bp
in B6 and 104bp in C3H mice. rs3666933 was analyzed by genotyping with primers TTGTTCAGCACAGCCCCAGC and TCATCCCTACATCCCTGTCC, followed by digestion with DpnI; PCR product from C3H, but not B6 mice, can be cut with DpnI. rs30102223 was analyzed by genotyping with primers CATGACTCTCAAGGGATCCACC and ATCCAAGGACAGCTTAAGATGC, followed by digestion with SphI; PCR product from C3H, but not B6 mice, can be cut with SphI.

**Animals**

Generation of *Dlg5<sup>−/−</sup>* mice (Nechiporuk et al., 2007) and *N-Cad<sup>flox/flox</sup>* mice (Kostetskii et al., 2005) has been previously described.

**Genotyping**

The T→C transition underlying the *Dlg5<sup>Lp</sup>* allele creates a restriction enzyme-resistant polymorphism. This mutant line is genotyped by amplification of a ~300bp fragment with primers GGCGAGTGTCACCCTGTGAG and GCCTTTCTTCCTGATATCTGGAG, followed by digestion with PvuII; only PCR product from wild type allele can be cut with PvuII. Genotyping for *Dlg5<sup>−/−</sup>* mice and *N-cad<sup>flox/flox</sup>* mice were performed as previously described (Kostetskii et al., 2005; Nechiporuk et al., 2007).

**Plasmids**
V5-DLG5 expression construct was previously described (Nechiporuk et al., 2007). This construct was modified by inserting gene for mCherry under control of CMV promoter to generate V5-DLG5mCherry. To generate V5-DLG5(L1642P)mCherry we used overlapping PCR with primers A:

GTGAAGGTGCAAGGGAAGCTAGAGC, B:

CTGGGCATTCTCATCCgGCTGCCAGGCATC, C:

GATGGCCTGGCAGCcGGATGAGAATGCCCAG and D:

TGTACTCCTGTGATCGATCTTCTGTGC.

shRNA targeting DLG5 and silencing-resistant DLG5 were constructed using LEMPRA based system as previously described (Zhou et al., 2006). shRNA against DLG5 was cloned into pLLX vector using primers tAGACAGAAGTTGTGGGAATT, ttcaagagaAATTCCACAACTTCTGTCTTTtttttgaac, tgcagttccaaaaaaAGACAGAAGTTGTGGGAATT and tctcttgaaAATTCCACAACTTCTGTCTTa. Silencing-resistant form DLG5 gene was generated by overlapping PCR using primers A:

ACGCGACCAGGTTCATCTCCGAGCTG, B:

CTCTCAATTTCtACgACcTCgGTCTCCAC, C:

GTGGGAGACGCgGTCATCTCCGAGCTG and D:

CATGCCGTAGCATGCCCTGGGTGA and cloned as SexAI/NheI fragment into DLG5 in pLEMPRA vector. Small letters indicate nucleotides that were changed in DLG5 sequence to generate silencing resistant form. Constructs expressing 3HA-SH3 and 6MYC-GUK of DLG5 were generated by PCR using primers for SH3 domain (amino acids 1595-1664)
atggccggcctGGAGACAGCTTCTACATCAG (forward) and
atggcgcgccCTAGAACTCTTGGTCCATCACGTATTTG (reverse) and primers for
GUK domain (aminoacids 1722-1910)
atggccggcctGACTCAGTGAGCCTGGCCTATCAG (forward) and
atggcgcgccCTATTGTTCTTGACTGACCATGG (reverse). Resulting PCR
products were cloned 3’ to 3 HA and 6 MYC epitopes in pCAGGS based vectors.
All constructs generated by PCR were verified with sequencing.

**In Situ Hybridization**

*In situ* hybridization was performed as previously described (Giger et al., 2000).
Generation of riboprobe for Dlg5 has been described elsewhere (Nechiporuk et al., 2007).

**Immunohistochemistry**

Immunostaining of tissue sections were performed as previously described
(Giger et al., 2000). Primary antibodies used were mouse anti-neurofilament
(2H3; 1:5000; Developmental Studies Hybridoma Bank), rabbit anti-Brn2 (1:500;
Santa Cruz), rabbit anti-Cux1 (1:500; Santa Cruz), rat anti-Ctip2 (1:500; Abcam)
and rabbit anti-Tbr1 (1:500; Santa Cruz). Species-specific secondary antibodies
(Invitrogen) were used at 1:500. Immunostaining of cultured neurons were
performed as previously described (Tran et al., 2009). Primary antibodies used
were chicken anti-GFP (1:1000; AVES), guinea pig anti-vGlut1 (1:1000;
Millipore), mouse anti-PSD95 (1:500; Millipore), rabbit anti-VGAT (1:1000;
Synaptic Systems), mouse anti-gephyrin (1:1000; Synaptic Systems), mouse
anti-β-catenin (1:100; Sigma), mouse anti-V5 (1:500; Invitrogen), and rabbit anti-
DsRed (1:1000; Living Colors). Species-specific secondary antibodies
(Invitrogen) were used at 1:1000.

**Golgi analysis**

Freshly dissected P14 and P21 mouse brains were incubated in Golgi solution
A+B (FD Rapid GolgiStain Kit, FD NeuroTechnologies) for 8 days. After
incubation, all brains were washed thoroughly with Solution C for 2-4 days at
room temperature, and embedded in OCT embedding medium (Tissue-Tek).
Coronal sections (100 µm) through the somatosensory cortex and hippocampus
were cut with a Leica CM3050 cryostat and mounted on 3% gelatin coated
slides. Staining procedures were followed as described (FD
NeuroTechnologies), and slides were dehydrated in ethanol, cleared in xylene,
and mounted with Permount (Fisher Scientific) for microscopy. Only Layer II-III
and V pyramidal neurons from the somatosensory cortex, hippocampal pyramidal
neurons from dorso-medial CA1, and granule cells from dorso-medial dentate
gyrus were included in our analyses.

**Primary neuronal cultures**

The day of plug was designated E0.5 for all timed-pregnancies. E13.5 cortices
were dissected in ice cold L-15 Leibovitz Medium (Sigma) and incubated in
trypsin-EDTA (0.05%; Gibco) at 37° C for 15 minutes. The tissues were washed
once at room temperature in Ca\(^{2+}\) - and Mg\(^{2+}\) - free Hank’s balanced salt solution (HBSS; Gibco) and dissociated with a fire-polished glass Pasteur pipette in HBSS containing 0.025% DNAse I. Dissociated cells were plated on poly-D-lysine (Sigma) coated 12mm coverslips at a density of 2\(\times\)10\(^5\) cells per coverslip and grown in 24-well plates in Neurobasal medium (Gibco) containing 2% B27 supplement (Gibco), 50 U/ml penicillin, 50 mg/ml streptomycin (Gibco), and 2 mM glutamax (Gibco) at 37° C, with medium changes every other day.

Transfection was performed on DIV 8, with 1µg DNA and 2µl Lipofectamine (Invitrogen) as described in manufacturer’s manual (Invitrogen). Following transfection, culture medium was switched to the aforementioned medium plus 2.5% FBS (Gibco), and cultured for another 10-13 DIV, with medium changes every other day.

**In Utero Electroporation**

*In utero* electroporation was performed as previously described (Tran et al., 2009). E13.5 embryos from timed-pregnant CD1 female mice were injected using pLLX (control group) and DLG5 shRNA and analyzed at P21. Dendritic spines of layer V somatosensory cortex were visualized using immunohistochemistry with anti-GFP antibody (Aves) detecting eEGFP encoded by constructs used for electroporation.

**Transmission electron microscopy**
P21 mice were fixed by transcardial perfusion with 3.0% formaldehyde/1.5% glutaraldehyde in 0.1 M Na⁺-cacodylate, 3 mM Ca²⁺ and 2.5% sucrose, at pH 7.4. Brains were dissected and the somatosensory cortex was trimmed and fixed overnight in the aforementioned fixative. The tissue was washed with 0.1 M Na⁺-cacodylate/2.5% sucrose, reduced with 2% OsO₄, then dehydrated in ethanol, infiltrated and flat embedded in EPON. Semi-thin and ultra-thin sections were collected using a Leica Ultracut microtome and analyzed with a Hitachi 7600 TEM. Only identified synapses on dendritic spines of layer V pyramidal neurons were included in these analyses.

**Electrophysiology**

Mutant mice and their wild-type littermates aged 3–4 weeks were anaesthetized by isoflurane inhalation and decapitated. Brains were quickly dissected in ice-cold buffer containing 212.7 mM sucrose, 10 mM glucose, 2.6 mM KCl, 1.23 mM NaH₂PO₄, 26 mM NaHCO₃, 0.5 mM CaCl₂ and 5 mM MgCl₂. Brains were vibratome-sectioned in the same solution at 300 µm and transferred to normal ACSF composed of 124 mM NaCl, 5 mM KCl, 1.23 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 2 mM CaCl₂ and 1 mM MgCl₂. Slices were recovered at 30°C for 1 h and then maintained at room temperature (22–25°C). Neurons were targeted for whole-cell patch-clamp recording with borosilicate glass electrodes having a resistance of 3–6 MΩ. The electrode internal solution was composed of 130 mM caesium methanesulphonate, 10 mM HEPES, 0.5 mM EGTA, 8 mM CsCl, 5 mM TEA-Cl, 1 mM QX-314, 10 mM Na phosphocreatine,
0.5 mM Na-GTP and 4 mM Na-ATP. Cortical pyramidal neurons were selected from layer V of the posterior medial barrel subfield of primary somatosensory cortex. For AMPA receptor-mediated miniature EPSCs, external solution was supplemented with the following: 1 µM tetrodotoxin, 50 µM d,l-APV (2-amino-5-phosphonovalerate) and 100 µM picrotoxin. Data were acquired with a Multiclamp 700A and Clampex 8 program (Molecular Devices) at 10 kHz. Prior to mEPSC detection and analysis, current traces were low-pass filtered at 1 kHz. mEPSCs were detected and analyzed using Mini Analysis (Synaptosoft) or Clampfit 10 program (Molecular Devices). Kinetic measurements were performed on scaled, mean EPSC traces using a monoexponential decay function. Rise times correspond to 10–90% of peak amplitude. A total of 25 wild-type neurons and 25 mutant neurons were recorded from layer V; n = 8 animals for wild type and n = 6 animals for mutant animals.

**Subcellular fractionation**

Freshly dissected mouse brains were homogenized in ice-cold 4 mM HEPES pH 7.4, 0.32 M sucrose, and protease inhibitors (Roche). Brain lysate was centrifuged at 2000 g for 10 min to give S1 (supernatant) and P1 (pellet) fractions. The S1 fraction was centrifuged at 37,000 g for 30 min to give S2 (supernatant) and P2 (pellet) fractions. The P2 pellet was resuspended in 4 mM HEPES and centrifuged at 82,500 g for 2 hours in a sucrose gradient consisting of 4 mM HEPES plus 0.85 M, 1.0 M, and 1.2 M sucrose. The synaptosomal fraction was harvested at the interface between the 1.2 M and 1.0 M part of the
gradient, centrifuged at 150,000 g for 30 min, and resuspended in 80 mM Tris-HCl pH 7.8. To isolate PSD fractions, synaptosomes were incubated with 40 mM Tris-HCl pH 8.0, 0.5% Triton-X for 15 min on ice, then centrifuged at 32,000 g for 20 min. The pellet was resuspended in 40 mM Tris-HCl pH 8.0 to give PSD I. The PSD I fraction was incubated again with 40 mM Tris-HCl pH 8.0, 0.5% Triton-X for 15 min on ice and centrifuged at 201,800 g for 1 hour. The pellet was resuspended in 40 mM Tris-HCl pH 8.0 with 0.3% SDS to give PSD II fraction. To isolate PSD III fraction, PSD I was incubated with 40 mM Tris-HCl pH 8.0, 3% sarkosyl for 15 min and centrifuged at 201,800 g for 1 hour. The pellet was resuspended in 40 mM Tris-HCl pH 8.0 to give PSD III. Protein concentrations were measured by BCA assay (Pierce) and mixed in Laemmlli’s sample buffer. Western blot analysis was performed on 5 µg of each fraction using standard methods.

Co-immunoprecipitation
Freshly dissected mouse brains were homogenized in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 20% SDS, 50 mM Tris-HCl pH 8.0) with protease inhibitor (Roche) on ice for 30 min. Co-immunoprecipitation (co-IP) was performed using standard methods with mouse anti-β-catenin antibody (4 µg, Sigma) and protein A/G agarose beads (Pierce). For in vitro co-IP, HEK 293T cells were transfected using Lipofectamine (Invitrogen) according to manufacturer’s manual. Cells were lysed in RIPA lysis buffer with protease inhibitor (Roche) on ice for 30 min. Co-IP was performed using standard
methods with anti-HA antibody-conjugated beads (Roche). Beads were washed three times with RIPA lysis buffer and mixed with Laemmli’s sample buffer. Western blot analysis was performed using standard methods.

**Surface protein biotinylation assay**

Cortical neurons and hippocampal neurons were cultured for 21 DIV, and then incubated with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS for 30 min on ice to label cell surface proteins. Unbound biotin was then quenched with 50 mM glycine in PBS. Cells were lysed in RIPA lysis buffer, and immunoprecipitation of surface protein was performed using standard methods with streptavidin agarose beads (Pierce).

**Western blot**

Western blot was performed using standard methods. Primary antibodies used were mouse anti-PSD95 (1:2000; Millipore), rabbit anti-synaptophysin (1:1000; Santa Cruz), mouse anti-β-catenin (1:1000; Sigma), mouse anti-HA (12ca5; 1:1000; Santa Cruz), mouse anti-myc (9e10; 1:2000; Sigma), rabbit anti-GFP (1:1000; Santa Cruz), mouse anti-actin (1:5000; Millipore), mouse anti-N-cadherin (1:1000; Invitrogen), mouse anti-GluR1 (1:2000; gift from Rick Huganir lab), and rabbit anti-neuropilin-2 (1:1000; Cell Signaling). Generation of rabbit anti-Dlg5 antibody has been described previously (Nechiporuk et al., 2007). Species-specific secondary antibody was used at 1:5000 (Jackson).
3.5 Acknowledgements

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3.6 References:


interacts with Neurexin1 and regulates excitatory synapse formation. Neuron 64, 799-806.


binding to the third PDZ domain of the scaffolding protein ZO-1. The Journal of biological chemistry 286, 43352-43360.


control spine distribution and morphogenesis in the postnatal CNS. Nature 462, 1065-1069.


Figure 3-1. A Forward Genetic Screen for Recessive Mutations Identifies Mutant Line With Enlarged Ventricles

(A) Breeding scheme for a forward genetic screen to identify recessive mutations affecting cortical neuronal morphology.

(B-C) Neurofilament immunostaining of postnatal day 5 (P5) coronal brain sections of control (A) and mutant (B) littermates from line #3007 showing enlarged lateral ventricles and an abnormal corpus callosum.

Scale bar in (B) = 1 mm for (B) and (C).
Figure 3-2. Mapping of a Novel Dlg5 Allele

(A) Schematic diagram of the Dlg5 locus highlighting exon 26 (red), in which single base substitution was identified in the Dlg5 mutant obtained in our genetic screen. Shown in black are coding exons from the largest predicted Dlg5 transcript ENSMUST00000090398 (Ensembl).

(B) Schematic diagram of the DLG5 protein. The L1642P substitution is located within the DLG5 SH3 domain.
(C) Primary DNA sequence data highlighting the T5395C mutation in DLG5 exon 26, resulting in the leucine\textsuperscript{1642} to proline mutation (Dlg5\textsuperscript{LP}).

(D) Western-blot analysis of DLG5 protein levels in total brain lysates prepared from \textit{Dlg5}\textsuperscript{+/+} and \textit{Dlg5}\textsuperscript{LP/LP} littermates, revealing reduced levels of DLG5 protein in \textit{Dlg5}\textsuperscript{LP/LP} brain lysates.

(E) Quantification of DLG5 protein levels from the total brain lysates prepared from 6 pairs of \textit{Dlg5}\textsuperscript{+/+} and \textit{Dlg5}\textsuperscript{LP/LP} littermates, normalized to \textit{Dlg5}\textsuperscript{+/+} protein levels: 1±0.18, \textit{Dlg5}\textsuperscript{LP/LP}: 0.57±0.025. Error bars represent standard deviation. **p<0.01 by two-tailed student t test.
Figure 3-3. Cortical Lamination is Normal in *Dlg5*<sup>LP/LP</sup> Mice

(A-F) P5 brain sections from *Dlg5*<sup>+/+</sup> (A, C, and E) and *Dlg5*<sup>LP/LP</sup> (B, D, and F) mice were stained for Brn2 to label cortical layers II/III and V (A and B), Cux1 to label layer II/III and Ctip2 to label layer V (C and D), and Tbr1 to label layers II/III and VI (E and F). *Dlg5*<sup>LP/LP</sup> mice exhibit normal cerebral cortex lamination.

Scale bar in (A) = 50 µm for (A) and (B), and in (C) = 50 µm for (C) to (F).
Figure 3-4. Expression Pattern of Dlg5 in the Mouse Brain

(A-F) In situ hybridization for Dlg5 on brain sections from E14.5 (A and B), P0 (C and D) and P10 (E and F) WT mice. Robust expression of Dlg5 is observed in the ventricular zone (VZ) and cortical plate (CP) at E14.5, cortex and striatum at P0, and cortex and hippocampus at P10. (A, C, and E) Antisense probe and (B, D, and F) sense probe.

Scale bar in (A) = 200 µm for (A-D), and in (E) = 200 µm for (E and F).
Figure 3-5. Subcellular Localization of DLG5 Protein in the Mouse Forebrain

Subcellular fractionation of P21 mouse forebrains was performed to generate fractions progressively enriched in postsynaptic membranes, as verified by immunoblotting for PSD-95 and synaptophysin (SYP). DLG5 protein is highly enriched in post-synaptic density (PSD) fractions. DLG5<sup>LP</sup> protein is also enriched in PSD fractions, however the protein level is significantly reduced.
Figure 3-6. DLG5 Regulates Dendritic Spine Density of Layer V Cortical Neurons

(A and B) Layer V pyramidal neurons from Dlg5+/+ (A) and Dlg5LP/LP (B) brains were examined using Golgi staining at P21. Dlg5LP/LP neurons show a significant reduction in dendritic spine density.

(C) Quantification of dendritic spine density in layer V pyramidal neurons (Dlg5+/+) apical dendrite: 0.516±0.033 spines/µm; side branches: 0.588±0.033 spines/µm; basal dendrites: 0.501±0.030 spines/µm. Dlg5LP/LP apical dendrite: 0.39±0.029 spines/µm; side branches: 0.441±0.029 spines/µm; basal dendrites: 0.385±0.034 spines/µm). Numbers of neurons quantified: n=80 neurons from 5 brains for Dlg5+/+; n=80 neurons from 6 brains for Dlg5LP/LP. Error bars represent SEM.

***p<0.001 by two-tailed student t test.

Scale bar in (A) = 3 µm for (A and B).
Figure 3-7. DLG5 Functions During Early Stages of Dendritic Spine Formation

(A and B) Layer V cortical pyramidal neurons from $Dlg5^{+/+}$ (A) and $Dlg5^{LP/LP}$ (B) brains were examined using Golgi staining at P14. $Dlg5^{LP/LP}$ neurons show a significant reduction in dendritic spine density.

(C) Quantification of dendritic spine density in layer V cortical pyramidal neurons at P14 ($Dlg5^{+/+}$ apical dendrite: $0.605 \pm 0.044$ spines/$\mu$m; side branches: $0.681 \pm 0.039$ spines/$\mu$m; basal dendrites: $0.636 \pm 0.05$ spines/$\mu$m. $Dlg5^{LP/LP}$ apical dendrite: $0.438 \pm 0.029$ spines/$\mu$m; side branches: $0.498 \pm 0.025$ spines/$\mu$m; basal dendrites: $0.461 \pm 0.027$ spines/$\mu$m). Number of neurons quantified: n=50 neurons from 3 brains for $Dlg5^{+/+}$; n=60 neurons from 4 brains for $Dlg5^{LP/LP}$. Error bars represent SEM. ***p<0.001 by two-tailed student t test.
Scale bar in (A) = 3 μm for (A and B).
Figure 3-8. DLG5 Regulates Dendritic Spine Density in Select Dendritic Segments of Layer II/III Cortical Neurons

(A and B) Layer II/III cortical pyramidal neurons from Dlg5+/+ (D) and Dlg5LP/LP (E) brains were examined using Golgi staining at P21. Dlg5LP/LP neurons show a significant reduction in dendritic spine density in apical dendrite and basal dendrites, but not in side branches that extend off of the apical dendrite.

(C) Quantification of dendritic spine density in Layer II/III cortical pyramidal neurons at P21 (Dlg5+/+ apical dendrite: 0.579±0.008 spines/μm; side branches: 0.546±0.012 spines/μm; basal dendrites: 0.559±0.01 spines/μm. Dlg5LP/LP apical dendrite: 0.462±0.012 spines/μm; side branches: 0.514±0.008 spines/μm; basal dendrites: 0.48±0.013 spines/μm). Number of neurons quantified: n=30 neurons from 3 brains for Dlg5+/+; n=40 neurons from 4 brains for Dlg5LP/LP. Error bars
represent SEM. ***p<0.001, *p<0.01, and N.S. p=0.22 by two-tailed student t test. Scale bar in (A) = 3 µm for (A and B).
Figure 3-9. DLG5 Does Not Regulate Dendritic Spine Density in Hippocampal Neurons

(G and H) Dentate gyrus granule cells from $Dlg5^{+/+}$ (G) and $Dlg5^{LP/LP}$ (H) brains were examined using Golgi staining at P21. There is no change in dendritic spine density in $Dlg5^{LP/LP}$ neurons.

(I and J) CA1 pyramidal neurons from $Dlg5^{+/+}$ (I) and $Dlg5^{LP/LP}$ (J) brains were examined using Golgi staining at P21. There is no change in dendritic spine density in $Dlg5^{LP/LP}$ neurons.

(K) Quantification of dendritic spine density in hippocampal dentate gyrus (DG) and CA1 neurons at P21. $Dlg5^{LP/LP}$ brains show normal hippocampal dendritic spine density. ($Dlg5^{+/+}$ DG: 0.705±0.021 spines/µm; CA1: 0.693±0.023 spines/µm. $Dlg5^{LP/LP}$ DG: 0.697±0.025 spines/µm; CA1: 0.676±0.021 spines/µm). Number of neurons quantified: n=35 DG neurons and 30 CA1 neurons from 3 brains for $Dlg5^{+/+}$; n=45 DG neurons and 35 CA1 neurons from 4 brains for $Dlg5^{LP/LP}$. Error bars represent SEM. N.S. p=0.82 (DG), and N.S. p=0.59 (CA1) by two-tailed student t test. Scale bar in (A) = 5 µm for (A to D).
Figure 3-10. Complementation Test Confirms $Dlg5^{LP}$ Mutation Causes Dendritic Spine Phenotype

(A and B) Genetic complementation test confirms that the decrease in dendritic spine density is caused by a mutation in $Dlg5$. Layer V pyramidal neurons from $Dlg5^{+/+}$ (D) and $Dlg5^{LP/null}$ (E) brains were examined using Golgi staining at P21. $Dlg5^{LP/null}$ neurons show a significant reduction in dendritic spine density.

(C) Quantification of dendritic spine density in layer V pyramidal neurons ($Dlg5^{+/+}$ apical dendrite: $0.532 \pm 0.021$ spines/µm; $Dlg5^{LP/null}$ apical dendrite: $0.364 \pm 0.021$ spines/µm). Numbers of neurons quantified: n=15 neurons from 3 brains for $Dlg5^{+/+}$; n=15 neurons from 3 brains for $Dlg5^{LP/null}$. Error bars represent SEM. ***$p<0.001$ by two-tailed student t test.

Scale bar in (D) = 3 µm for (D) and (E).
Figure 3-11. Reduction of Dendritic Spine Density in *Dlg5* Mutants Is Not A Secondary Effect of Enlarged Ventricles
(A and B) Cortical neurons derived from E13.5 \textit{Dlg5}^{+/+} (A) and \textit{Dlg5}^{LP/LP} (B) embryos were cultured for 8 DIV and then transfected with a GFP-expressing construct, to visualize dendritic spines, and cultured for a total of 18 DIV. \textit{Dlg5}^{LP/LP} cortical neurons with pyramidal morphology show a significant reduction in dendritic spine density \textit{in vitro}, confirming that the effect of \textit{Dlg5}^{LP} on dendritic spine density is not a secondary effect of enlarged ventricles.

(C) Quantification of dendritic spine density in cultured cortical neurons at 18 DIV (\textit{Dlg5}^{+/+}: 0.454±0.015 spines/µm; \textit{Dlg5}^{LP/LP}: 0.345±0.011 spines/µm). Number of neurons quantified: n=39 neurons from 3 independent cultures for \textit{Dlg5}^{+/+}, and n=60 neurons from 3 independent cultures for \textit{Dlg5}^{LP/LP}. Error bars represent SEM. ***p<0.001 by two-tailed student t test.

(D) Quantification of dendritic spine length in cultured cortical neurons at 18 DIV (\textit{Dlg5}^{+/+}: 1.572±0.067 µm; \textit{Dlg5}^{LP/LP}: 1.726±0.078 µm). Number of spines quantified: n=141 spines from 19 neurons from 3 independent cultures for \textit{Dlg5}^{+/+}, and n=114 spines from 18 neurons from 3 independent cultures for \textit{Dlg5}^{LP/LP}. Error bars represent SEM. P=0.137 by two-tailed student t test.

(E) Quantification of dendritic spine head area in cultured cortical neurons at 18 DIV (\textit{Dlg5}^{+/+}: 0.528±0.057 µm²; \textit{Dlg5}^{LP/LP}: 0.489±0.054 µm²). Number of spines quantified: n=141 spines from 19 neurons from 3 independent cultures for \textit{Dlg5}^{+/+}, and n=114 spines from 18 neurons from 3 independent cultures for \textit{Dlg5}^{LP/LP}. Error bars represent SEM. P=0.625 by two-tailed student t test.

(F and G) Cortical neurons derived from E13.5 \textit{Dlg5}^{+/+} (A) and \textit{Dlg5}^{-/-} (B) embryos were transfected with GFP at 8 days \textit{in vitro} (DIV) to visualize dendritic
spines and cultured for a total of 18 DIV. $Dlg5^{+/}$ mutant neurons show a significant reduction in dendritic spine density comparable to $Dlg5^{LP/LP}$ neurons, confirming that $Dlg5^{LP}$ is a null or strong loss-of-function allele.

(H) Quantification of dendritic spine density in cultured cortical neurons at 18 DIV ($Dlg5^{+/+}$: $0.449\pm0.022$ spines/µm; $Dlg5^{-/-}$: $0.336\pm0.011$ spines/µm). Number of neurons quantified: n=20 neurons from 3 independent cultures for $Dlg5^{+/+}$, and n=20 neurons from 3 independent cultures for $Dlg5^{-/-}$. Error bars represent SEM. ***p<0.001 by two-tailed student t test.
Figure 3-12. shRNA Knockdown Effectively Reduces DLG5 Protein Level

(A) Western-blot analysis of whole cell extracts prepared from HEK293T cells transfected with constructs expressing DLG5, shRNA targeting Dlg5 and a silencing resistant Dlg5 shRNA (srDLG5). Immunoblotting with DLG5 antibody demonstrates efficient reduction in DLG5 protein levels in extracts prepared from cells transfected with the Dlg5 shRNA construct and no reduction following transfection with srDLG5. Actin provides the loading control.

(B and C) Cortical neurons derived from E13.5 embryos were transfected with constructs expressing GFP and V5-Dlg5 (E), or Dlg5 shRNA and V5-Dlg5 (F), at 8DIV and cultured for a total of 18 DIV. Immunostaining for V5 (blue) shows efficient reduction in DLG5 protein levels in neurons transfected with Dlg5 shRNA.
Figure 3-13. DLG5 Functions Cell-autonomously

(A to C) Cortical neurons derived from E13.5 wild type embryos were transfected with constructs expressing GFP (A), *Dlg5* shRNA and GFP (B), *Dlg5* shRNA, silencing-resistant *Dlg5* (sr*Dlg5*), and GFP (C) at 8 DIV and cultured for a total of 18-21 DIV. *Dlg5* knockdown with *Dlg5* shRNA resulted in a significant reduction in dendritic spine density. Rescue with a sr*Dlg5* restored dendritic spine density to wild type levels.
(D) Quantification of dendritic spine density in cultured cortical neurons at 18-21 DIV. (GFP: 0.455±0.029 spines/µm; Dlg5 shRNA and GFP: 0.257±0.024 spines/µm; srDlg5 shRNA and GFP: 0.417±0.024 spines/µm). Number of neurons quantified: n=15 neurons from 3 independent cultures for GFP, n=15 neurons from 3 independent cultures for Dlg5 shRNA, and n=16 neurons from 3 independent cultures for Dlg5 shRNA rescue. Error bars represent SEM. ***p<0.001 and N.S. p=0.313 by two-tailed student t test.

(E and F) Layer V cortical neurons of wild type mice were sparsely transfected with GFP (F) or Dlg5 shRNA (G) by in utero electroporation at E13.5 and analyzed by GFP immunostaining at P21. Dlg5 knockdown using shRNA resulted in a significant reduction in dendritic spine density in vivo.

(G) Quantification of dendritic spine density in cortical neurons labeled by in utero electroporation at P21 (GFP: 0.481±0.02 spines/μm. Dlg5 shRNA: 0.359±0.033 spines/μm). Number of neurons quantified: n=10 neurons for GFP, and n=10 neurons for Dlg5 shRNA. Error bars represent SEM. **p<0.01 by two-tailed student t test.

Scale bar in (A) = 10µm for (A to C), and in (E) = 10µm for (E and F)
Figure 3-14. DLG5 Regulates Excitatory, But Not Inhibitory Synapse Density

(A and B) Cortical neurons derived from E13.5 Dlg5+/+ (A) and Dlg5LP/LP (B) embryos were cultured for 21 DIV. Excitatory synapses were visualized using antibodies recognizing vGlut1 (green) and PSD95 (red), presynaptic and postsynaptic excitatory markers, respectively. Dlg5LP/LP neurons show a reduction in excitatory synapses. Shown are representative images for Dlg5+/+ (A) and Dlg5LP/LP (B) dendritic segments.
(C) Quantification of excitatory synapses from Dlg5\(^{+/+}\) (A) and Dlg5\(^{LP/LP}\) (B) cultured neurons. Values are normalized to Dlg5\(^{+/+}\) neurons:

- Dlg5\(^{+/+}\): 1±0.056 vGlut1 puncta/\(\mu\)m; Dlg5\(^{LP/LP}\): 0.812±0.051 vGlut1 puncta/\(\mu\)m.
- Dlg5\(^{+/+}\): 1±0.0455 PSD95 puncta/\(\mu\)m; Dlg5\(^{LP/LP}\): 0.822±0.0361 PSD95 puncta/\(\mu\)m.
- Dlg5\(^{+/+}\): 1±0.049 vGlut1/PSD95 colocalized puncta/\(\mu\)m; Dlg5\(^{LP/LP}\): 0.729±0.05 vGlut1/PSD95 colocalized puncta/\(\mu\)m.

Number of neurons quantified: n=23 neurons from 3 independent cultures for Dlg5\(^{+/+}\), and n=30 neurons from 3 independent cultures for Dlg5\(^{LP/LP}\). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001 by two-tailed student t test.

(D and E) Cortical neurons derived from E13.5 Dlg5\(^{+/+}\) (A) and Dlg5\(^{LP/LP}\) (B) embryos were cultured for 21 DIV. Inhibitory synapses were visualized using antibodies that recognize VGAT (green) and gephyrin (red), presynaptic and postsynaptic inhibitory markers, respectively. Dlg5\(^{LP/LP}\) neurons show no reduction in inhibitory synapse density. Representative images for Dlg5\(^{+/+}\) (A) and Dlg5\(^{LP/LP}\) (B) dendritic segments are shown.

(F) Quantification of inhibitory synapse density from Dlg5\(^{+/+}\) (A) and Dlg5\(^{LP/LP}\) cultured cortical neurons. Values are normalized to Dlg5\(^{+/+}\) neurons:

- Dlg5\(^{+/+}\): 1±0.054 VGAT puncta/\(\mu\)m; Dlg5\(^{LP/LP}\): 1.027±0.066 VGAT puncta/\(\mu\)m; Dlg5\(^{+/+}\): 1±0.056 gephyrin puncta/\(\mu\)m; Dlg5\(^{LP/LP}\): 0.818±0.059 gephyrin puncta/\(\mu\)m; Dlg5\(^{+/+}\): 1±0.073 VGAT/gephyrin colocalized puncta/\(\mu\)m; Dlg5\(^{LP/LP}\): 1.028±0.077 VGAT/gephyrin colocalized puncta/\(\mu\)m.

Number of neurons quantified: n=18 neurons from 3 independent cultures for Dlg5\(^{+/+}\), and n=19 neurons from 3 independent cultures for Dlg5\(^{LP/LP}\). Error bars represent SEM. P=0.032 for
gephryn puncta/µm. Scale bar in (A) = 10 µm for (A and B), and in (D) = 10 µm for (D and E).
Figure 3-15. DLG5 Regulates Excitatory Synapse Density But Not Synapse Morphology In Vivo

(A and B) Transmission electron microscopy (TEM) analysis of layer V cerebral cortex at P21 reveals a significant reduction of excitatory synapse density in $D_{lg5}^{LP/LP}$ brains (B) compared to $D_{lg5}^{+/+}$ brains (A). Red asterisks represent excitatory synapses.

(C and D) Higher magnification TEM views of $D_{lg5}^{+/+}$ (C) and $D_{lg5}^{LP/LP}$ (D) cortical neuron dendritic spines. Synapses morphology appears normal in $D_{lg5}^{LP/LP}$ mice.

(E) Quantification of excitatory synapse density by TEM ($D_{lg5}^{+/+}$: 11.719±0.534 synapses/µm²; $D_{lg5}^{LP/LP}$: 8.75±1.048 synapses/µm²). Number of synapses quantified: n=75 for $D_{lg5}^{+/+}$ and n=56 for $D_{lg5}^{LP/LP}$. Error bar represents SEM. *p<0.05 by two-tailed student t test.

(F) Quantification of mean dendritic spine area by TEM ($D_{lg5}^{+/+}$: 73699.39±891.08 nm²; $D_{lg5}^{LP/LP}$: 84903.79±685.4.25 nm²). Number of spines quantified: n=47 from 3 brains for $D_{lg5}^{+/+}$, and n=62 from 3 brains for $D_{lg5}^{LP/LP}$. Error bar represents SEM. N.S. p=0.17 by two-tailed student t-test.

(G) Quantification of mean PSD length by TEM ($D_{lg5}^{+/+}$: 201.14±10.27 nm; $D_{lg5}^{LP/LP}$: 211.35±8.34 nm). Number of synapses quantified: n=42 from 3 brains for $D_{lg5}^{+/+}$, and n=67 from 3 brains for $D_{lg5}^{LP/LP}$. Error bar represents SEM. N.S. p=0.7 by two-tailed student t-test.

(H) Quantification of mean number of presynaptic vesicles by TEM ($D_{lg5}^{+/+}$: 20.1±2.26; $D_{lg5}^{LP/LP}$: 24.26±3.4). Number of synapses quantified: n=30 from 3
brains for Dlg5+/+, and n=34 from 3 brains for Dlg5LP/LP. Error bar represents SEM. N.S. p=0.36 by two-tailed student t-test.

Scale bar in (A) = 500nm for (A), and (B), and in (C) = 50nm for (C) and (D).
Figure 3-16. Reduced mEPSC Frequency and Modest Change in mEPSC Amplitude and Kinetics in Dlg5LP/LP Cortical Neurons

(A and B) Layer V cortical neuron mEPSCs were recorded from Dlg5+/+ and Dlg5LP/LP somatosensory cortical slices. Sample traces from Dlg5+/+ (D) and Dlg5LP/LP (E) are shown.

(C) The cumulative plot shows an increase in mEPSC inter-event interval in Dlg5LP/LP layer V cortical neurons. Statistically significant by Kolmogorov-Smirnoff test.

(D) mEPSC frequency is reduced in Dlg5LP/LP layer V cortical neurons (Dlg5+/+: 5.254±0.17 Hz; Dlg5LP/LP: 3.37±0.132 Hz.). Number of neurons recorded: n=25 neurons from 8 mice for Dlg5+/+, and n=25 neurons from 6 mice for Dlg5LP/LP. Error bars represent SEM. ***p<0.001 by two-tailed student t test.

(E) Layer V cortical neuron mEPSCs were recorded from Dlg5+/+ and Dlg5LP/LP somatosensory cortical slices. The cumulative plot shows a modest but significant increase in mEPSC amplitude in Dlg5LP/LP layer V cortical neurons. Statistically significant by Kolmogorov-Smirnoff test.

(F) mEPSC amplitude is modestly increased in Dlg5LP/LP layer V cortical neurons (Dlg5+/+: 11.216±0.01 pA; Dlg5LP/LP: 12.523±0.106 pA). Error bars represent SEM. ***p<0.001 by two-tailed student t test.

(G) Kinetic analysis shows a modest increase in mEPSC tau decay in Dlg5LP/LP layer V cortical neurons (Dlg5+/+: 4.676±0.156 msec; Dlg5LP/LP: 5.21±0.144 msec). Error bars represent SEM. ***p<0.001 by two-tailed student t test.
(H) Kinetic analysis shows a modest increase in mEPSC rise time in $Dlk5^{LP/LP}$ layer V cortical neurons ($Dlk5^{+/+}$: 1.367±0.019 msec; $Dlk5^{LP/LP}$: 1.644±0.02 msec). Error bars represent SEM. ***p<0.001 by two-tailed student t test.
Figure 3-17. DLG5LP Mutation Disrupts DLG5 SH3 Domain and GUK Domain Interaction

(A) Crystal structure of human ZO-1 SH3-GUK domain presented as ribbon diagram (Nomme et al., 2010). Isoleucine 562, homologous to DLG5 leucine 1642 and tryptophan 799 are highlighted.

(B) HEK 293T cells were transfected with HA-tagged wild type SH3, and Myc-tagged GUK, or with HA-tagged SH3L1642P and Myc-tagged GUK. Cell lysates were immunoprecipitated with HA antibody-conjugated beads and immunoblotted for HA and Myc. The amount of GUK coimmunoprecipitated with SH3L1642P was greatly reduced compared to that coimmunoprecipitated by the wild type DLG5 SH3 domain.
Figure 3-18. DLG5<sup>LP</sup> Mutation Disrupts DLG5 and β-catenin Interaction

(A) Coimmunoprecipitation of wild type and mutant DLG5 by β-catenin.

Forebrain lysates from P21 Dlg5<sup>+/+</sup> and Dlg5<sup>LP/LP</sup> mice were immunoprecipitated by β-catenin antibody and immunoblotted for DLG5 and β-catenin. The input is 5% of the lysate used for coimmunoprecipitation.

(B) Quantification reveals decreased binding of mutant DLG5<sup>LP</sup> to β-catenin.

The amount of DLG5 or DLG5<sup>LP</sup> protein coimmunoprecipitated by β-catenin antibody was normalized to the amount of DLG5 or DLG5<sup>LP</sup> in the input. n=4 Dlg5<sup>+/+</sup> brains and 4 Dlg5<sup>LP/LP</sup> brains from 4 independent experiments. Error bar represents SEM. *p<0.05 by two-tailed student t test.
Figure 3-19. DLG5 Regulates Synaptic Localization of β-catenin in Cortical Neurons

(A) Cortical neurons derived from E13.5 $Dlgs^{+/+}$ and $Dlgs^{LP/LP}$ embryos were cultured for 21 DIV and stained for VGlut1 and β-catenin. $Dlgs^{LP/LP}$ neurons show a decrease in dendritic β-catenin puncta and co-localized β-catenin/VGlut1 puncta density.

(B) Quantification of β-catenin and co-localized VGlut1/β-catenin puncta density in dendrites of cortical neurons cultured for 21 DIV ($Dlgs^{+/+}$ β-catenin: $0.76\pm0.03$ puncta/μm; VGlut1/β-catenin: $0.464\pm0.026$ puncta/μm. $Dlgs^{LP/LP}$ β-catenin: $0.546\pm0.022$ puncta/μm; VGlut1/β-catenin: $0.331\pm0.016$ puncta/μm). Number of neurons quantified: n=27 from 3 independent cultures for $Dlgs^{+/+}$, and n=27 from 3 independent cultures for $Dlgs^{LP/LP}$. Error bar represents SEM. ***p<0.001 by two-tailed student t test.

Scale bar in (A) = 10 μm.
Figure 3-20. DLG5 Regulates Surface Localization of N-cadherin in Cortical Neurons but not Hippocampal Neurons

(A) Cortical neurons from E13.5 $Dlg5^{+/+}$ and $Dlg5^{LP/LP}$ embryos were cultured for 21 DIV. Surface proteins were biotinylated and precipitated using streptavidin beads. The precipitate and the input (5% of the lysate used for
immunoprecipitation), was immunoblotted for N-cadherin to reveal surface N-
cadherin and total N-cadherin, respectively. Surface N-cadherin is decreased in
\( Dlg5^{LP/LP} \) neurons, whereas total N-cadherin remains the same.

(B) Quantification shows greatly decreased surface N-cadherin level in \( Dlg5^{LP/LP} \)
neurons. Error bar represents SEM. \( n=5 \) independent cultures for \( Dlg5^{+/+} \), and 5
independent cultures \( Dlg5^{LP/LP} \). **p< 0.001 by two-tailed student t test.

(C) Cortical neurons from E13.5 \( Dlg5^{+/+} \) and \( Dlg5^{LP/LP} \) embryos were cultured for
21 DIV. Surface proteins were biotinylated and precipitated using streptavidin
beads. The precipitate and the input (5% of the lysate used for
immunoprecipitation) was immunoblotted for GluR1 and Nrp2 to reveal surface
and total GluR1 and Nrp2, respectively. There is change in surface GluR1 or
Nrp2 in \( Dlg5^{LP/LP} \) neurons.

(D) Hippocampal neurons from E16.5 \( Dlg5^{+/+} \) and \( Dlg5^{LP/LP} \) embryos were
cultured for 21 DIV. Surface proteins were biotinylated and precipitated using
streptavidin beads. The precipitate and the input (5% of the lysate used for
immunoprecipitation), was immunoblotted for N-cadherin to reveal surface N-
cadherin and total N-cadherin, respectively. There is no change in surface N-
cadherin in \( Dlg5^{LP/LP} \) hippocampal neurons.
Figure 3-21. N-cadherin Knockdown Decreases Dendritic Spine Density in Cortical Neurons

(A-C) Cortical neurons derived from E13.5 WT embryos were transfected with constructs expressing GFP (A), N-cadherin shRNA and GFP (B), or scrambled shRNA and GFP (C) at 8 DIV and cultured for a total of 21 DIV. N-cadherin knockdown causes a significant decrease in dendritic spine density, while scrambled shRNA has no effect.

(D) Quantification of dendritic spine density in cortical neurons at 21 DIV (GFP: 0.451±0.016 spines/µm; N-cadherin shRNA: 0.241±0.019 spines/µm; scrambled shRNA: 0.488±0.016 spines/µm). Number of neurons quantified: n=24 for GFP from 3 independent cultures, n=24 for N-cadherin shRNA from 3 independent cultures, and n=10 for scrambled shRNA from 3 independent cultures. Error bar represents SEM. ***p<0.001 by two-tailed student t-test. Scale bar in (A) = 10µm for (A) to (C)
Figure 3-22. Overexpression of N-cadherin Rescues Dendritic Spine Phenotype of $Dlgs^{LP/LP}$ Neurons In Vitro

(A to C) Cortical neurons from E13.5 $Dlgs^{+/+}$ and $Dlgs^{LP/LP}$ embryos were transfected with constructs expressing GFP or GFP–N-cadherin at 8DIV and then cultured for a total of 21DIV. N-cadherin overexpression rescues the dendritic spine density defect in $Dlgs^{LP/LP}$ neurons.

Scale bar in (A) = 10µm for (A) to (C).

(D) Quantification of dendritic spine density in cultured cortical neurons at 21 DIV. ($Dlgs^{+/+}$: 0.653±0.018 spines/µm; $Dlgs^{+/+}$+N-cadhrin: 0.653±0.021 spines/µm; $Dlgs^{LP/LP}$: 0.509±0.015 spines/µm; $Dlgs^{LP/LP}$+N-cadhrin: 0.684±0.021 spines/µm). Number of neurons quantified: n=27 from 3 independent cultures for $Dlgs^{+/+}$, n=27 from 3 independent cultures for $Dlgs^{+/+}$+N-cadhrin, n=27 from 3 independent cultures for $Dlgs^{LP/LP}$, and n=27 from 3 independent cultures for
$Dlg5^{LP/LP}+N$-cadhrin. Error bar represents SEM. ***p<0.001, N.S. p=0.27 by two-tailed student t test.
Figure 3-23. DLG5 and N-cadherin Genetically Interact In Vivo

(A to D) Layer V pyramidal neurons from wild type (A), Dlg5^{LP/+} (B), Ncad^{+/−} (C), and Dlg5^{LP/+}; Ncad^{+/−} (D) brains were examined by Golgi staining at P28. Dlg5^{LP/+}; Ncad^{+/−} neurons show a significant reduction in dendritic spine density, whereas Dlg5^{LP/+} and Ncad^{+/−} neurons have normal spine density.

Scale bar in (A)= 3µm for (A to D)

(E) Quantification of dendritic spine density in layer V pyramidal neurons. (Wild type: 0.608±0.012 spines/µm; Dlg5^{LP/+}: 0.63±0.015 spines/µm; Ncad^{+/−}: 0.622±0.019 spines/µm; Dlg5^{LP/+}; Ncad^{+/−}: 0.507±0.011 spines/µm). The number of neurons quantified was: n=30 neurons from 3 brains for wild type, n=30 neurons from 3 brains for Dlg5^{LP/+}, n=30 neurons from 3 brains for Ncad^{+/−}, and n=30 neurons from 4 brains for Dlg5^{LP/+}; Ncad^{+/−}. Error bars represent SEM. ***p<0.001 by two-tailed student t test.
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Table 3-1. Genes Located Between SNP Markers D14Mit207 And rs30102223
Chapter 4

Concluding Remarks

In this thesis, I provide insight into the molecular mechanisms that regulate dendritic spine development. We identify a novel function of Semaphorin 5A signaling in restricting dendritic spine density in the hippocampus. Further studies are required to understand the precise role of Sema5A signaling on a cellular level. It is not known whether Sema5A restricts dendritic spine density by decreasing dendritic filopodial motility, inducing retraction of dendritic filopodia or spines, or by disassembling axon-dendritic contacts. These questions can be answered by performing time lapse imaging to quantify the distance traveled for each dendritic filopodia, and the number of newly formed and retracted filopodia or spine within a given length of time.

Moreover, further studies are required to identify downstream pathways for Sema5A signaling. Previous studies have identified Rho GTPases as important regulators of dendritic spine and synapse development (Tolias et al., 2011). Moreover, a recent study has shown that the Rac GAP, β-chimaerin, binds to neuropilin-2 to mediate axon pruning (Riccomagno et al., 2012). It is likely that Sema5A also signals through Rho GTPases to restrict dendritic spine density. As a first step to identify downstream signaling molecules of Sema5A, an in situ hybridization screen can be performed to search for mammalian Rho GEF’s and Rho GAP’s that are expressed in the postnatal hippocampus. Next,
we can look for Rho GEF’s and Rho GAP’s that bind to plexinA family members by co-immunoprecipitation. Alternatively, we can take a non-biased approach, such as yeast-two hybrid screen or protein microarrays, to search for proteins that bind to plexinA family members. Then we can select candidates that are expressed in the postnatal hippocampus. Eventually, to ascertain which candidate mediates Sema5A signaling, we should obtain knockout mice to examine in vivo phenotypes and perform functional assays to investigate whether mutant neurons are resistant to the effects of Sema5A.

Interestingly, our study shows that Sema5A−/− mice exhibit behavioral abnormalities that reflect key social interaction defects in autism patients. How this relates to the increased dendritic spine density and altered synaptic properties in the hippocampus is unclear. The hippocampus is best known for its role in learning and memory, but it is also connected to the prefrontal cortex and subcortical structures of the limbic system. For instance, the ventral hippocampus has been shown to modulate the firing pattern of medial prefrontal cortex during anxiety-related behavioral tasks (Adhikari et al., 2010, 2011). Future studies using single unit recordings in the hippocampus and prefrontal cortex will be required to detect differences in activity pattern of these two brain regions in Sema5A−/− and wild type mice during social interaction or other behavioral tasks.

It is also possible that Sema5A−/− mice exhibit defects in synaptic function or plasticity in other brain regions. Recently, perturbed metabotropic glutamate receptor (mGluR)-dependent plasticity has been observed in various mouse
models of autism, including Neuroligin3−/−; Tsc2+/−; and Fmr1−/− mice (Auerbach et al., 2011; Baudouin et al., 2012), suggesting a shared pathophysiology underlying both syndromic and non-syndromic autism spectrum disorders. It would be interesting to examine whether Sema5A−/− mice also show defects in mGluR-dependent synaptic plasticity, and whether the behavioral deficits observed in these mice can be corrected by mGluR5 inhibitors (Michalon et al., 2012). Taken together, Sema5A−/− mice could serve as a valuable model for investigating the developmental and synaptic mechanisms underlying social behavior impairment in autism spectrum disorders.

In addition, we addressed important mechanisms that regulate subcellular localization of cell-surface proteins during synaptogenesis. We identified DLG5 as a key regulator of N-cadherin cell-surface localization. The availability of N-cadherin at the cell surface during dendritic spine formation is likely to be an important determinant of dendritic spine density. Dendritic spines are specialized subcellular compartments where specific synaptic molecules are concentrated. The correct localization of these proteins is indispensable for normal development and function of the synapse. DLG5 was first identified as an important regulator of N-cadherin localization in epithelial tight junctions. Likewise, other scaffolding proteins involved in cell junction formation may also play important roles in regulating the localization of synaptic proteins during synaptogenesis.

Interestingly, DLG5 is required for correct apical-basal polarity of epithelial cells but not neurons. In epithelial cells, the correct localization of N-cadherin and formation of tight junctions could be important for segregating membrane
proteins specific for the apical and basolateral domains. However, cell polarity in mature neurons may be determined by distinct mechanisms. One of the most important steps in neuronal polarization is the formation of a single axon among multiple undifferentiated neurites. TGF-β mediated par6 phosphorylation and the LKB1-SAD kinase pathway have been shown to be required for axon formation in vivo (Barnes et al., 2007; Yi et al., 2010). The polarized distribution of extracellular cues, such as TGF-β, could therefore ensure the correct orientation and alignment of axons and dendrites (Yi et al., 2010).

Another interesting finding in this study relates to the extent that formation of dendritic spines is dependent on DLG5. Loss of DLG5 results in a 25% decrease in dendritic spine density. It would be interesting to determine whether certain excitatory synapses are more dependent on DLG5 regulation of N-cadherin localization or whether dendritic spine loss in Dlg5 mutants is purely stochastic. Previous studies have shown that loss of PSD-95 or PSD-93 silences non-overlapping subsets of excitatory synapses in adult mice (Elias et al., 2006). Given the heterogeneity of cortical excitatory synapses, it is possible that DLG5 is important for regulating the formation of only a subset of excitatory synapses. To address this question, we first need to develop DLG5 antibodies that are compatible with immuno-electron microscopy or array tomography to examine whether DLG5 protein is localized in subsets of excitatory synapses. Next, we could use monosynaptic retrograde trans-synaptic tracing techniques to identify presynaptic partners of a given cortical neuron (Wickersham et al., 2007), and then examine if there is a preferential loss of certain synaptic inputs.
DLG5 is expressed in the postnatal hippocampus; however, we do not observe any change in hippocampal dendritic spine density in $Dlgs^{LP/LP}$ mice. Interestingly, cell surface N-cadherin localization is not decreased in cultured $Dlgs^{LP/LP}$ hippocampal neurons. This finding reinforces the idea that DLG5 regulates dendritic spine density by controlling surface localization of N-cadherin. What could be the role of DLG5 in hippocampal neurons? Previous studies have shown that N-cadherin is required for dendritic spine stabilization and enlargement induced by LTP in hippocampal neurons (Bozdagi et al., 2010; Mendez et al., 2010). It is possible that DLG5 could be involved in activity-dependent N-cadherin delivery in hippocampal neurons. Future studies are required to examine the effects of DLG5 loss on dendritic spine morphology and LTP.

DLG5 is a multi-domain protein and could have multiple binding partners important for neuronal function. Non-biased approaches, such as yeast-two-hybrid screen or protein microarray screening, could be performed to identify additional interacting proteins of DLG5 and gain a more comprehensive understanding of its function.

Throughout this study, we have focused our analysis on cortical neurons and hippocampal neurons, in part because the molecular mechanisms regulating development and physiology of excitatory synapses in these classes of neurons have been subject to intense study. However, DLG5 is highly expressed in the postnatal striatum, thalamus, and cerebellum, while Sema5A is expressed in the postnatal cerebellum. It would be interesting to investigate whether the
molecular mechanisms regulating synaptic density discussed in this study are also involved in the development of excitatory synapses in other brain regions. Moreover, we have limited our in vivo analysis to excitatory synapses, using dendritic spines as a morphological marker. Compared to excitatory synapses, less is known about the development of inhibitory synapses. Given the important role of inhibitory synaptic transmission in a wide array of neuropsychiatric disease, such as Parkinson’s disease, schizophrenia, depression, and drug addiction, further efforts should be invested on studying the cellular and molecular events involved in the development of these synapses in the mammalian brain.

References:


Appendix

A Forward Genetic Screen to Search for Genes Regulating Neuronal Connectivity in the Postnatal Mouse Brain

To search for novel genes involved in the development of neural circuitry, we devised a forward genetic screen to identify recessive mutations that affect brain development in mice. Forward genetic screens in mice have proven effective for the identification of molecules involved in diverse biological processes, such as embryonic patterning and morphogenesis (Garcia-Garcia et al., 2005), regulation of circadian rhythm (Vitaterna et al., 1994), and peripheral axon navigation (Lewcock et al., 2007; Merte et al., 2010). In this study, we focus our attention on mutants with defects in major axon tracts in the CNS.

In order to develop an efficient mutagenesis protocol for the genetic screen, we experimented with various methods to induce random insertional mutations in the mouse genome, including mobilization of L1 retrotransposons (An et al., 2006; Han and Boeke, 2004) and injection of lentivirus into single-cell mouse embryos (Lois et al., 2002). However, after extensive work we determined that none of these methods achieved the mutagenic potency of ethynitrosourea (ENU). To establish G1 lines for our genetic screen, a standard dose of ENU (300mg/Kg body weight) was injected into 50 C57Bl/6 males, and a total of 167 G1 lines were established. At this dosage, each G1 male is expected to harbor around 30 gene-inactivating mutations, according to previously
reported specific locus test (Hitotsumachi et al., 1985). Therefore, in our screen we recovered approximately 5000 gene-inactivating mutations.

Our screen was conducted at postnatal day 5 (P5), when the majority of axon tracts in the mouse brain are developed. Sagittal sections were cut at 150µm and immunostained with neurofilament antibody (2H3). This allowed us to examine overall brain architecture as well as numerous axon pathways, including the corticospinal tract, corticothalamic tract, anterior commissure, corpus callosum, hippocampal commissure, and neural circuits of the limbic system. To increase the efficiency of our screen, we designed an enlarged sectioning platform that enabled us to cut one litter of brains simultaneously on the microtome. Additionally, we designed and had manufactured in-house a staining tray that fits into 24-well tissue culture plates for performing immunohistochemistry. Thus, we were able to stain large numbers of sections at the same time by moving the trays between 24-well plates containing different solutions. Overall, we were able to screen 30 litters of G3 pups each week. Throughout the course of this project, we screened approximately 9000 pups from 1100 G3 litters, which were derived from the original 167 G1 lines.

Several interesting mutant lines were identified through our genetic screen. Lines #3005 and #3007 showed identical phenotypes, including enlarged ventricles and disrupted midline structures. These two lines were mapped to the identical missense mutation, Leu1642Pro, in discs large-5 (Dlg5) gene. Characterization of this mouse line and investigation of DLG5 function in development of neuronal connectivity is described in Chapter 3.Interestingly,
line #3005 and #3007 were derived from the same ENU-injected male. ENU injection results in a period of infertility due to near-depletion of spermatogonia. The small percentage of surviving spermatogonia will repopulate the testes. Therefore, the germ cells of ENU-injected male mice that have regained fertility consist of a small number of clones. It is possible that G1 #3005 and #3007 are derived from the same spermatogonia clone.

Line #4182 showed defects in the anterior commissure (AC) and fasciculus retroflexus (FR). The anterior limb of the AC is greatly reduced and misprojected ventrally (Figures A-1A and B), while the posterior limb of the AC is absent (Figures A-1C and D). Additionally, the FR is severely defasciculated. This line was mapped to a G to A transition in codon 1379, leading to a nonsense mutation, Trp460Stop, in the second CUB domain of the neuropilin-2 coding region. The axon guidance phenotypes of line #4182 are identical to those described for neuropilin-2-/- mice (Giger et al., 2000).

Several mutant lines showed an absence or severe attenuation of the corpus callosum, including lines #4033, #4479, and #4783 (Figure A-2). However, linkage analysis conducted by comparing SNP’s between C57Bl/6 and C3H/Hej mice failed to find any chromosome region linked to the phenotype in these lines. Formation of the corpus callosum is a complicated developmental event, and several molecular cues are involved in the guidance of callosal axons, including netrin (Serafini et al., 1996), Sema3C (Niquille et al., 2009), EphrinB (Mendes et al., 2006), and draxin (Islam et al., 2009). Interestingly, corpus callosum defects have also been described in certain inbred mouse strains. For
instance 50% of balb/c mice and 70% of 129/J mice have corpus callosum
defects. The genetic lesions causing abnormal callosal axon guidance in these
mouse strains have not been identified (Livy and Wahlsten, 1991, 1997). It is
possible that the corpus callosum defects in lines #4033, #4479, and #4783 are
not caused by single genetic mutations, precluding further mapping by linkage
analysis.

Additionally, several G1 lines showed early postnatal lethality. We
examined the neuromuscular junction (NMJ) in mice derived from these lines at
P0 by immunostaining with neurofilament to label motor axons and α-
bungarotoxin to label acetylcholine receptor (Ach R) clusters. Lines #4044 and
#4176 showed an absence of postsynaptic Ach R clusters and also overshooting
of motor axon terminals. Both lines were mapped to LDL-receptor-related protein
4 (Lrp4); line #4044 is a missense mutation, while line #4176 is a nonsense
mutation. Lrp4 was recently identified as an agrin receptor and is required for
postsynaptic development of the NMJ (Kim et al., 2008; Zhang et al., 2008).
Lines #4044 and #4176 show NMJ and limb development phenotypes identical to
those observed in Lrp4\textsuperscript{mitt} mutants, as described previously (Kim et al., 2008;
Weatherbee et al., 2006).

Overall, the percentage of lines that showed a phenotype of interest in our
genetic screen was lower than previously published screens (Merte et al., 2010;
Garcia-Garcia et al., 2005; Lewcock et al., 2007). This could be due to several
factors. First of all, we are searching for specific phenotypes in neural
connectivity. The number of genes involved in this process may be much smaller
than those involved in more generalized developmental processes, such as dorsal-ventral patterning of the embryo (Garcia-Garcia et al., 2005). Secondly, by performing a postnatal screen, we were not able to identify mutant lines that show embryonic lethality. Recent studies have highlighted the similarity between axon guidance and angiogenesis, and some of these molecules have been shown to play important roles in both processes (Carmeliet and Tessier-Lavigne, 2005). For instance, neuropilin-1 is a receptor for both Sema3A and VEGF, and *neuropilin-1*−/− mice show embryonic lethality due to cardiovascular defects (Kawasaki et al., 1999). It is conceivable that other axon guidance molecules, or signaling molecules downstream of axon guidance receptors, may also be involved in cardiovascular development or other important aspects of embryogenesis. Thirdly, although we have focused our screen on a few axon tracts that are readily detectable with neurofilament immunostaining, the neuroanatomy of the postnatal mouse brain is quite complex and shows considerable individual variability. Thus, subtle phenotypes may not have been easily detected in our genetic screen. This is particularly relevant since ENU induces point mutations that can lead to hypomorphic mutations. Nevertheless, the identification of a *neuropilin-2* mutant in our screen provides proof of principle that genes affecting neuronal connectivity can be identified using this approach. A simpler screening method involving fewer processing steps may lead to a higher yield in the future.
Reference:


Mendes, S.W., Henkemeyer, M., and Liebl, D.J. (2006). Multiple Eph receptors and B-class ephrins regulate midline crossing of corpus callosum fibers in the
developing mouse forebrain. The Journal of neuroscience : the official journal of
the Society for Neuroscience 26, 882-892.

Merte, J., Wang, Q., Vander Kooi, C.W., Sarsfield, S., Leahy, D.J., Kolodkin,
Sema3A(K108N), which binds to neuropilin-1 but cannot signal. The Journal of
neuroscience : the official journal of the Society for Neuroscience 30, 5767-5775.

Niquille, M., Garel, S., Mann, F., Hornung, J.P., Otsmane, B., Chevalley, S.,
neuronal populations are required to guide callosal axons: a role for semaphorin

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R.,
commissural axon guidance in the developing vertebrate nervous system. Cell
87, 1001-1014.

Vitaterna, M.H., King, D.P., Chang, A.M., Kornhauser, J.M., Lowrey, P.L.,
McDonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W., and Takahashi, J.S.
(1994). Mutagenesis and mapping of a mouse gene, Clock, essential for

related protein 4 is crucial for formation of the neuromuscular junction.
Development 133, 4993-5000.
Figure A-1. Line #4182 Shows Defects in Anterior Commissure (AC)

(A and B) The anterior limb of the AC is reduced and misprojected ventrally in #4182 mutants.

(C and D) The posterior limb of the AC is absent in #4182 mutants.
Figure A-2. Line #4033 Shows Defects in the Corpus Callosum

(A and B) The lateral part of the corpus callosum is relatively normal in #4033 mutants.

(C and D) The corpus callosum is reduced and shows an abnormal morphology in the midline of #4033 mutants.
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