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Molecular and Cellular Mechanisms of Reelin Signaling in the Adult Hippocampus

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Molecular and Cellular Mechanisms of Reelin Signaling in the Adult Hippocampus

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Dedication

This dissertation is dedicated to the countless individuals that have enabled my metamorphosis into a scientist through their endless support and thoughtful mentorship. Getting to this point would not have been possible had my parents, Jerry and Kelly Trotter, not fostered my early entrance into a career path in science by funding and supporting my many successes (and failures) in science fair competitions throughout grade school. Although I had countless wonderful science teachers and science research instructors, no two were more instrumental to my success than Amy McCormick and Jean Almeida, who exhibited vast patience for my youthful lack of focus and rampant curiosity.

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Abstract

The Reelin signaling pathway is critical for neuronal migration during development and the function of excitatory synapses in the adult forebrain. Despite a growing body of evidence implicating impaired Reelin signaling in the pathogenesis of numerous neuropsychiatric and neurodegenerative disorders, including Schizophrenia and Alzheimer’s disease, little is known still regarding the specific molecular and cellular mechanisms whereby Reelin signaling modulates the function of synapses to enable normal learning and memory. In this dissertation, we addressed these knowledge gaps by identifying mechanisms of Reelin proteolysis following synaptic potentiation (Chapter 2) and dissociated the synaptic function of Reelin signaling at excitatory (Chapter 3) and inhibitory synapses (Chapter 4). In the adult brain, Reelin is secreted by GABAergic interneurons into the extracellular space, after which it is cleaved by unknown proteases to generate active fragments that signal downstream. In Chapter 1, we demonstrate that tissue plasminogen activator (tPA) and its major \textit{in vivo} substrate, plasminogen, cleave Reelin under cell-free conditions to generate major Reelin fragments found \textit{in vivo}. Since manipulation of tPA levels under basal conditions had no effect on Reelin processing, we hypothesized that synaptic activity may be required to render Reelin susceptible to proteolysis by tPA. Indeed, the modulation of Reelin processing by synaptic potentiation of \textit{ex}
vivo hippocampal slices required the presence of tPA. These data are the first to
demonstrate a specific context in which Reelin signaling may be initiated in the
intact brain and further emphasize that extracellular proteolysis of Reelin by tPA
and other yet-to-be identified proteases is important to consider when trying to
understand how altered Reelin processing and/or expression contribute to
cognitive impairments associated with disease states. In Chapters 2 and 3 of this
dissertation, we describe recent attempts by our lab to elucidate cellular
mechanisms of Reelin signaling in the adult brain. To do this, we generated two
conditional knockout mutants that lack the obligate downstream adaptor protein,
Disabled-1 (Dab1), specifically in postnatal excitatory neurons (eKO) or
GABAergic interneurons (iKO). Despite some overlap of Reelin and Dab1 in a
subset of GABAergic interneurons, we found that their expression was generally
juxtaposed, with Dab1 being primarily expressed by principle neurons and a
more widespread population of Reelin-negative GABAergic interneurons. While
eKO mice exhibited normal forebrain lamination, dendritic architecture, and
dendritic spine density, they did have reductions in spine volume and a loss of
basal and activity-dependent Akt and MAPK activation. These changes
culminated in impairments in short-term and long-term synaptic plasticity, as well
as impairments in associative learning and spatial memory. Taken together, our
observations in the eKO mice are the first to definitively establish a synaptic
function of Reelin signaling in the adult hippocampus. While characterizing the
eKO mice, we also observed that GABAergic interneurons expressed Dab1,
which motivated us to explore the inhibitory synapse as a novel locus of Reelin
signaling (Chapter 4). Although loss of Dab1 in GABAergic interneurons did not affect forebrain development or the overall patterning of inhibitory synapses, iKO mice presented with an ataxic gait, resting tremor and cerebellar hypoplasia. Interestingly, loss of Dab1 in interneurons led to altered expression of some major glutamatergic synapse proteins (i.e. NMDA receptor subunits NR1 and NR2B), while other excitatory and inhibitory synapse proteins were normal (e.g. NR2A, GAD67/65, and gephyrin). Hippocampal field recordings further demonstrated that even partial loss of Dab1 expression in iHET mice, led to enhanced presynaptic activation and impaired theta-burst induced LTP. These data establish the inhibitory synapse as a novel locus of Reelin signaling in the developing and adult brain. Taken together, data discussed herein should prove useful for understanding and treating disorders associated with Reelin signaling impairments (e.g. AD and Schizophrenia).
Despite significant progress in our understanding of the basic molecular mechanisms of learning and memory (Baudry et al., 2011; Kandel, 2012; Zovkic et al., 2013), the identification of drug targets and development of effective therapeutics for treating cognitive disorders has been slow. One reason for this is because many genes that are important in the adult brain are also vital for neurodevelopment, limiting the utility of conventional genetic knockout strategies to dissociate developmental and adult functions of a gene of interest. A classic example of this is the Reelin signaling pathway, the study of which has been hindered by the severe neurodevelopmental abnormalities that characterize existing genetic mouse models, including the reeler (D'Arcangelo et al., 1995) and scrambler mutants (Sheldon et al., 1997b). Although other traditional knockout and knockin strategies have been useful for identifying Reelin receptors and ascertaining their basic function, these mouse models do not allow definitive dissociation of developmental and adult functions. Notwithstanding, the Reelin signaling pathway has been implicated in numerous processes in the adult brain,
including the modulation of excitatory neurotransmission (Beffert et al., 2005; Chen et al., 2005; Sinagra et al., 2005; Qiu et al., 2006a; Groc et al., 2007; Qiu and Weeber, 2007; Teixeira et al., 2011), dendritic spine formation (Niu et al., 2008; Rogers et al., 2011), synaptic plasticity (Weeber et al., 2002; Beffert et al., 2005; Marrone et al., 2006; Rogers et al., 2011; Stranahan et al., 2011), and learning and memory (Weeber et al., 2002; Beffert et al., 2005; Qiu et al., 2006b; Rogers et al., 2011).

The importance of these findings is underscored by the fact that Reelin signaling impairments are associated with several neuropsychiatric and pathological disorders, including schizophrenia (Impagnatiello et al., 1998; Chen et al., 2002; Wedenoja et al., 2010), major mood disorders (Fatemi et al., 2000; Lussier et al., 2011; Teixeira et al., 2011), autism (Fatemi et al., 2000; Persico et al., 2001; Zhang et al., 2002) and Alzheimer’s disease (Chin et al., 2007; Seripa et al., 2008; Chen et al., 2010). The association of developmental, adult-onset and age-related neurological disorders with Reelin signaling impairments emphasize the urgency of establishing a better temporal framework within which we understand Reelin signaling. Here, we will discuss known mechanisms of Reelin signaling in the brain by reviewing the literature according to distinct ontological phase of Reelin signaling spanning brain development through adulthood. These phases are important, as the specific mechanisms that govern the Reelin signaling pathway during development may be distinct from those that dictate its function later in life.
The study of Reelin signaling and its role in cortical development is responsible for the large preponderance of known signaling mechanism. For that reason, section one will consist of an overview of cortical development followed by a more specific discussion of major and aspiring players in the Reelin signaling pathway. We will also briefly discuss other considerations important to understanding Reelin signaling, such as Reelin processing. Since most of the molecular details of Reelin signaling during development have been derived from the study of projection neuron migration, we will also discuss other less explored brain regions and cell types that may be critical to consider when understanding the role of Reelin signaling in disease states, such as GABAergic interneurons.

Individual sections of this chapter have been dedicated for the discussion of other developmental processes, including dendritic morphogenesis, synaptogenesis, and synapse maturation. Since comparatively less is known about Reelin signaling in the adult, the sections on synaptic plasticity and learning and memory will also try to emphasize important future directions in the Reelin field that may be pursued with the advent of conditional transgenic models. The primary goal of this review is to provide a guide for future experiments that carefully consider unique contributions of Reelin signaling to distinct aspects of nervous system development and adult function, which we predict will be a prerequisite to understanding the role of Reelin signaling perturbations in neurological disorders, particularly those associated with cognitive impairments.
The glycoprotein Reelin is secreted by Cajal-Retzius cells of the developing marginal zone, where it signals to projection neurons to control their radial migration (Hammond et al., 2001; Olson et al., 2006; Franco et al., 2011; Jossin and Cooper, 2011). Radially migrating neurons utilize glia-independent somal translocation to enter the developing cortical plate (CP) via movement of their somas along their leading processes, which first encounters Reelin in the marginal zone (Nadarajah et al., 2001; Tabata and Nakajima, 2003). The inside-out layering of the developing cortex requires that later-born neurons migrate further utilizing additional modes of migration (e.g. glia-guided) and ultimately employing glia-independent somal translocation when their leading process reaches the marginal zone (Tabata and Nakajima, 2003). The specific step at which secreted Reelin influences neuronal positioning of early- and late-born neurons is glia-independent somal translocation (Franco et al., 2011), as it does not influence other migrational modes (Franco et al., 2011; Jossin and Cooper, 2011).

In *reeler* mice, which carry a null mutation in Reelin, cortical development has been described as roughly-inverted (Landrieu and Goffinet, 1981). However
this viewpoint is not entirely complete, as early-born neurons shift from a deep laminar position and amass in a superficially-positioned superplate, while late-born neurons exhibit broad and irregular distribution (Boyle et al., 2011). The *scrambler* and *yotari* mutants resemble the *reeler* phenotype, but carry null mutations in the gene encoding Disabled-1 (Dab1) (Sheldon et al., 1997a; Ware et al., 1997; Rice et al., 1998). Targeted-disruption of Dab1 also generated a reeler-phenotype (Howell et al., 1997b), providing early evidence that Reln and Dab1 were part of the same molecular signaling pathway. Numerous studies have since identified Dab1 as the obligate downstream adaptor protein that transduces the extracellular Reelin signal via its association with the Reelin receptors, apolipoprotein E receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999). Indeed, the combined absence of ApoER2 and VLDLR leads to abnormal neuronal migration throughout the forebrain and cerebellar cortex, mirroring both reeler and scrambler mice (Trommsdorff et al., 1999).

Binding of Reelin to ApoER2 and VLDLR induces receptor clustering (Strasser et al., 2004) and association of the Dab1 phosphotyrosine-binding domain (PTB) with receptor NPxY internalization motifs (Howell et al., 1999b; Morimura et al., 2005). Loss of Dab1 binding to ApoER2 via a knock-in loss-of-function mutation in its NPxY motif leads to abnormal neuronal positioning that, when combined with VLDLR deficiency, resembles a reeler-phenotype (Beffert et al., 2006). The Dab1 PTB domain also contains a conserved Pleckstrin homology-like subdomain, which allows Dab1 to simultaneously associate with...
the plasma membrane via phosphoinositide-binding (Stolt et al., 2003), which, in turn, is required both for the association of Dab1 with its downstream effectors and transduction of the Reelin signal (Huang et al., 2005; Stolt et al., 2005; Xu et al., 2005).

Following clustering of Dab1 on the receptor cytoplasmic tails, it associates with the Src Homology 2 (SH2) domains of the non-receptor tyrosine kinases, Src family kinases (Howell et al., 1997a). The SFKs, Src and Fyn, then activate Dab1 through phosphorylation of its several highly conserved tyrosine residues (i.e. Y185, Y198, Y220, and Y232) (Howell et al., 1999a; Arnaud et al., 2003b; Benhayon et al., 2003; Bock and Herz, 2003). Although individual loss of Src or Fyn does not generate a reeler phenotype, likely because of compensation by other SFK members, site-directed mutagenesis of all four major Dab1 tyrosine residues does (Howell et al., 2000; Keshvara et al., 2001). However, combined genetic deficiency of Fyn and Src or pharmacological blockade of Src kinases in cortical explant cultures does result in reeler phenotype (Jossin et al., 2003; Kuo et al., 2005). Thus, tyrosine phosphorylation of Dab1 by SFKs is critical for downstream Reelin signaling. Some evidence suggests that there is a hierarchy of Dab1 phosphorylation sites; in particular, Y198 and Y220 are considered the major Reelin-induced phosphorylated tyrosine residues (Keshvara et al., 2001). Moreover, a recent study suggested that Dab1 tyrosine sites act in a coordinated fashion, with Y185/198 and Y220/232 behaving as couplets. Phosphorylation of one member of each pair is
generally sufficient for Reelin to promote proper neuronal positioning during development (Morimura and Ogawa, 2009).

Fyn is also a critical regulator of Dab1 protein levels, as phosphorylation of Dab1 by Fyn leads to its eventual polyubiquitination and degradation by the proteasome (Arnaud et al., 2003b), a process which represents a potential negative feedback mechanism following Reelin stimulation (Bock et al., 2004). In fact, tyrosine and serine/threonine phosphorylation of Dab1 both affect its propensity for polyubiquitination and degradation (Arnaud et al., 2003a). In particular, phosphorylation of Dab1 by Fyn leads to its association with the E3 ubiquitin ligase component Cullin 5 (Cul5), which in complex with SOCS (suppressor of cytokine signaling) proteins, ubiquitinates Dab1 and targets it for destruction (Feng et al., 2007). Loss of Cul5 during development leads to an accumulation of Dab1 protein, excessive migration, and the buildup of neurons at the top of the cortical plate (Feng et al., 2007), suggesting that the termination of the Reelin signal via Dab1 degradation, is also critical for neuronal positioning.

Tyrosine phosphorylation of Dab1 also results in the activation of SFKs (i.e. Src and Fyn) (Bock and Herz, 2003). However, it is still not known whether SFK substrates, other than Dab1, are important for Reelin signaling during development. Phosphorylated Dab1 activates phosphatidylinositol 3-kinase (PI3K) through its association with the p85alpha regulatory subunit (Bock et al., 2003). Activation of PI3K by Dab1 leads to a host of downstream signaling activities, including the activation of the serine/threonine kinase Akt (also known as Protein Kinase B) and inhibition of glycogen synthase kinase 3beta, or GSK-
Indeed, the activation of PI3K and Akt by Reelin is essential for cortical migration (Bock et al., 2003). Although Reelin-stimulated Akt also activates the mTOR (mammalian target of rapamycin)-S6K1 (S6 Kinase 1) pathway and suppresses GSK-3β, these targets are not involved in the modulation of neuronal migration by Reelin (Jossin and Goffinet, 2007).

Another major downstream component of the Reelin signaling pathway involves the association of tyrosine phosphorylated Dab1 with the Crk adaptor protein family. Specifically, Dab1 phosphorylated at Y220 and Y232 binds the Crk-family proteins (CrkL, Crkl, and CrkII), and indirectly binds Dock1 (Ballif et al., 2004; Chen et al., 2004). Crk adaptor proteins appear to be critical for Dab1 to activate C3G, the guanine nucleotide exchange factor (GEF) for the small GTPase Rap1, as well as Akt (Ballif et al., 2004; Park and Curran, 2008; Feng and Cooper, 2009). Combined absence of Crk and Crk-like (CrkL) results in impaired Reelin-dependent neuronal migration (Park and Curran, 2008) and dendritogenesis (Matsuki et al., 2008), confirming that the association of Dab1 with Crk adaptor proteins represents a critical arm of downstream Reelin signaling.

Crk-I and Crk-II may also stimulate phosphorylation of Dab1 on Y220 by SFKs (Huang et al., 2004), although this has not yet been widely confirmed. A recent study demonstrated that the Dab1-Crk/CrkL-C3G-Rap1 pathway is required for the inside-out activation of α5β1-integrin, which in turn, is critical for neuronal positioning (Sekine et al., 2012). Moreover, activation of Rap1 by Reelin has been found to stabilize the leading process of migratory projection neurons in
the marginal zone through promoting its association with Cajal-Retzius via the adhesion molecules, Cdh2 (Franco et al., 2011) and nectins 1 and 3 (Franco et al., 2011; Gil-Sanz et al., 2013). Specifically, Reelin signaling promotes the assembly of adhesion sites via nectin-dependent stabilization of cadherin function through afadin, Rap1, and p120ctn (Gil-Sanz et al., 2013). Taken together, these data suggest that the association of Dab1 with Crk adaptor proteins is fundamental to downstream Reelin signaling during development, and likely is involved in Reelin signaling in other contexts.

In this section, we have extensively reviewed major players of the Reelin signaling pathway that have clear and established roles downstream of Reelin in promoting proper neuronal positioning. We will next discuss aspiring players whose roles in Reelin signaling have not yet been firmly established.

**Aspiring Players**

*Cyclin-Dependent Kinase 5 (CDK5)*

In the brain, Dab1 is phosphorylated at serine-400 and -491 by CDK5 (Keshvara et al., 2002; Sato et al., 2007), a proline-directed serine/threonine kinase that is critical for brain development. In fact, loss of CDK5 or combined loss of its neuronal activators, p35 and p39, results in perturbed neuronal migration, generating a phenotype that partially resembles *reeler* and *scrambler* mice (Ko et al., 2001; Ohshima et al., 2001). Ectopic neurons in CDK5 KO mice
have reduced reelin signaling during late cortical development (Keshvara et al., 2002), suggesting that CDK5 and Reelin signaling may converge at some level. In further support of this idea, impairments in neuronal positioning in p35 KO mice are worsened by partial loss of Reelin signaling (Ohshima et al., 2001; Beffert et al., 2004).

Existing data suggests that CDK5 phosphorylates Dab1 independently of Reelin signaling (Keshvara et al., 2002) and that Reelin signaling itself does not promote the activation of CDK5 (Beffert et al., 2004). One possibility is that CDK5 permissively modulates Reelin signaling by regulating the stability and/or propensity of Dab1 to be tyrosine phosphorylated. Supporting this idea, absence of CDK5 in primary neurons both abolishes Dab1 serine phosphorylation and increases its tyrosine phosphorylation in response to Reelin treatment. Interestingly, while serine phosphorylation of the predominant p80 Dab1 isoform negatively regulate its propensity for tyrosine phosphorylation (Ohshima et al., 2007), serine phosphorylation of p45 enhances its tyrosine phosphorylation. Thus, CDK5 may fine-tune Reelin signaling by regulating the serine phosphorylation of Dab1 prior to its association with ApoER2/VLDLR.

Non-phosphorylated Dab1 interacts with CIN85, an SH3-containing adaptor protein involved in endocytosis, as well as CPalpha/CPbeta, subunits of barbed end F-actin-capping proteins (CP). CDK5-dependent serine phosphorylation of Dab1 inhibits its association with CIN85, which in turn may affect actin dynamics (Sato et al., 2007). In a recent study, binding of non-phosphorylated Dab1 to CIN85 was found to enable its association with the
plasma membrane and ApoER2. Moreover, Reelin promotes the interaction of CIN85 with tyrosine-phosphorylated Dab1 (Fuchigami et al., 2013). Thus, CDK5-mediated Dab1 phosphorylation may act as a switch mechanism, preventing the association of Dab1 with CIN85 and the ability of Reelin to signal downstream. Knowledge of the actual sequence of phosphorylation events (serine and tyrosine) in developing and mature neurons will better explain the significance of crosstalk between CDK5 and Reelin signaling.

**β-Amyloid Precursor Protein (APP)**

APP is a type I transmembrane glycoprotein that is most known for its causative role in Alzheimer’s disease. APP KO mice have only subtle alterations in neuronal migration that are worsened by the additional loss of its family members, amyloid precursor-like protein 1 (APLP1) and APLP2 (von Koch et al., 1997; Heber et al., 2000). Recently, in utero knock-down of APP with RNAi, demonstrated that APP is important for migration of neurons in the developing cortical plate in a manner that required Dab1 (Young-Pearse et al., 2007), mediated in part by Dab1 binding to the APP NPxY motif (Homayouni et al., 1999). A genetic interaction between Dab1 and APP has also been noted in Dab1 hypomorphic mice, whose neuronal positioning deficits are worsened by overexpression of mutant human APP(swe) and partially-rescued by APP deficiency (Pramatarova et al., 2008). Additional studies have recently suggested that Reelin might bind to APP directly, wherethrough it regulates its trafficking
and processing (Rice et al., 2013), as well as association with beta1-integrins (Hoe et al., 2009). Further work will be required to establish whether APP represents a core component of the canonical Reelin signaling pathway during neuronal migration, or a point of divergence.

**Integrins**

Integrins represent an important class of adhesion molecules that respond bi-directionally to regulate the interaction between migrating cells and their surrounding environment. The N-terminal region of Reelin has been shown to pull-down with α3β1-integrins, while Dab1 may bind to the NPxY motifs on the beta-integrin cytoplasmic domain (Schmid et al., 2005). Another study found that phosphorylation of Dab1 at Y220 and Y232 regulates α3-integrin levels and promotes the timely detachment of migrating neurons from radial glia (Sanada et al., 2004). However, recent data have challenged the notion that Reelin regulates glia-dependent neuronal migration via α3β1-integrins (Belvindrah et al., 2007). Consistent with an increasingly-accepted role of Reelin in glia-independent migration, Reelin has been shown to activate inside-out α5β1-integrin signaling by utilizing the Dab1-Crk/CrkL-C3G-Rap1 pathway, thereby promoting the adhesion of the leading process to fibronectin in the marginal zone and controlling terminal translocation and proper neuronal positioning (Sekine et al., 2012). The significance of Dab1 binding NPxY motifs in the cytoplasmic domain
of β1-integrins has not yet been established, but likely reflects some role of Dab1 in regulating the endocytosis of this important adhesion molecule.

_Ephrins and EphBs_

Ephrin Bs function primarily through binding to the EphB family of transmembrane tyrosine kinases, which is responsible for tyrosine kinase-mediated signals in Eph-expressing (forward) and ephrin-expressing (reverse) cells. This form of transcellular signaling is critical for processes requiring cell adhesion/repulsion responses, such as axon guidance and synapse development (Klein, 2004; Pasquale, 2008). The existence of a receptor responsible for localizing SFKs to the ApoER2/VLDLR/Dab1 complex has been speculated about for over a decade. Recently, ephrin B receptors have been identified as candidate receptors to perform such a function during brain development. Ephrin Bs were found to bind Reelin and associate at the membrane with ApoER2/VLDLR (Senturk et al., 2011). Clustering of ephrin Bs was sufficient to induce Dab1 phosphorylation, while loss of ephrin Bs prevented Reelin-stimulated Dab1 activation. Consistent with a role for ephrin Bs in Reelin signaling, absence of ephrin B1-3 results in a _reeler_ phenotype, while activation of ephrin Bs can rescue migration errors in _reeler_ mice (Senturk et al., 2011).

A recent study also found that the N-terminal Reelin fragment directly binds to EphB, inducing receptor clustering and forward signaling in a manner that doesn’t depend on ApoER2/VLDLR (Bouche et al., 2013). The activation of
EphB by Reelin leads to cytoskeletal arrangements in vitro, suggesting that Reelin-mediated EphB forward signaling is a form of non-canonical Reelin signaling that is also important for neuronal migration. In fact, similar displacements of CA3 pyramidal cells are seen in EphB1/2 KO mice and reeler mice. Even though this study could repeat the previously reported binding of Reelin to Ephrin B3 (Senturk et al., 2011), it did not find that EphB3 promoted Dab1 phosphorylation (Bouche et al., 2013).

Together, Ephrins and EphBs may represent important new members of the canonical and non-canonical Reelin signaling pathway, respectively. Future studies will likely be required before the roles of these receptors in the developmental and adult function of the Reelin signaling pathway becomes widely-accepted.

Notch

The Notch signaling pathway has a well-characterized role in both neurogenesis (Yoon and Gaiano, 2005) and dendritogenesis (Louvi and Artavanis-Tsakonas, 2006). During cortical development, inhibition of Notch generates a reeler phenotype in the dentate gyrus (DG), characterized by a failure of the radial glial scaffold to form and dispersion of granule cells. Additional experiments have demonstrated that Reelin acts through Notch to promote the formation of the radial glia scaffold and DG development (Sibbe et al., 2009). This is supported by in vitro data demonstrating that the Reelin
canonical pathway promotes radial glia maturation via Notch activation (Keilani and Sugaya, 2008). The activation of Notch by Reelin is also required for proper neuronal migration in the cortex, where Reelin signaling was found to inhibit the degradation of the Notch intracellular domain, known to translocate to the nucleus to initiate transcription (Hashimoto-Torii et al., 2008).

Conclusion

Taken together, this section has described emerging roles of several important signaling cascades that may act downstream of Reelin during development. Many aspects of how Reelin signaling engages these and other unidentified partners is likely conserved, and may be relevant to our understanding of how Reelin influences other aspects of development and adult brain function.

Other Considerations

When considering above-mentioned details of Reelin signaling during development, it is important to remember that there are countless other factors that serve to fine-tune the Reelin signal, including restricted expression and/or alternative splicing of individual Reelin receptors, differential processing of Reelin, competition with other lipoprotein receptors ligands, etc. It is not the goal of this review to discuss each of these regulatory mechanisms in detail, as their relevance to developmental and adult processes are still poorly understood.
Instead, for this sub-section, we will focus on Reelin proteolysis, which is essential for Reelin’s function during development (Jossin et al., 2007) and perturbed in a number of disease states, particularly epilepsy (Duveau et al., 2011; Tinnes et al., 2011; Tinnes et al., 2013).

During cortical development, metalloproteinase inhibition prevents Reelin processing and downstream activation of Dab1, resulting in impaired neuronal migration (Lambert de Rouvroit et al., 1999; Jossin et al., 2007). This finding implies that Reelin is tethered to the extracellular matrix following secretion, where it remains inactive until liberated by proteases. Indeed, Reelin undergoes proteolytic cleavage at two main sites, between EGF-like repeats 2-3 and 6-7 (Jossin et al., 2004). Full-length Reelin and the 5 potential fragments generated by Reelin cleavage can be observed in the developing and adult brain. The specific function of Reelin processing and the contexts in which it occurs is not known.

The Reelin N-terminal region (N-R2) is important for protein homopolymerization and signaling (D’Arcangelo et al., 1997), but is not essential for lipoprotein receptor binding (Jossin et al., 2004). Disruption of Reelin aggregation with the CR-50 antibody impairs neuronal migration (Nakajima et al., 1997). The N-terminal region of Reelin has been reported to bind α3β1-integrins (Dulabon et al., 2000) and EphBs (Bouche et al., 2013). On the other hand, the central region of Reelin (R3-6) is responsible for binding both ApoER2 and VLDLR (D’Arcangelo et al., 1999), as well as APP (Hoe et al., 2009), and may be sufficient for regulating Reelin dimerization (Yasui et al., 2011). Lastly, the highly-
charged C-terminal region (R7-C) may be involved in Reelin folding, secretion (D'Arcangelo et al., 1997; de Bergeyck et al., 1997), and signaling efficacy (Nakano et al., 2007).

Despite the existence of several Reelin fragments *in vivo*, only fragments containing R5-6 are capable of binding ApoER2 and VLDLR. Both Lys-2360 and Lys-2467, found in Reelin-R6, are directly responsible for coordinated binding of Reelin to the conserved ligand binding domains of ApoER2/VLDLR (Yasui et al., 2007; Yasui et al., 2010). In agreement with these findings, application of Reelin fragments containing R5-6 to *reeler* cortical explants is sufficient to induce Dab1 phosphorylation and normalize cortical lamination (Jossin et al., 2004). However, exogenous Reelin application bypasses the need for local secretion and extracellular proteolysis, which is underscored by findings that the broad-spectrum metalloproteinase inhibitor GM6001, which blocked Reelin processing between EGF-like repeats 2-3, could prevent endogenous Reelin processing, Dab1 activation, and normal cortical lamination in cortical explant cultures (Jossin et al., 2007). Thus, although Reelin fragments containing R3-6 are capable of binding ApoER2/VLDLR, extracellular proteolysis is the penultimate step for Reelin regulation at least during development, and likely in the adult.

Recent studies have demonstrated that a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) processes Reelin (Hisanaga et al., 2012; Krstic et al., 2012). The p50 isoform of ADAMTS-4 cleaves Reelin at its N-terminal cleavage site, whereas the p75 isoform cleaves Reelin at both sites. In addition to ADAMTS-4, another study found that
ADAMTS-5 more broadly degrades Reelin into smaller and presumably inactive fragments (Krstic et al., 2012). The significance of ADAMTS-4/5-mediated Reelin processing has not yet been established. Also, the urokinase plasminogen activator (uPA) (Lugli et al., 2003) and tissue plasminogen activator (tPA) (Krstic et al., 2012) have both been found to cleave Reelin at its C-terminal site, but the function of this cleavage event is not yet known. Although there are now several candidate Reelin cleaving enzymes, the identity of the specific enzymes that mediate developmental processing of reelin remains elusive.

Another important example of how the processing of Reelin by metalloproteinases may dictate its function occurs in the epileptic hippocampus. Granule cell dispersion (GCD) in the dentate gyrus is considered a major hallmark of temporal lobe epilepsy and in rodents, GCD can be induced by administering the excitotoxin Kainic acid (KA) (Haas et al., 2002; Gong et al., 2007). Importantly, blocking Reelin signaling in the hippocampus is sufficient to induce GCD in the absence of epileptiform activity (Heinrich et al., 2006), while enhancement of Reelin signaling can mitigate GCD caused by KA (Muller et al., 2009). In addition to reduced Reelin expression levels, there is an apparent increase in the levels of full-length Reelin and reduction in its major fragments in the epileptic hippocampus (Tinnes et al., 2011). Supporting a role for MMP inhibition in blocking Reelin processing, treatment with TIMP-1 or chemical inhibition of metalloproteinases is sufficient to cause GCD (Tinnes et al., 2011; Tinnes et al., 2013). Thus, Reelin processing is critical in the adult brain, at least
in the context of pathological increases in synaptic activity as seen in epilepsy models.

*Other Systems*

In addition to cortical projection neurons, Reelin signaling is also critical for the development of other structures in the nervous system, including (but not limited to) the spinal cord (Phelps et al., 2002; Yip et al., 2004; Yip et al., 2007), retina (Rice et al., 2001; Trotter et al., 2011), olfactory bulb (Hellwig et al., 2012), and midbrain (Rossel et al., 2005). Most studies have focused on the development of excitatory neurons, but comparatively few have considered other cell types including GABAergic interneurons and radial glia. In this subsection, we will briefly consider the role of Reelin signaling in the development of GABAergic interneurons.

The placement of GABAergic interneurons, similar to their excitatory counterparts, is severely perturbed in the forebrain of *reeler* mice, while their dendrites are significantly larger (Yabut et al., 2007). However, the displacement of interneurons in the *reeler* mutants appears to be an indirect result of projection neuron displacement, as *scrambler*-derived GABAergic interneurons (early and late) position normally in the wild-type cortex (Pla et al., 2006). A separate study found that the layer acquisition of late born neurons requires Reelin, but not of early-born neurons (Hammond et al., 2006). Despite evidence that Dab1 is widely-expressed by developing GABAergic interneurons (Pla et al., 2006), there
have been no follow-up studies to address the extent to which Reelin signaling influences the development of GABAergic interneurons.

**Dendritic Morphogenesis**

While neuronal positioning largely occurs during embryogenesis, the development of morphologically-mature neurons requires a period of protracted postnatal development. Tightly-regulated morphological development of neurons is required for their eventual ability to receive signals, integrate them, and propagate output to target cells. Both axonal and dendritic compartments are generated through developmental neuronal polarization, which begins with the formation of an axonal process followed by the asymmetric development of dendrites (Arimura and Kaibuchi, 2005). Although there is some evidence that Reelin influences axonal development (Borrell et al., 2007; Leemhuis et al., 2010; Matsuki et al., 2010), this section will focus primarily on the established role of Reelin signaling in dendritogenesis.

The morphology and orientation of dendrites is severely-perturbed in *reeler* and *scrambler* mice, which suggested initially that Reelin signaling may regulate both neuronal positioning and dendritic development (D'Arcangelo et al., 1995). Supporting this notion, loss of Reelin signaling in cultured neurons leads to reduced dendritic outgrowth and branching (Niu et al., 2004; MacLaurin et al., 2007). Further strengthening the argument that Reelin signaling separately influences neuronal positioning and dendritogenesis, neurons position normally
in layer 6 of the reeler cortex, but require Reelin for proper neuronal orientation and dendritogenesis (Nichols and Olson, 2010; O'Dell et al., 2012). Also, conditional early-postnatal deletion of Dab1 reduces dendritic branching of normally-positioned CA1 pyramidal cells (Matsuki et al., 2008).

The specific mechanisms through which Reelin acts to facilitate dendritogenesis are still poorly understood. A seminal study in the field established that Reelin promotes dendritic development by binding lipoprotein receptors and promoting the downstream activation of Dab and SFKs (Niu et al., 2004). Recent studies have identified additional requirements for Reelin-dependent dendritogenesis, including the activation of the PI3K/Akt/mTOR pathway (Jossin and Goffinet, 2007), association of Dab1 with the Crk adaptor proteins (Matsuki et al., 2008), and regulation of the interaction between APP and α3β1-integrins (Hoe et al., 2009). Interestingly, the N-terminal Reelin fragment, which does not act through the canonical Reelin pathway, promotes the dendritic maturation of cortical layer II/III pyramidal neurons (Chameau et al., 2009). Additional experiments will be required to better understand how the proteolytic processing of Reelin into functionally-distinct fragments regulates dendritogenesis.

Although its role in dendrite development is not yet well understood, Reelin signaling also promotes the deployment of the Golgi apparatus into developing dendrites (Matsuki et al., 2010). A recent study found that Reelin-mediated Golgi deployment requires the activation of the Cdc42/Rac1 guanine nucleotide exchange factor, αPIX/Arhgef6 (Meseke et al., 2013). The deployment
of the Golgi apparatus may represent a penultimate output of Reelin signaling during dendritogenesis, as the Golgi apparatus is hypothesized to provide the increased materials needed to support a growing membrane surface, including lipids and glycosylated proteins such as adhesion molecules and receptors (Ye et al., 2007).

In addition to principle neurons, there are other cell types that may require Reelin for proper dendritogenesis, including GABAergic interneurons and adult-born granule cells of the dentate gyrus (DG). In reeler mice, the positioning and dendritic development of cortical GABAergic interneurons is severely-perturbed (Yabut et al., 2007). Although recent data indicate that interneuron displacement in Reelin signaling deficient mice emerges from abnormal positioning of projection neurons (Pla et al., 2006), the extent to which Reelin regulates interneuron dendritic development has not been studied previously. In the adult brain, neural progenitors residing in the subgranular zone of the DG generate immature granule cells that undergo distinct phases of migration, axon and dendrite development, and synaptic maturation prior to their integration into the hippocampal synaptic pathway. Over-expression of Reelin accelerates dendritic maturation of adult-born dentate granule cells, while loss of Dab1 expression in developing granule cells leads to aberrant migration, decreased dendritic development, and ectopic dendrite formation in the hilus (Teixeira et al., 2012). More detailed study of GABAergic interneuron and GC dendrite development may reveal whether Reelin signaling is universally-required for dendrite development.
**Synapse Formation**

A critical stage of postnatal brain development is synaptogenesis, an intricate process that interconnects vastly complicated neuronal networks through the successive generation of synaptic competence and apposition of presynaptic and postsynaptic anatomical structures (Craig et al., 2006). In previous sections, we comprehensively reviewed the mechanisms of Reelin signaling during neuronal positioning and dendrite development. Here we will review known functions of Reelin signaling in synaptogenesis.

Throughout postnatal synaptic development and on through adulthood, Reelin is expressed primarily by a subset of GABAergic interneurons positioned throughout forebrain circuitry (Pesold et al., 1999). Reelin, ApoER2 and Dab1 have been localized to the postsynaptic density of excitatory synapses (Rodriguez et al., 2000; Beffert et al., 2005; Dumanis et al., 2011), implicating this site as a major locus of Reelin signaling at synapses. Importantly, the possibility that Reelin interacts with other synaptic compartments has not been addressed previously, which is why this section will focus exclusively on the development of excitatory glutamatergic synapses.

In hippocampal area CA1, partial or complete absence of Reelin signaling leads to a profound reduction in the formation of dendritic spines (Liu et al., 2001; Dumanis et al., 2011) and reduced expression of excitatory postsynaptic proteins (i.e. NR2A, PSD95) (Niu et al., 2008; Ventruti et al., 2011). Observed reductions in spine density in the heterozygous *reeler* mouse (HRM) occurs without
detectable alterations in neuronal positioning or dendritic branching (Liu et al., 2001; Niu et al., 2008). Thus far, it seems that Reelin promotes dendritic spine formation through Dab1, ApoER2/VLDLR, and SFKs (Niu et al., 2008), while other established mechanisms of Reelin signaling during development have not yet been tested.

Although the positioning of the neuronal soma and dendritic arborization is relatively fixed following the completion of development, synapses continue to undergo dynamic morphological changes throughout life. It is possible that Reelin exerts similar effects on newly formed synapses in the developing and adult brains. Existing data agree with this notion, as wild-type mice intraventricularly-injected with Reelin have increased spine density in CA1 (Rogers et al., 2011). Although mice ectopically overexpressing Reelin in pyramidal cells do not have increased spine density, they do have increased presynaptic terminal density and dendritic spine hypertrophy (Pujadas et al., 2010). In these mice, hypertrophic spines were associated with an increased number of synaptic junctions and morphologically-resembled large mushroom spines that form following learning or long-term potentiation (LTP) (Yuste and Bonhoeffer, 2001). Inducible loss of Reelin-overexpression in these mice led to a complete reversal of presynaptic density and spine morphology changes, as well as a significant reduction in dendritic spine density. The change in spine density only after reducing ectopically over-expressed Reelin, suggests that the formation of dendritic spines is highly sensitive to Reelin levels. Taken together these data suggest that Reelin signaling is a critical determinant of glutamatergic synapse structure and density
in the adult brain. Future studies should address the mechanisms wherethrough Reelin signaling modulates glutamatergic synapses, as well as determine if other types of synaptic structures, particularly inhibitory synapses, are sensitive to Reelin signaling.

**Synaptic Maturation**

The final step of synaptic development is maturation, which enables synapses to stabilize their presynaptic and postsynaptic properties. Newly generated synapses contain primarily NR1/NR2B NMDA receptor subunits, whereas mature synapses contain mostly NR1/NR2A subunits (van Zundert et al., 2004). This switch in subunit composition is essential for the completion of synapse maturation, synaptic plasticity, and establishing normal neural network function in the adult (Bear, 2003; Collingridge et al., 2004). Several factors have been identified that regulate the maturational switch of NMDAR composition, including Reelin (Sinagra et al., 2005; Groc et al., 2007; Qiu and Weeber, 2007). In this section, we will summarize evidence suggesting that synaptic maturation is an important ontological window of Reelin signaling.

In dissociated neuronal cultures, Reelin has been found to regulate the maturation of both somatic and synaptic NMDARs (Sinagra et al., 2005; Groc et al., 2007; Qiu and Weeber, 2007). Interestingly, Reelin promotes the down-regulation of somatic NMDARs via the canonical cascade, requiring lipoprotein receptor binding and SFK activation (Sinagra et al., 2005; Qiu and Weeber,
On the other hand, Reelin promotes the maturational switch of synaptically-expressed NR1/NR2B in a manner that requires β1-integrins, but not the canonical cascade. These data imply that individual Reelin fragments, capable of divergent downstream signaling via integrins and lipoprotein receptors, exert differential influence on synaptic maturation.

A recent study provided evidence that impairments in Reelin-mediated synaptic maturation leads to profound reductions in dendritic spine density and synaptic plasticity in the prefrontal cortex (Iafati et al., 2013). These impairments were completely normalized following administration of the NMDAR antagonist ketamine, or specific inhibition of NR2B (Iafati et al., 2013), providing evidence that Reelin mediates synaptic maturation in the intact postnatal brain. Mice that over-express Reelin exhibit increased resilience to behavioral phenotypes of schizophrenia and bipolar disorders, including reduced depressogenic behavior following corticosterone treatment, reduced behavioral sensitization to cocaine, and reduced paired-pulse inhibition deficits induced via NMDAR antagonism (Teixeira et al., 2011). Interestingly, these mice also have a marked resistance to stress-mediated up-regulation of NR2B-mediated synaptic transmission in the hippocampus, a finding which further strengthens the notion that the modulation of NMDAR composition by Reelin is also critical throughout adulthood.

Another determinant of glutamatergic synapse maturation is the insertion of AMPARs, which render “silent synapses” that only contain NMDARs active (Isaac et al., 1995). In addition to promoting NMDAR compositional maturation, Reelin also facilitates AMPAR insertion in a manner that depends on lipoprotein
receptor binding and SFK activation (Qiu and Weeber, 2007). A role for integrins in the unsilencing of synapses has not been explored previously, though this should be explored in the context of non-canonical Reelin signaling.

An additional locus of Reelin signaling may be at the glutamatergic presynaptic site, where Reelin has been suggested to influence neurotransmitter release (Hellwig et al., 2011). In the reeler hippocampus, although the density of presynaptic boutons is normal, there is an increase in the number of neurotransmitter vesicles. Altered vesicle number is likely driven by impairments in the machinery for synaptic vesicle exocytosis, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Indeed, reeler mice have reduced SNAP25 expression, but no change in other SNARE components. Reelin was able to rescue SNAP25 expression and vesicle number via activation of non-canonical signaling via integrins (Hellwig et al., 2011), while canonical signaling was not required. Further supporting an involvement of Reelin in presynaptic maturation, as mentioned in an earlier section, ectopic over-expression of Reelin leads to a profound increase in the density of presynaptic terminals throughout the hippocampus (Pujadas et al., 2010). Although these studies support a role for Reelin signaling in the maturation and function of presynaptic terminals, additional studies will be required to better establish the mechanism of Reelin action.
Adulthood

Synaptic Plasticity

So far, we have discussed the role of Reelin signaling across a continuum of ontologically-distinct developmental periods, spanning neuronal migration during corticogenesis to postnatal synaptic maturation. Although the positioning of neurons and their dendrites is relatively fixed in the postnatal brain, the generation and maturation of synapses continues throughout adult life. Changes in the strength of individual synapses are thought to be required for the formation and maintenance of long-term memories. Underpinning altered synaptic strength, is a multiplicity of structural, biochemical, and electrical modifications that enable sustained short-term and long-term changes in synapses, or plasticity. In this section we will discuss a form of long-term synaptic plasticity that has been studied now for over 40 years, long-term potentiation (LTP) (Bliss and Lomo, 1970).

LTP is a form of persistent synaptic strengthening that represents the prominent cellular model of synaptic plasticity. Indeed, LTP is widely hypothesized to utilize similar mechanisms as those responsible for changes in synaptic strength during memory formation and consolidation (Tsien et al., 1996; Whitlock et al., 2006). Several studies have found that Reelin signaling may be important for LTP, which could explain why mice with partial reductions in Reelin signaling have memory deficits (Weeber et al., 2002; Beffert et al., 2005; Beffert
et al., 2006; Qiu et al., 2006b). In this section, we will consider the role of Reelin signaling in synaptic plasticity, which comprises a critical ontological phase of Reelin signaling.

A seminal study in the field reported that perfusion of wild-type hippocampal slices with partially-purified Reelin led to a significant enhancement of LTP. Moreover, in the absence of either ApoER2 or VLDLR, slices failed to respond to Reelin treatment (Weeber et al., 2002). An important function of Reelin signaling in LTP was further confirmed by findings of impaired LTP in both ApoER2 KO (Weeber et al., 2002) and heterozygous reeler mice (Qiu et al., 2006b). Evidence that activation of Dab1 is necessary for the enhancement of LTP by Reelin comes from a study describing ApoER2 knock-in mice with a mutated NPxY motif that prevents Dab1 binding, which show impaired LTP and only minimal response to Reelin (Beffert et al., 2006). Although important, all early studies utilized mouse models with known developmental abnormalities (Trommsdorff et al., 1999; Niu et al., 2008; Dumanis et al., 2011), which means that reported changes in synaptic plasticity are confounded by underlying development impairments. Overcoming the limitation of traditional knock-out or knock-in approaches will require the use of conditional transgenic strategies, which afford more precise spatiotemporal control of target gene expression.

The alternatively-spliced exon19 of ApoER2 allows it to physically couple to the NMDAR complex via binding the scaffold protein, post-synaptic density 95 (PSD-95) (Beffert et al., 2005; Hoe et al., 2006). Unlike ApoER2 KO and NPxY mutants, mice deficient in ApoER2 exon19 do not have detectable changes in
neuronal positioning, but could still have altered synaptic development. While absence of ApoER2 exon19 does not affect LTP by itself, it does prevent the enhancement of LTP by Reelin (Beffert et al., 2005). The same study also demonstrated that ApoER2 (containing the exon19 insert) physically associates with the NMDAR complex, allowing Reelin to increase the phosphorylation of NR2A and NR2B subunits and overall activation of NMDARs (Beffert et al., 2005). The upstream mechanism throughout which Reelin enhances LTP thus appears to require the binding of Reelin to ApoER2 and VLDLR and enhancement of NMDAR activation (Beffert et al., 2005). Importantly, the absence of basal impairments in LTP in ApoER2 exon19-deficient mice suggests that Reelin may not be absolutely required for LTP at the Schaffer collateral / CA1 synapse.

What mechanisms are responsible for the enhancement of LTP by Reelin? Although this has not yet been firmly established, it appears that Reelin enhances the activation of NMDARs via SFK-mediated phosphorylation of the NR2A and NR2B subunits (Qiu et al., 2006a), which is known to increase calcium influx (Kohr and Seeburg, 1996). Indeed, Reelin treatment increases glutamate-triggered calcium influx through NMDARs, in a manner that also requires receptor binding and activation of Dab1 and SFKs (Chen et al., 2005). Reelin also increases AMPAR-mediated neurotransmission by promoting the surface insertion of AMPARs. In contrast to the activation of NMDARs, the effect on AMPARs requires lipoprotein receptor binding and Akt activation, but not the activation of SFKs (Qiu et al., 2006a). The enhancement of both NMDAR and
AMPAR activities likely underlies at least some of Reelin’s ability to enhance LTP, as both NMDA and AMPA receptors are critical components of LTP at glutamatergic synapses.

A recent study reported that Reelin-dependent maturation of NMDAR subunit composition may also contribute to LTP. They found that the maintenance of LTP was reduced in the prefrontal cortex of juvenile HRM, a change which was normalized by blocking NMDARs with ketamine or the NR2B subunit specifically, 24 hours earlier (Iafriati et al., 2013). Additional studies will likely uncover other mechanistic ties between Reelin signaling and the molecular machinery that underlie the expression of long-term synaptic plasticity. An emphasis, however, should be placed on testing proposed mechanisms in adult hippocampal slices in the context of stimulated LTP; this is important because most mechanistic insight in the adult has been obtained using primary dissociated neurons or treatment of hippocampal slices without electrical stimulation.

The most impactful limitation of all of the mouse models that have been discussed thus far, is that they are constitutive in nature, and thus, changes observed could simply reflect subtle alterations in neuronal positioning or the development of synapses, and not an active and ongoing role of Reelin signaling in synaptic plasticity. Several findings, however, support an active and ongoing function of Reelin in regulating synaptic plasticity in the adult brain. At 5 days following a single bilateral, intraventricular injection of Reelin into the wild-type brain, our lab has observed increased dendritic spine density and enhanced LTP
(Rogers et al., 2011). We have also reported that HRM have reduced LTP (Qiu et al., 2006b; Rogers et al., 2013), which can be recovered following a single intraventricular dose of Reelin (Rogers et al., 2013). Finally, mice that overexpress Reelin also have dramatic enhancements of LTP (Pujadas et al., 2010). Even though these findings demonstrate that an increase of Reelin level in adult wild-type mice can enhance LTP, they still do not confirm an active and acute role of Reelin in the molecular processes that underlie LTP. This is because these studies evaluated LTP long after initial exposure to increased Reelin levels. Only through utilizing conditional knockout strategies, which afford precise spatiotemporal control of target gene expression, will we be able to elucidate the role of Reelin signaling in adult synaptic plasticity.

Recent studies have found that the modulation of LTP by Reelin is impaired in mouse models for Alzheimer’s disease (Chen et al., 2010). Neurotoxic oligomers of the amyloid-beta (Aβ) peptide, which are found in the brains of Alzheimer’s patients, impair LTP by activating protein phosphatases which dephosphorylate and promote the endocytosis of AMPAR/NMDAR complexes (Snyder et al., 2005; Hsieh et al., 2006). Reelin can counteract the effects of Aβ oligomers on LTP by activating SFKs and normalizing the surface expression of glutamate receptor subunits (Durakoglugil et al., 2009). Mice carrying the human apoE4 allele (Chen et al., 2010), a major genetic risk factor for late-onset Alzheimer’s disease, have reduced surface expression of ApoER2 and do not exhibit the normal enhancement of LTP by Reelin or the ability of Reelin to mitigate Aβ neurotoxicity (Chen et al., 2010). This is in contrast to mice
carrying either the apoE2 (protective) or apoE3 (neutral) alleles, which respond to Reelin with enhanced LTP and protection from Aβ neurotoxicity (Chen et al., 2010).

**Learning and Memory**

Successful learning results in memory formation, a process which requires a multitude of complex, coordinated changes in synaptic structure and function. We have demonstrated previously that mice deficient in either ApoER2 or VLDLR (Weeber et al., 2002), as well as the HRM (Qiu et al., 2006b), have impairments in hippocampal-dependent fear associative learning. Moreover, mice lacking ApoER2 exon19 (Beffert et al., 2005) or expressing ApoER2 that is unable to bind Dab1 (Beffert et al., 2006) also have deficits in fear associative learning, as well as spatial memory. Recently, it was shown that the lipoprotein receptor antagonist receptor-associated protein (RAP) can interfere with spatial learning in the hidden-platform watermaze (HPWM) task when injected into the lateral entorhinal cortex, which serves as the primary input to the hippocampus via the perforant pathway (Stranahan et al., 2011). RAP interferes non-selectively with all lipoprotein receptors by blocking their ligand-binding domains, so this study cannot conclusively associate its findings with a Reelin signaling impairment. However, the ability of acute interference of lipoprotein receptor signaling to impair learning, suggests that Reelin and other lipoprotein receptors ligands may be actively involved in the molecular processes underlying memory formation.
Further supporting this notion, we recently demonstrated that intraventricular administration of Reelin can enhance fear associative and spatial learning in wild-type mice (Rogers et al., 2011) and can recover learning deficits in the HRM model (Rogers et al., 2013). These data strongly suggest that Reelin signaling is important for the molecular processes of hippocampal-dependent contextual fear learning, and that learning impairments observed in HRM may reflect a tonic decrease in Reelin levels rather than perturbed synaptic development. However, a significant time gap did exist between Reelin administration and the learning and memory tests, suggesting that while Reelin may have persistent effects on synapses involved in memory formation, the time and site of its action remains a mystery.

Future studies should utilize region-specific and/or inducible deletion of Reelin signaling components as an approach for ascertaining the extent to which Reelin influences behavior, particularly the formation of long-term memories. Also, fear associative learning is poised as a paradigm to serve as a model system for bridging the large gap in understanding of molecular mechanisms of Reelin signaling in the adult brain. Ultimately, ascertaining how Reelin signaling influences the formation and maintenance of memories, may lead to better understanding and treatment of disorders associated with impaired Reelin signaling, particularly Alzheimer’s disease and schizophrenia.
Concluding Remarks

In this chapter, we comprehensively reviewed several distinct ontological phases of Reelin signaling, ranging from cortical migration during development to synaptic plasticity in the adult brain. Our primary goal was to contextualize Reelin signaling, so that similarities and differences could be drawn between this important signaling pathway in distinctively different stages of nervous system function. For example, it should be clear that the study of Reelin signaling during cortical development has identified several downstream signaling events that may also be helpful for understanding how Reelin signals in the adult brain, but have not yet been tested in this context. Many facets of Reelin signaling are likely to be conserved across spatiotemporal boundaries. For example, the mechanisms whereby Reelin promotes the maintenance or formation of dendritic spines, which is currently unknown, will likely share similarities to how Reelin stabilizes the leading process of migratory neurons in the marginal zone. However useful, what we describe as ontological phases of Reelin signaling, will eventually prove to be too general, as it is very likely that within each of these contexts, Reelin acts upon numerous types of cells, synapses, and even non-synaptic structures. Nonetheless, with the advent of conditional transgenic models that allow unprecedented spatiotemporal control of target gene expression, the study of Reelin signaling within each of the ontological stages defined by this review is now possible.
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Chapter 2

Extracellular Proteolysis of Reelin by Tissue Plasminogen Activator Following Synaptic Potentiation

Abstract

The secreted glycoprotein reelin plays an indispensable role in neuronal migration during development and in regulating synaptic function in the adult. The upstream mechanisms responsible for initiating and regulating the duration and magnitude of reelin signaling are largely unknown. Here we report that reelin is cleaved between EGF-like repeats 6-7 (R6-7) by tissue plasminogen activator (tPA) under cell-free conditions. No changes were detected in the level of reelin and its fragments in the brains of tPA knockouts, implying that other unknown proteases are responsible for generating reelin fragments found constitutively in the adult brain. Since forskolin treatment has been previously shown to lead to tPA- and NMDAR-dependent long-term potentiation (LTP), we evaluated its effects on reelin expression and processing. We found that forskolin treatment resulted in altered processing of reelin in hippocampal area CA1, an effect not observed in tPA knockouts. Induction of NMDAR-independent LTP with the potassium channel blocker tetraethylammonium chloride (TEA-Cl) led to a
specific up-regulation of reelin processing at R6-7. However, no changes in reelin expression and processing were observed in tPA knockouts following TEA-Cl treatment. These results demonstrate that synaptic potentiation results in tPA-dependent reelin processing and suggest that extracellular proteolysis of reelin may regulate reelin signaling in the adult brain.

**Introduction**

The inside-out layering of the mammalian cortex requires the extracellular glycoprotein reelin, which is secreted by Cajal-Retzius cells (D’Arcangelo et al., 1995; Hirotsune et al., 1995). Loss of reelin or downstream signaling components (ApoER2/VLDLR, Dab1, etc.) leads to a roughly inverted cortex, perturbed hippocampal lamination, and cerebellar hypoplasia (Trommsdorff et al., 1999). In addition to playing a pivotal role in neuronal migration, reelin signaling is necessary for dendritic morphogenesis (Niu et al., 2004), synapse development (Groc et al., 2007; Qiu and Weeber, 2007; Niu et al., 2008; Rogers et al., 2011; Trotter et al., 2011), and synaptic plasticity (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011). Changes in reelin expression (Impagnatiello et al., 1998; Botella-Lopez et al., 2006; Chin et al., 2007; Herring et al., 2012), processing (Botella-Lopez et al., 2010; Duveau et al., 2011; Tinnes et al., 2011) and glycosylation (Botella-Lopez et al., 2006) have been associated with the pathoetiology of a range of neuropsychiatric and neurodegenerative diseases,
underscoring the importance of understanding mechanisms that regulate reelin in the adult brain.

Reelin is processed at two main sites (Fig. 1A), between EGF-like repeats 2-3 (R2-3) and 6-7 (R6-7) (Jossin et al., 2004). Full-length reelin and the 5 potential fragments generated by reelin cleavage can be observed in both the developing and adult brain (Jossin et al., 2007; Krstic et al., 2012). The N-R2 region of reelin (Fig. 1A) is important for protein homopolymerization and signaling (D'Arcangelo et al., 1997), but is not essential for lipoprotein receptor binding (Jossin et al., 2004). In fact, disruption of reelin aggregation with the CR-50 antibody, which binds the CR-50 region between N-R2 (Fig. 1A), perturbs neuronal migration \textit{in vivo} (Nakajima et al., 1997). The N-R2 region has also been reported to bind \( \alpha_3\beta_1 \)-integrins (Dulabon et al., 2000). The preponderance of known reelin functions require the R3-6 region (Fig. 1A), which is responsible for binding apoE receptor 2 (ApoER2), very low-density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999), and amyloid precursor protein (APP) (Hoe et al., 2009). Consistent with these findings, application of reelin fragments containing R5-6 to \textit{reeler} cortical explants is sufficient to induce Dab1 phosphorylation and normalize cortical lamination (Jossin et al., 2004). The highly-charged C-terminal region (R7-C) may be involved in reelin folding, secretion (D'Arcangelo et al., 1997; de Bergeyck et al., 1997), and signaling efficacy (Nakano et al., 2007), but is not known to bind to receptors.

While significant progress has been made in delineating downstream mechanisms of reelin signaling, upstream mechanisms operative in the
developing and adult nervous system remain elusive. Similar to other extracellular signaling molecules, a critical locus of reelin signaling regulation is at the level of transcription (Erbel-Sieler et al., 2004; Wang et al., 2004; Chen et al., 2007; Miller and Sweatt, 2007; Cubelos et al., 2008), although receptor availability (Duit et al., 2010; Hong et al., 2010) and secretion (Duveau et al., 2011) may also play a role. However, these mechanisms only serve to adjust the level of reelin signaling, as they are not sufficient to initiate the reelin signal by themselves. In support of this view, preventing extracellular proteolysis of reelin by inhibiting metalloproteinases blocks signaling in the developing cortex and disrupts corticogenesis (Lambert de Rouvroit et al., 1999; Jossin et al., 2007). These findings imply that reelin is tethered to the extracellular matrix following secretion, where it remains inactive until liberated by proteolysis to initiate downstream signaling.

Recent \textit{in vitro} studies have identified a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4/5) and tissue plasminogen activator (tPA) as candidate enzymes capable of generating major reelin fragments (Krstic et al., 2012). Although little is known about the function of ADAMTS-4 and -5 in the brain, tPA represents a promising reelin protease candidate due to its high expression in the hippocampus and important role in learning and memory (Qian et al., 1993; Barnes and Thomas, 2008). Moreover, activity-dependent secretion and activation of tPA has been found to be critical for synapse formation and hippocampal synaptic plasticity (Pang et al., 2004; Nagappan et al., 2009), processes which also require reelin signaling (Weeber et
al., 2002; Pujadas et al., 2010; Rogers et al., 2011). The extent to which tPA cleaves reelin in vivo and its potential significance has not been previously addressed. In this study we present evidence that tPA is a critical regulator of reelin processing following synaptic potentiation.

Materials and Methods

Animals

Both C57BL/6J (wild-type) and tPA knockout mice (B6.129S2_Plat^{tm1mlg}/j) were obtained from Jackson Laboratory. For experiments, males at approximately 4 months of age were used. Animals were grouped housed in a standard 12 hour light/dark cycle and fed ad libitum standard mouse chow. All animal care protocols were followed in accordance with the Institutional Animal Care and Use Committee of the University of South Florida.

Chemicals and Reagents

Plasminogen (P7397), activated plasmin (P1867), Diisopropylfluorophosphate (DIFP; D0879), tetraethylammonium chloride (TEA-Cl; 86616) and forskolin (F3917) were obtained from Sigma. Recombinant tissue plasminogen activator (tPA; #176) was obtained from American Diagnostica Inc. Plasminogen activator inhibitor-1 (PAI-1, 528205) and aprotinin (616371) were
purchased from Calbiochem. HyClone Dulbecco’s Phosphate Buffered Saline (DPBS) without magnesium and calcium and Halt Protease Inhibitor Cocktail (78425) were obtained from Thermo Scientific. Mammalian protein extraction reagent (M-PER) was obtained from Thermo Pierce. Reelin G10 antibody (MAB5364) was obtained from Millipore and was used to visual full-length reelin and the 370 and 180 kDa fragments. Reelin ab14 antibody was generously provided by Andre Goffinet. Beta-actin (#4967) and p-ERK1/2 antibodies (Tyr202/Tyr204; #4370S) were obtained from Cell Signaling.

**Recombinant Protein Production**

HEK293 cells were transfected with full-length reelin pCrl vector to produce recombinant reelin as described previously (Qiu and Weeber, 2007).

**Cell-Free Reelin Processing Assay**

Partially-purified reelin (50 nM) was incubated with recombinant tPA (0, 5, 25, 100, 200, and 400 nM) for 15 minutes at 37°C in DPBS. The reactions were terminated by combining them with equal volume of Laemmli Sample Buffer (Bio-Rad) with 5% 2-mercaptoethanol. The Halt protease inhibitor cocktail (without EDTA) was used to block Reelin processing by 200 and 400 nM of tPA. The cocktail was used at a 1X concentration, resulting in final concentrations of 1 mM AEBSF, 800 nM aprotinin, 50 μM Bestatin, 15 μM E64, 29 μM Leupeptin, and 10
µM Pepstatin A. A time course of reelin processing by tPA and plasminogen was established by incubating reelin (50 nM) with tPA (50 nM), plasminogen (18 µg/µl), tPA and plasminogen, or active plasmin (0.5 U/ml) in DPBS at 37°C for 15 and 45 minutes. The aforementioned reactions were also mixed separately with aprotinin (40 µM), DIFP (100 µM), PAI-1 (1 ng/ul) for 45 minutes at 37°C to establish the role of serine protease activity in reelin processing by the tPA/plasminogen system. Reactions were mixed with CR-50 (0.02 µg/µl) for 45 minutes at 37°C to determine the role of reelin dimerization in its processing. All in vitro experiments were performed in triplicate.

**Ex vivo Slice Culture Assay**

Mice were euthanized and their brains were dissected as described previously (Weeber et al., 2002). Brains were sectioned horizontally in ice-cold cutting solution at 400 µm. The hippocampus was dissected and acclimated in 50:50 solution (cutting:ACSF) for 10 minutes at room temperature. Sections were then recovered in ACSF at 32 °C for 1.5 to 2 hours. To measure the effect of tPA on reelin processing in the intact brain, hippocampal slices (n = 4 per treatment group) were treated with tPA (50 or 100 nM) diluted in ACSF for 45 minutes. For this and subsequent experiments, CA1 was dissected in ice-cold cutting solution and snap-frozen on dry ice. Samples were stored at -80°C until processing for Western blot analysis. To determine the effects of PKA activation on reelin expression and processing, slices were incubated with 50 µM forskolin for 5, 15,
and 45 minutes. For the tPA KO mice the hippocampal slices were exposed to forskolin for 45 minutes. Chemical long-term potentiation was induced by exposing slices to tetraethylammonium chloride (TEA-Cl, 25 mM) diluted in ACSF (adjusted for osmolarity) for 10 minutes. Slices were then recovered in normal ACSF and collected 5, 15, and 45 minutes post-treatment. The tPA KO slices were treated with TEA-Cl for 10 min and recovered in ACSF for 15 min. All experiments were performed with a minimum of 6 hippocampal slices total obtained from at least 2 animals.

**Western Blot Analysis**

For all *ex vivo* slice culture assays, total protein was extracted from dissected hippocampal area CA1 by mixing with 25 µl M-PER supplemented with Halt protease inhibitor cocktail. For evaluating Reelin levels *in vivo*, supplemented M-PER was used to isolate proteins (1 ml / 80 mg) from the hippocampus, cortex, and cerebellum of adult wild-type and tPA knockout mice (*n* = 6 per genotype). Samples were incubated on ice for 30 minutes followed by clarification at 15,000 x g for 15 minutes. Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Scientific). Samples were adjusted to equal protein concentrations and combined with equal volume Laemmli Sample Buffer with 5% of 2-mercaptoethanol. Unboiled samples were electrophoretically-separated on 4-15% TGX gels (Bio-Rad) and transferred to PVDF blotting membranes (Millipore). The membranes were blocked in blotting solution
containing 0.1M tris-buffered saline with 0.1% Tween-20 and 5% nonfat milk. The membranes were then incubated with primary antibody diluted at 1:1000 in blotting solution overnight. The next day, the membranes were washed and then incubated with anti-Mouse-HRP or anti-Rabbit-HRP secondary antibodies (Southern Biotech) diluted at 1:2000 in blotting solution. For Western blot analysis of tissue lysates, beta-actin was also probed to normalize for variation in protein loading. Blots were detected using Pierce ECL Western Blotting reagent (Thermo Scientific) for chemiluminescence on autoradiography X-ray film.

The films were digitized and optical densities were determined using a computerized image analysis system with a high powered scanner and the software program Image J (v1.43u, National Institutes of Health). Single autoradiographic signal bands of appropriate molecular weights were identified and quantified. An experimenter who was blind to the treatment conditions measured the optical density of the bands.

**Statistical Analysis**

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, v 16.0, Chicago, IL, USA). We used independent samples t-tests (two-tailed) to examine the statistical significance of differences between the different brain regions of tPA KO mice and wild-type mice when comparing protein levels. We also examined the effects between the non-treated group and each treatment group with independent samples t-tests (two-tailed) to
examine the difference in protein levels following the tPA, forskolin and TEA-Cl treatments. The criterion for statistical significance was set at $p < 0.05$. All data are represented as means ± the standard error of the mean.

Results

Processing of Reelin by tPA under Cell-Free Conditions.

To evaluate the effects of tPA on reelin processing, we combined partially-purified reelin (50 nM) with varying concentrations of tPA (0-400 nM) for 15 minutes at 37°C (Fig. 1B). The reelin G10 antibody was used to look at full-length reelin and the 370 kDa (N-R6) and 180 kDa (N-R2) fragments; whereas Ab14 was used to evaluate the 80 kDa (R7-8) fragment (Fig. 1A). We found that tPA dose-dependently increased reelin processing at R6-7, revealed by increased levels of both the 370 and 80 kDa fragments. The level of the 180 kDa fragment was not significantly affected by tPA treatment, suggesting that tPA is unable to affect cleavage at the R2-R3 site. Because generation of the 370 and 80 kDa fragments both reflect cleavage of reelin at R6-7, all later experiments evaluated reelin processing using the reelin G10 antibody. To block the effect of tPA we used the Halt broad-spectrum protease inhibitor cocktail (PIC) without EDTA, at a concentration that effectively inhibits aspartic acid, cysteine and serine proteases. Inclusion of the PIC significantly inhibited tPA-mediated reelin processing (Fig. 1C).
The major *in vivo* substrate of tPA is the zymogen plasminogen, which is processed into the serine protease plasmin (Silverstein et al., 1984). As most established tPA functions in the adult brain are mediated through plasmin (Pang et al., 2004; Chen et al., 2008; Echeverry et al., 2010), we determined the effects of both tPA and plasmin on reelin processing (Fig. 2A) by incubating reelin (50 nM) with tPA (50 nM), plasminogen (18 µg/µl), plasminogen and tPA or purified plasmin (0.5 U/ml) for 15 and 45 minutes. Plasminogen alone had no effect on reelin levels, whereas both active plasmin and the tPA/plasminogen combination resulted in a complete degradation of full-length reelin and the 370 kDa fragment into the 180 kDa fragment and three sub-180 kDa fragments. Both tPA-activated plasminogen and purified active plasmin generated the same spectrum of reelin fragments. Reelin degradation by plasmin progressed slightly from the 15 to 45 minute time-points, but revealed no additional reelin fragments. These findings suggest that tPA may influence reelin processing in both a plasminogen-independent and –dependent fashion.

We next examined the specificity of reelin processing by the tPA/plasminogen system using various serine protease inhibitors. The bovine pancreatic trypsin inhibitor, aprotinin, is capable of blocking the fibrinolytic effects of active plasmin (Kang et al., 2005) but does not directly affect tPA protease activity (Lottenberg et al., 1988). Consistent with these findings, aprotinin (40 µM) was unable to prevent tPA-dependent processing of reelin after 45 minutes (Fig. 2B), but potently blocked plasmin-mediated reelin degradation. The plasminogen/reelin combination was used as a negative control because
plasminogen alone has no effect on reelin processing. Diisopropylfluorophosphatase (DIFP) is a general serine protease inhibitor that can be used to inhibit plasmin activity (Liu and Gurewich, 1991; Novak et al., 1997). We found that DIFP (100 µM) effectively inhibited reelin processing by both tPA and plasmin. Plasminogen activator inhibitor (PAI-1) is a member of the serpin family of serine protease inhibitors and is the principle inhibitor of both uPA and tPA (Ranby and Brandstrom, 1988). We found that PAI-1 (1 ng/µl) led to a specific inhibition of tPA- and tPA/plasminogen-mediated reelin processing, whereas it did not affect processing of reelin by active plasmin. These results confirm that both tPA and plasmin differentially affect reelin processing, and that their effects can be dissociated using aprotinin, DIFP, and PAI-1.

Secreted reelin forms homodimeric complexes comprised of more than 40 monomers (Kubo et al., 2002). Blocking reelin dimerization using the CR-50 antibody reduces its signaling efficacy (Utsunomiya-Tate et al., 2000). To determine if dimerization is required for reelin proteolysis we co-incubated reactions with the CR-50 antibody (0.02 µg/µl). Inhibition of dimerization had no effect on reelin processing by tPA or plasmin (Fig. 2B).

Processing of Reelin by tPA in Acute Hippocampal Slices.

Cell-free conditions are devoid of extracellular factors which may ultimately dictate the propensity of reelin to be processed in vivo. For this reason, we examined the effects of exogenous tPA application on reelin processing in the
hippocampus of wild-type mice (Fig. 3). Acute hippocampal slices were treated with tPA for 60 minutes at 50 and 100 nM (n = 4 per treatment). We used 50 nM of tPA as it is sufficient to enhance long-term potentiation in hippocampal area CA1 (Zhuo et al., 2000). There were no significant differences in the levels of full-length reelin and the 370 kDa (N-R6) and 180 kDa (N-R2) fragments following any tested concentration of tPA. Higher concentrations of tPA (up to 400 nM) were also tested (data not shown) and had no effect on reelin processing, suggesting that the R6-7 cleavage site may not be readily accessible in the native tissue environment.

Reelin Levels in the Brains of Adult tPA KO versus Wild-Type Mice.

Since reelin fragments are observed under basal conditions in the adult brain (Krstic et al., 2012), we next determined if tPA deficiency in vivo altered reelin processing. The levels of full-length reelin and fragments were evaluated in 4-month old tPA KO and wild-type mice (Fig. 4, n = 6 per genotype). We found no significant differences in the levels of full-length reelin or fragments in the cortex, hippocampus, and cerebellum; brain regions demonstrated to have high levels of tPA expression in the adult (Thewke and Seeds, 1999; Teesalu et al., 2004). These findings suggest that under basal conditions, tPA has minimal effect on reelin processing in the adult brain.
Modulation of Reelin Processing by Forskolin.

The adenylyl cyclase activator, forskolin, has been demonstrated previously to induce a form of synaptic potentiation in the hippocampus that requires tPA and NMDA receptor (NMDAR) activation (Baranes et al., 1998). For this reason, we determined whether forskolin treatment of acute hippocampal slices could modulate reelin processing in a tPA-dependent manner. To establish a time course of forskolin-mediated changes in reelin processing, wild-type hippocampal slices were treated with 50 µM forskolin for 5, 15, and 45 minutes. An overall ANOVA revealed a significant main effect for the full length and the 370 kDa reelin fragment, \( F(3,21) = 3.269, p <0.05 \) and \( F(3,21) = 3.970, p <0.05 \), respectively. Post-hoc analysis revealed a significant decrease for the 45 minute forskolin treatment group when compared to the non-treatment, 5 and 15 minute exposure groups for both the full-length and 370 kDa fragments (all \( p \)-values < 0.05). (Fig. 5A). As a positive control, we examined the effects of forskolin on the activation of extracellular signal-related kinases 1 and 2, since forskolin-mediated increases in cAMP lead to Ras-dependent activation of ERK (Ambrosini et al., 2000). There were increases in the activity of both p-ERK1 and p-ERK2 following forskolin treatment (Fig. 5B). An ANOVA revealed a significant effect of forskolin treatment on p-ERK1 and p-ERK2 levels \( F(3,20) = 6.519, p <0.05 \) and \( F(3,20) = 4.082, p <0.05 \), respectively. Post-hoc analyses showed that there was a significant increase in p-ERK1 levels for the 15 and 45 minute time points when compared to the non-treatment control group, the 45 minute time point was also
significantly higher than the 5 minute time point (all $p$-values $< 0.05$, respectively). For the p-ERK2 levels, the 45 min group was significantly higher than the non-treated control group ($p < 0.05$).

Because of the large effect of forskolin on reelin processing at the 45 minute time point in wild-type slices, we used this duration of forskolin treatment to examine reelin processing in tPA KO hippocampal slices. We found that there were no significant differences in the levels of full-length reelin or its fragments following forskolin treatment (Fig. 6A). Similar to wild-type slices, we found a significant increase in the activation of p-ERK2 after 45 min of forskolin exposure, ($t(15) = -2.246, p < 0.05$; Fig. 6B), indicating that tPA KO slices respond biochemically to forskolin stimulation.

**Effect of Chemical LTP on Reelin Processing.**

The potassium channel blocker TEA-Cl induces a robust Ca$^{2+}$-dependent and NMDAR-independent LTP in hippocampal area CA1 (Aniksztejn and Ben-Ari, 1991). For this reason, we evaluated changes in reelin processing in area CA1 at 5, 15, and 45 minutes following a 10 minute exposure to TEA-Cl (Fig. 7A). We found a significant increase in the levels of the 370 kDa fragment in the 15 and 45 minute recovery groups versus the non-treated group [$t(13.852) = -2.287, p < 0.05$ and $t(28) = -2.240, p < 0.05$, respectively]. Because induction of LTP by TEA-Cl also requires activation of PKA and ERK1/2, we measured p-ERK1/2 levels as a positive control for TEA-Cl treatment (Kanterewicz et al.,
The p-ERK1/2 levels were significantly elevated by 5 minutes which remained elevated but decreased back towards baseline levels at the 15 and 45 minute time points (Fig. 7B). Independent samples t-tests revealed that the 5 minute recovery group has significantly higher levels of p-ERK1 compared to the non-treatment control group [(t(24) = -3.436, p < 0.05)]. When examining p-ERK2 we found that the 5, 15 and 45 min recovery groups had significantly higher levels compared to the non-treatment group [(t(24) = -10.207, p < 0.001), (t(25) = -5.567, p < 0.001) and (t(14.267) = -2.854, p < 0.05), respectively].

The effects of TEA-Cl treatment on reelin processing in tPA KO hippocampal slices was examined following the 15 minute recovery period (Fig. 8A). No differences were found in the levels of full-length reelin or fragments. However, tPA knockout slices still responded to TEA-Cl treatment with significant increases in p-ERK1 and p-ERK2 [(t(15) = -2.246, p < 0.05) and (t(15) = -2.246, p < 0.05), respectively].

**Discussion**

The secreted glycoprotein reelin performs diverse roles in the developing and adult brain, including regulation of neuronal migration (D'Arcangelo et al., 1995), dendritogenesis (Niu et al., 2004), synapse development (Niu et al., 2008; Rogers et al., 2011; Ventruti et al., 2011), hippocampal synaptic plasticity (Weeber et al., 2002; Rogers et al., 2011; Trotter et al., 2011), and learning and memory (Weeber et al., 2002; Qiu et al., 2006; Rogers et al., 2011). Recent
studies have suggested that changes in reelin expression and processing may contribute to cognitive deficits associated with several debilitating neuropsychiatric and neurodegenerative disorders, including schizophrenia (Impagnatiello et al., 1998; Fatemi et al., 2000), depression (Fatemi et al., 2000; Lussier et al., 2009; Lussier et al., 2011), and Alzheimer’s disease (Chin et al., 2007; Botella-Lopez et al., 2010; Herring et al., 2012). Although the downstream mechanisms of reelin signaling have been extensively studied, there is still little known about the upstream mechanisms that control the initiation, magnitude, and duration of the reelin signal. Here we present evidence that extracellular proteolysis by tPA could serve as a critical upstream regulator of reelin signaling following synaptic potentiation. Specifically, we demonstrate that reelin is processed by tPA at a single site between R6-7. Although tPA is not involved in cleaving reelin under basal conditions, induction of NMDAR-independent and -dependent forms of LTP with TEA and forskolin, respectively, revealed dynamic changes in reelin expression and processing that required tPA.

The binding of full-length reelin and fragments containing R5-6 to the conserved ligand-binding modules of apolipoprotein E receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR) (Yasui et al., 2010) promotes receptor clustering and tyrosine phosphorylation of Disabled-1 by Src family kinase members (Hiesberger et al., 1999; Trommsdorff et al., 1999). Blocking cleavage of reelin through metalloproteinase inhibition prevents Dab1 activation and disrupts cortical development in embryonic cortical explants (Jossin et al., 2007). Although the specific metalloproteinases responsible for reelin cleavage in
vivo have not been identified, a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4 and -5) are both capable of cleaving reelin at N- and C-terminal cleavage sites in vitro (Hisanaga et al., 2012; Krstic et al., 2012). Despite the ability of ADAMTS-4 to cleave reelin, it is not the enzyme responsible for reelin processing in cultured cortical neurons (Hisanaga et al., 2012), highlighting an important limitation of cell culture models in which reelin and proteases are secreted freely. This emphasizes the need for in vivo models to identify and test proteases that regulate reelin processing in its native, extracellular matrix (ECM) bound state.

In the brain, reelin is enriched in the golgi apparatus, axonal secretory pathways, and throughout the extracellular matrix of dendrite-rich neuropils (Rodriguez et al., 2000; Ramos-Moreno et al., 2006). Potential mechanisms responsible for the initiation of the reelin signaling pathway may involve changes in its transcription and secretion, alterations in the availability of reelin receptors, or extracellular proteolysis. Although not studied extensively, both reelin expression and secretion do not appear to be regulated by synaptic activity (Lacor et al., 2000; Tinnes et al., 2011). Moreover, acute hippocampal slices respond to reelin stimulation with enhancement of LTP (Weeber et al., 2002), reflecting ready availability of receptors on the cell surface. We hypothesized that extracellular proteolysis of reelin by activity-dependent proteases may initiate reelin signaling following synaptic potentiation.

A potential candidate for the activity-dependent processing of reelin is tPA, which was recently found to cleave reelin between R6-7 in vitro (Krstic et al.,
tPA is both secreted and activated in an activity-dependent fashion (Pang et al., 2004; Nagappan et al., 2009), allowing it to activate substrates which influence synaptic plasticity and learning and memory. In agreement with the recently published study (Krstic et al., 2012), we found that tPA cleaves reelin between R6-7. The ability of PAI-1 to inhibit the cleavage of reelin by tPA suggests that it could serve as an endogenous inhibitor of Reelin processing and signaling in the brain. We also found that plasmin was capable of cleaving reelin at several sites, effectively degrading both full-length reelin and the 370 kDa fragment. This is unlike the brain-derived neurotrophic factor (BDNF), whose maturation is promoted by a single cleavage of pro-BDNF by plasmin (Pang et al., 2004). Although the current study did not focus on plasmin-mediated reelin processing, the additional cleavage sites that are sensitive to plasmin may represent a more complex mechanism for the control of the duration and magnitude of reelin signaling.

The inability of tPA to modulate reelin processing in ex vivo slices cultures, even at concentrations known to enhance LTP (Zhuo et al., 2000), may be due to the cleavage site being sterically-hindered under basal conditions in intact tissue. To determine if tPA deficiency affects reelin processing in vivo, we evaluated reelin fragment levels in tPA KOs. No changes were observed in the levels of full-length reelin or its fragments in the adult cortex, hippocampus, and cerebellum. The lack of obvious changes in reelin processing under basal conditions is consistent with a requirement of high levels of synaptic activity to drive the release and activation of tPA (Lochner et al., 2006; Lochner et al., 2008).
Notwithstanding, we did still observe that the reelin N-R2 (370 kDa) and N-R6 (180 kDa) fragments were expressed in all tested regions, implying that other proteases expressed in the adult brain are responsible for reelin processing under basal conditions.

Because we did not observe any effect of tPA on reelin processing under basal conditions, we hypothesized that synaptic potentiation could drive activation of tPA and processing of reelin at R6-7. The adenylyl cyclase activator forskolin has been previously reported to induce tPA- and NMDAR-dependent LTP (Baranes et al., 1998). We found that wild-type hippocampal slices treated with forskolin for 45 minutes had a significant reduction in full-length reelin and fragments, whereas tPA knockout slices did not. The decrease of full-length reelin observed in wild-type slices would require extracellular proteolysis by both the N- and C-terminal cleaving enzymes, the former of which remains unidentified. Activation of ERK1/2 in both wild-type and tPA KOs suggests that the absence of changes in Reelin expression and processing do not reflect an inability of tPA KO slices to respond to forskolin.

Due to the inability of forskolin to induce a stable potentiation of CA1 synapses (Otmakhov et al., 2004), we used the potassium channel blocker tetraethylammonium chloride (TEA-Cl), which induces a robust, Ca$^{2+}$-dependent and NMDAR-independent chemical LTP (cLTP) in hippocampal area CA1 (Aniksztejn and Ben-Ari, 1991). Unlike electrical stimulation, which allows activation of only restricted bundles of Schaffer collaterals (SC), TEA-Cl induces a more uniform activation of the SC, culminating in LTP throughout area CA1.
The selective generation of the 370 kDa (N-R6) fragment reveals that either proteolysis does not always occur sequentially or that the fragment levels are differentially regulated depending on the mode of synaptic potentiation. Together these findings show that two independent approaches to altering synaptic potentiation both affect reeling proteolysis by tPA at the R6-R7 site.

Here we present a novel mechanism whereby activity-dependent release and activation of tPA, and possibly other proteases, results in the processing of reelin into active, receptor-binding fragments. The generation of fragments that bind to and activate unique receptor pathways offers a potential mechanism to explain the robust effect of reelin on synaptic plasticity and learning and memory. As illustrated in Fig. 9, we predict that activity-dependent processing of Reelin by tPA is critical for the generation of the ApoER2/VLDLR-binding R3-6 region, which initiates signaling downstream through Dab1 and modulates excitatory neurotransmission through promoting NMDAR activation (Chen et al., 2005). Understanding these mechanisms may have particular relevance to AD, as impaired regulation of tPA has been observed in post-mortem AD tissue and in mouse models (Liu et al., 2011), a change which could contribute to deficits in reelin signaling and synaptic dysfunction. Future experiments should further explore mechanisms of activity-dependent reelin processing and establish its relevance to the regulation of synaptic function and learning and memory, particularly in the context of pathological alterations in reelin proteases such as tPA.
References


Figure 2.1. Modulation of Reelin Processing by tPA. (A) Reelin is cleaved between epidermal growth factor (EGF) repeats 2-3 (R2-3) and 6-7 (R6-7), resulting in 5 potential fragments (370, 270, 190, 180, and 80 kDa). The anti-reelin G10 primary antibody detects full-length reelin (450 kDa), and the 370 and 180 kDa fragments. Ab14 was used to detect the 80 kDa fragment. (B) Recombinant reelin (50 nM) was incubated with 0-400 nM tPA at 37°C for 15 minutes. tPA dose-dependently increased processing of recombinant reelin between R6-7. Both the 370 and 80 kDa reelin fragments were increased, while the level of full-length reelin was decreased by tPA treatment. The 180 kDa fragment was not altered. (C) Inclusion of the broad-spectrum Halt protease inhibitor cocktail (1X P.I.C.) with tPA (200 nM and 400 nM) inhibited reelin processing. Experiments were performed in triplicate.
Figure 2.2. Effects of tPA and Plasminogen on Reelin Processing under Cell-Free Conditions. Recombinant reelin (50 nM) was incubated with tPA (50 nM), plasminogen (Plg, 18 µg/µl), plasminogen (18 µg/µl)/tpa (50 nM), and activated plasmin (0.5 U/ml) at 37°C for 15 and 45 minutes under cell-free conditions. (A) At both the 15 and 45 minute time points, tPA promoted the generation of the 370 fragment. Plasminogen alone had no effect. The Plg/tPA combination and active plasmin converted reelin to 180 kDa and sub-180 kDa fragments. (B) The above-mentioned reactions were co-incubated with serine protease inhibitors or CR-50 for 45 minutes at 37°C. Plasminogen activator inhibitor (PAI-1, 1 ng/µl) blocked the cleavage of reelin by tPA and the plg/tPA combination, whereas it had no effect on already active plasmin. DIFP (100 µM)
inhibited the processing of reelin by all protease combinations. Aprotinin (40 µM) blocked the effects of tPA-activated plasmin and purified plasmin, but did not prevent tPA-mediated reelin processing. The dimerization-inhibiting CR-50 antibody (0.02 µg/µl) had no effect on reelin processing by tPA, plg/tPA, or active plasmin. Experiments were performed in triplicate.
Figure 2.3. tPA does not Modulate Reelin Processing in Acute Hippocampal Slices. Wild-type hippocampal slices (n = 4 per treatment) were treated with 50 or 100 nM tPA for 60 minutes. No significant differences were found in full-length reelin or fragment levels.
Figure 2.4. Loss of tPA in vivo does not Affect Reelin Processing. The level of full-length reelin and the 370 and 180 kDa fragments were measured in the brains of 4 month old wild-type (n = 6) and tPA knockout (n = 6) mice. No significant differences were found in the levels of full-length reelin or fragments in the cortex (A), cerebellum (B), or hippocampus (C). The values are shown as a relative density and error bars represent the mean ± the standard error of the mean.
Figure 2.5. Forskolin Promotes Reelin Processing. Acute hippocampal slices from wild-type mice (at least 2 slices from 3 different mice) were treated with forskolin (50 µM) for 5, 15, and 45 minutes. Proteins from dissected hippocampal area CA1 were evaluated for changes in reelin processing and ERK1/2 activation. (A) The levels of the 370 kDa and 180 kDa fragments were significantly reduced following 45 minutes of forskolin treatment compared to the non-treated group. (B) The levels of p-ERK1 (44 kDa) were increased at 5, 15, and 45 minutes compared to the non-treated group. The levels of p-ERK2 (42 kDa) were significantly increased at 15 and 45 minutes compared to the non-treated group. The values are shown as a relative density and error bars represent the mean ± the standard error of the mean. (*) denotes p < 0.05 as indicated by the independent samples two-tailed t-test.
Figure 2.6. tPA is Required for Modulation of Reelin Processing by Forskolin. Acute hippocampal slices from tPA KO mice (at least 3 slices from 2 different mice) were treated with forskolin (50 µM) for 45 minutes. Proteins from dissected hippocampal area CA1 were evaluated for changes in reelin processing and ERK1/2 activation. (A) The levels of full-length reelin and the 370 and 180 kDa fragments were not affected by forskolin treatment. (B) Forskolin treatment significantly increased p-ERK2 (42 kDa) levels compared to the non-treated group. p-ERK1 (44 kDa) levels were not affected by forskolin treatment. The values are shown as a relative density and error bars represent the mean ± the standard error of the mean. (*) denotes p < 0.05 as indicated by the independent samples two-tailed t-test.
Figure 2.7. cLTP Induces Reelin Processing. Acute hippocampal slices from wild-type mice (at least 2 slices from 3 different mice) were treated with TEA-Cl (25 mM) for 10 minutes and recovered in aCSF for 5, 15, and 45 minutes. Proteins from dissected hippocampal area CA1 were evaluated for changes in reelin processing and ERK1/2 activation. (A) The level of the 370 kDa fragment was significantly increased at 15 and 45 minutes following TEA-Cl treatment. (B) The level of p-ERK1 (44 kDa) was increased at 5 minutes post-treatment compared to the non-treated group. The levels of p-ERK2 (42 kDa) were significantly increased at 5, 15, and 45 minutes following TEA-Cl treatment. The values are shown as a relative density and error bars represent the mean ± the standard error of the mean. (*) denotes p < 0.05 as indicated by the independent samples two-tailed t-test.
Figure 2.8. tPA is Required for Modulation of Reelin Processing by cLTP.
Acute hippocampal slices from tPA KO mice (at least 3 slices from 2 different mice) were treated with TEA-Cl (25 mM) for 10 minutes and recovered in aCSF for 15 minutes. Proteins from dissected hippocampal area CA1 were evaluated for changes in reelin processing and ERK1/2 activation. (A) There were no significant changes in full-length reelin or either of the fragments in tPA KO mice 15 minutes following TEA-Cl treatment. (B) The levels of p-ERK1 (44 kDa) and p-ERK2 (42 kDa) were significantly increased 15 minutes following TEA-Cl treatment. The values are shown as a relative density and error bars represent the mean ± the standard error of the mean. (*) denotes p < 0.05 as indicated by the independent samples two-tailed t-test.
Figure 2.9. Schematic Representation of the Activity-Dependent Processing of Reelin by tPA. Activation of the presynaptic terminal by high-frequency stimulation causes release of neurotransmitters and tPA from dense-core synaptic vesicles. In the potentiated synapse, tPA cleaves full-length reelin between the R6-7. We also predict that another unidentified protease (as indicated by X) cleaves reelin at R2-3 and is critical for the generation of the ApoER2/VLDLR-binding R3-6 fragment. Lipoprotein receptor binding then initiates signaling downstream through phosphorylation of Dab1 and increases in LTP. Binding of the N-R2 fragment to integrins may also contribute to changes in synaptic function. The generation of fragments that bind to and activate unique receptor pathways offers a potential mechanism to explain the robust effect of reelin on synaptic plasticity and learning and memory. Abbreviations: apolipoprotein E receptor 2 (ApoER2); very low density lipoprotein receptor (VLDLR), phosphorylated disabled-1 (dab1-p), long-term potentiation (LTP), glycosaminoglycans (GAG), and tissue plasminogen activator (tPA).
Chapter 3

Dab1 is Required for Synaptic Plasticity and Associative Learning

Note to Reader: These results have been previously published (Trotter et al., 2013) and have been included with permission of the publisher (Appendix 1).


Abstract

Disabled-1 (Dab1) is an adaptor protein that is an obligate effector of the Reelin signaling pathway, and is critical for neuronal migration and dendrite outgrowth during development. Components of the Reelin pathway are highly expressed during development, but also continue to be expressed in the adult brain. Here we investigated in detail the expression pattern of Dab1 in the postnatal and adult
forebrain, and determined that it is expressed in excitatory as well as inhibitory neurons. Dab1 was found to be localized in different cellular compartments, including the soma, dendrites, and pre- and postsynaptic structures. Mice that are deficient in Dab1, Reelin or the Reelin receptors ApoER2 and VLDLR exhibit severely perturbed brain cytoarchitecture, limiting the utility of these mice for investigating the role of this signaling pathway in the adult brain. In this study, we developed an adult forebrain- and excitatory neuron-specific conditional knockout (cKO) mouse line, and demonstrated that Dab1 is a critical regulator of synaptic function and hippocampal-dependent associative and spatial learning. These dramatic abnormalities were accompanied by a reduction in dendritic spine size, and defects in basal and plasticity-induced Akt and ERK1/2 signaling. Deletion of Dab1 led to no obvious changes in neuronal positioning, dendrite morphology, spine density or synaptic composition. Collectively, these data conclusively demonstrate an important role for Reelin-Dab1 signaling in the adult forebrain, and underscore the importance of this pathway in learning and memory.

Introduction

Disabled-1 (Dab1) is an adaptor protein that is essential for neuronal migration and maturation in response to the extracellular protein Reelin (see D'Arcangelo, 2005 for review). Spontaneous mutant mice lacking Reelin (reeler)
or Dab1 (scrambler), and Dab1 knockout mice present with widespread defects in cellular layer formation (D'Arcangelo et al., 1995; Howell et al., 1997; Sheldon et al., 1997; Howell et al., 2000). A developmental delay in dendrite and axon branching, as well as spine formation and synaptogenesis, has been also reported in the hippocampus of Reelin and Dab1 mutant mice (DelRio et al., 1997; Niu et al., 2004; Borrell et al., 2007; Niu et al., 2008). Reelin signaling continues to perform an important role in the adult brain by promoting excitatory synapse maturation (Qiu and Weeber, 2007; Ventruti et al., 2011) and modulating synaptic plasticity and learning and memory (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011).

During forebrain development, Reelin is secreted by Cajal-Retzius cells in superficial cortical layers (D'Arcangelo et al., 1995; Ogawa et al., 1995), and targets principal neurons, which express apolipoprotein E receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999; Hiesberger et al., 1999). Receptor binding leads to the activation of Src family kinases (SFKs) and tyrosine phosphorylation of Dab1 (Hiesberger et al., 1999; Howell et al., 1999). Phosphorylated Dab1 propagates Reelin signaling, regulating neuronal migration through the recruitment of Crk/CrkL (Chen et al., 2004; Park and Curran, 2008), Rap1, cadherins and integrin α5β1 (Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2012). The molecular mechanism underlying the control of postnatal developmental processes, such as dendrite outgrowth and spine formation, also requires ApoER2/VLDLR and Dab1 (Niu et
al., 2004; Niu et al., 2008) and the downstream activity of phosphatidylinositol-3 kinase (PI3K)/Akt (Beffert et al., 2002) and mTOR (Jossin and Goffinet, 2007).

In the adult forebrain, Reelin is secreted by a subset of inhibitory neurons (Alcantara et al., 1998b; Pesold et al., 1998), and it is believed to be important for synaptic function since Reelin haploinsufficiency or loss of either VLDLR or ApoER2 leads to learning and memory deficits (Weeber et al., 2002; Qiu et al., 2006b). However, at least one study did not report similar defects in heterozygous reeler mice (Krueger et al., 2006). An ApoER2 isoform capable of binding PSD-95 has been further implicated in synaptic plasticity and cognition through a mechanism involving the NMDA receptor (Beffert et al., 2005a). The role of Dab1 in the adult brain has not yet been investigated due to the absence of an animal model that lacks developmental abnormalities. To overcome this limitation, we generated a conditional knockout mouse with selective deletion of Dab1 in excitatory neurons of the adult forebrain. Here we present evidence that Dab1 plays a critical role in mediating the synaptic function of Reelin, and that it is required for hippocampal synaptic plasticity and learning and memory.

Materials and Methods

Mouse Colonies

All animals used for this study were handled in accordance with protocols approved by the Association for Assessment and Accreditation of Laboratory
Animal Care committee at Rutgers, the State University of New Jersey, and by the Institutional Animal Care and Use Committee of the University of South Florida. Animals of either sex were group housed in a standard 12 hour light/dark cycle and fed *ad libitum* standard mouse chow. The $Dab1^{flox/flox}$ founder mice were genotyped as described previously (Franco et al., 2011). CamKIIα-Cre transgenic driver mice (B6.Cg-Tg(Camk2a-cre)T29-1Stl/J), tdTomato reporter mice (B6.Cg-Gt(ROSA)26Sor$t^{tm9(CAG-tdTomato)Hze}$J), spontaneous $Dab1$ mouse mutant *scrambler* (A/A-$Dab1^{scm}$/J), and wild type C57Bl6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mutant and transgenic mice were genotyped by PCR as suggested by the distributor. Constitutive $Dab1$ knockout (KO) mice were obtained from J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) and genotyped as described previously (Howell et al., 1997).

**Tissue Histology and Immunostaining**

Mice were deeply anesthetized with Avertin (2,2,2-tribromoethanol dissolved in tertiary amyl alcohol and distilled water) or isoflurane and perfused transcardially with phosphate buffered saline (PBS; pH 7.2) or saline solution (0.9% NaCl), followed by 4% paraformaldehyde (PFA) in PBS. Brains were dissected, post-fixed in 4% PFA overnight at 4°C, and cryoprotected by incubation at 4°C in 30% sucrose in PBS. Brains were mounted onto a sliding microtome using OCT (Tissue-Tek) and sectioned either coronally (for
immunohistochemistry) or sagitally (for histology) at 25-30 µm. To evaluate
neuroanatomical organization, sections were processed for thionin staining (FD
Neurotechnologies, Columbia, MD) according to the manufacturer’s protocol.

For immunoperoxidase staining, brain sections were immersed in 3%
H$_2$O$_2$ solution for 15 minutes at room temperature (RT). Sections were washed
with PBS and then permeabilized and blocked with 4% normal goat serum (NGS)
in PBS (supplemented with 1.83% lysine and 0.2% Triton X-100) for 30 minutes
at RT. Sections were incubated overnight at RT with anti-Dab1 B3 antibody at
1:1000 (provided by Dr. Brian Howell, SUNY Upstate Medical University) diluted
in PBS with 0.2% Triton X-100 and 4% NGS. After washing with PBS, sections
were incubated with biotinylated goat anti-rabbit IgG (1:3000; Southern Biotech)
diluted in PBS with 0.2% Triton X-100 and 4% NGS. Sections were washed in
PBS and then incubated with ABC reagent (Vectashield) for 1 hour at RT,
followed by 3 washes in PBS and 1 wash in fresh TBS. Sections were incubated
with 1.4 mM diaminobenzidine in TBS with 0.03% hydrogen peroxide and 0.5%
nickel ammonium sulfate in PBS for 5 min. Finally, stained sections were
mounted on positively-charged glass slides, dehydrated, and coverslipped with
DPX mounting media. Imaging was performed with a Zeiss Axio Scope A1
Microscope.

Fluorescence immunostaining was performed as described previously
(Trotter et al., 2011). The following primary antibodies were used: anti-Dab1 B3
(1:500), anti-Reelin (1:1000; MAB5364, Millipore), anti-NeuN (1:2000; MAB377,
Millipore), anti-PSD-95 (1:2000; P78352, NeuroMab), anti-synaptophysin (1:400;
5461, Cell Signaling), and anti-GAD67 (1:2000; MAB5406, Millipore). The following secondary antibodies were used: goat anti-mouse IgG-Alexa 546, goat anti-rabbit IgG-Alexa 488, goat anti-rabbit IgG-Alexa 633 (all used at 1:500 and obtained from Invitrogen). Sections were mounted on positively-charged glass slides and coverslipped with ProLong Gold Antifade Reagent with DAPI (Invitrogen). Sections were imaged using the Olympus FV10i confocal microscope. For comparison between genotypes, images were taken with the same exposure settings and were adjusted similarly for brightness and contrast in Adobe Photoshop.

**Protein Extracts and Crude Synaptosome Preparation**

For developmental analysis, the cortex, hippocampus, and cerebellum were dissected from C57Bl6 mice at several postnatal days and snap-frozen. The cortices and hippocampi of homozygous constitutive *Dab1* knock out (KO) mice or conditional *Dab1* KO (cKO) mice and their wild type (WT) littermates were dissected and processed for total lysates or synaptoneurosome (SNS) fractions. Frozen cell pellets from primary neuronal cultures or hippocampal slices were similarly processed for total lysate extraction. All centrifugation steps were performed at 4°C. To prepare total lysates, the tissue or cell pellets were homogenized in ice-cold RIPA buffer (50 mM Tris pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA) supplemented with complete protease and phosphatase inhibitor cocktails and cleared by centrifugation at
15,000 × g for 10 minutes at 4ºC. SNS fractions were prepared as described previously with minor modifications (Hoe et al., 2009; Ventruti et al., 2011). Tissue was homogenized in ice-cold homogenization buffer (5 mM HEPES pH 7, 1 mM MgCl₂, 0.5 mM CaCl₂) containing protease inhibitor and phosphatase inhibitor as mentioned above. The total homogenate was centrifuged at 1,500 × g for 10 minutes and the supernatant (S1) was collected. The pellet was re-suspended in homogenization buffer and centrifuged again at 800 × g for 10 minutes. The supernatant was collected, combined with S1, and cleared by centrifugation at 13,800 × g for 10 minutes to obtain the homogenate sample. The remaining pellet, containing SNS fraction, was re-suspended in homogenization buffer. Synaptic fractionation was performed on freshly dissected forebrains from 3 month-old C57Bl6 mice as described previously (Dumanis et al., 2011).

**Western Blot Analysis**

For brain tissue analysis, protein concentration was determined using the Bradford or BCA Protein Assay (Bio-rad or Thermo Scientific). Samples were adjusted to equal protein concentration and combined with Laemmli Sample Buffer containing 5% 2-mercaptoethanol. Samples were boiled for 5 minutes at 95ºC, except when analyzing Reelin expression. Protein lysates were separated electrophoretically onto 8% or 4-15% Tris-Glycine SDS-PAGE gels (Invitrogen or Bio-rad) and transferred to nitrocellulose or PVDF blotting membrane. The membranes were blocked in a solution containing 0.1M tris-buffered saline (TBS)
with 0.1% Tween-20 and 5% nonfat milk, and then incubated overnight at 4°C primary antibodies diluted in blocking solution. Primary antibodies include: monoclonal anti-Dab1 (a gift of Dr. André M Goffinet, Université Catholique de Louvain, Belgium), anti-p-Akt (Ser473; #3787, Cell Signaling), anti-total Akt (#2966, Cell Signaling), anti-p-ERK1/2 (Thr202/Tyr204; #4370, Cell Signaling), anti-total ERK1/2 (#4696, Cell Signaling), anti-NR2A (#07-632, Millipore), anti-NR2B (#06-600, Millipore), anti-NR1 (#05-432, Millipore), anti-synapsin IIa (#610667, BD Transduction labs), anti-ribosomal protein S6 (#2317, Cell Signaling), anti-PSD-95 (P78352, NeuroMab), anti-synaptophysin (#5461, Cell Signaling), anti-Reelin (MAB5364, Millipore), anti-ApoER2 (ab108208, Abcam), anti-GADPH (H86903M, Meridian Life Science), and anti-β-actin (#4967, Cell Signaling). The next day, membranes were washed and incubated with anti-mouse IgG-HRP or anti-rabbit IgG-HRP secondary antibodies (Sigma or Southern Biotech) diluted at 1:2000 in blocking solution. Proteins were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific) and autoradiography. The films were digitized and optical densities were measured using a computerized image analysis system with a high-powered scanner and the software program Image J (v1.43u, National Institutes of Health) or Alphaimager (ProteinSimple).
Golgi Impregnation

Golgi-Cox impregnation was performed by FD Neurotechnologies (Columbia, MD), using the GD Rapid GolgiStain Kit (#PK401). Briefly, Dab1 cKO and WT controls were perfused for 5 min with PBS, followed by 4% PFA in PBS for 15 min. Brains were immersed in impregnation solution (equal volumes of Solutions A and B, containing mercuric chloride, potassium dichromate and potassium chromate), and stored at room temperature. Impregnation solution was replaced after 24 hrs. After two weeks, brains were transferred to Solution C and stored at 4°C for 48 hrs, with the solution replaced after 24 hrs. The brain was sectioned sagitally at 100 µm using a cryostat and sections were mounted on gelatin-coated microscope slides with Solution C. Slides were rinsed twice in distilled water (2 min each), and then placed in a mixture of 1 part Solution D, 1 part Solution E, and 2 parts distilled water for 10 minutes. After rinsing with distilled water, sections were dehydrated in 50%, 75%, and 95% ethanol (4 min each), and then were further dehydrated in 100% ethanol 4 times (4 min each). Sections were cleared in xylene 3 times (4 min each) and coverslipped with Permount solution.

Dendrite and Spine Analysis

Comparable Golgi-stained sections obtained from wild type and Dab1 cKO mice were imaged by an experimenter blinded with respect to genotype. To
examine the dendrite branching pattern of hippocampal pyramidal neurons in area CA1, z-stack bright field images were collected at 1 µm increments using an inverted microscope (Olympus IX50) with 10× and 20× objectives. The images were then flattened, and the number of secondary apical branches was measured. To determine the orientation of apical dendrites and the soma, the shortest line between hippocampal surfaces was drawn, and the angle between this line and the longest axis corresponding to either the primary apical dendrite or the soma was measured as described previously (O'Dell et al., 2012). A total of 26 WT and 17 cKO neurons from 4-5 animals of each genotype were traced for dendrite analysis and 40 WT and 34 cKO neurons for soma analysis. For spine analysis, secondary apical dendrites in the stratum radiatum of the CA1 region were imaged in bright field with a 60x Plan Apo oil immersion objective (NA 1.4). z-stack images were generated at 0.2 µm increments. Dendrite length was traced through the z-stack using the open source Simple Neurite Tracer, a Segmentation Plugin in ImageJ (FIJI image processing package). The number of dendritic spines was quantified from multiple dendrite segments, and spine density values were averaged by the number of neurons analyzed (WT = 46 neurons; cKO = 57 neurons). Data were obtained from multiple brain sections per animal (4-5 animals per genotype). A subset of z-stack images was randomly selected and further used to analyze dendritic spine area. Dendritic spine area (µm²) was measured using a 4x digital zoom and the Freehand Selection Tool in ImageJ. Data were averaged by neuron (n = 50 neurons per genotype).
Dendritic spine density and area analyses were performed by an experimenter blinded with respect to genotype.

**Ex vivo Slice Preparation and cLTP Induction**

Hippocampal slices were prepared from 2 month-old Dab1 cKO and WT controls as described previously (Weeber et al., 2002). Briefly, brains were sectioned horizontally in ice-cold cutting solution at 400 µm. The hippocampus was dissected and acclimated in 50:50 solution (cutting:ACSF) for 10 minutes at room temperature. Sections were then recovered in ACSF at 32 ºC for 2 hours. Chemical long-term potentiation (LTP) was induced by exposing slices to tetraethylammonium chloride (TEA-Cl, 25 mM) diluted in ACSF (adjusted for osmolarity) for 10 minutes. Slices were recovered in normal ACSF and collected at 5 and 45 minutes post-treatment. Hippocampal area CA1 was rapidly removed under a dissecting microscope and snap-frozen until further analysis.

**Electrophysiology**

Hippocampus slices were prepared from 3-6 month-old Dab1 cKO and WT mice as previously reported (Weeber et al., 2002). The brain was rapidly dissected and placed in ice-cold, oxygenated cutting solution containing (in mM) 110 sucrose, 60 NaCl, 3 KCl, 28 NaHCO₃, 1.25 NaH₂PO₄, 5 glucose, 0.6 ascorbate, 7 MgCl₂, and 0.5 CaCl₂. Horizontal 350 µm sections were generated
in cutting solution using a vibratome. The hippocampus was carefully dissected and transferred to room temperature cutting solution diluted 1:1 with artificial cerebral spinal fluid (ACSF). ACSF contains, in mM, 125 NaCl, 2.5 KCl, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 25 glucose, 1 MgCl$_2$, and 2 CaCl$_2$. Slices were maintained in this solution with constant 95% O$_2$/5% CO$_2$ perfusion for 10 min before being transferred to the brain slice recording chamber (Fine Science Tools, San Francisco, California, USA) or maintained in a holding container. Slices were recovered for a minimum of 1 hour before recording. The recording chamber was held at 30° ± 0.5°C with a ACSF flow rate of 1 ml/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded from stratum radiatum in hippocampal area CA1 via glass micropipettes pulled to an approximate 1 µm tip diameter (1–4 MΩ) and loaded with ACSF. Responses were generated by stimulation of Schaffer collaterals arising from the CA3 region. Stimulating electrodes consisted of formvar-coated nichrome wire, which was used to deliver biphasic stimulus pulses (1–15 V, 100 μsec duration, 0.05 Hz). Delivery of stimulation, controlled by pClamp 9.0 software (Axon Instruments, Forster City, California, USA), was via the Digidata 1322A interface (Axon Instruments) and a stimulus isolator (model 2200, A-M Systems, Sequim, Washington, USA). Signals were amplified using a differential amplifier (model 1800, A-M Systems), filtered at 1 kHz and digitized at 10 kHz. For all experiments, baseline stimulus intensity was set at the level that elicited approximately 50% of the maximum fEPSP response as determined from the input-output curve. The input-output relationship was determined by stimulating
slices from 0-18 mV at 0.5 mV increments. Short-term plasticity was measured via paired-pulse facilitation, which was induced by stimulating slices at half-max intensity with sequential pulses spaced at 20 ms intervals from 20-300 ms. Long-term potentiation (LTP) was induced by a theta-burst protocol, which consisted of five trains of four pulse bursts at 200 Hz separated by 200 ms, repeated six times with an inter-train interval of 10 s. High frequency stimulation (HFS) was also used to induce LTP, comprising 2 one-second trains of 100 Hz stimulation with a 20 second inter-train interval. To evaluate Reelin’s effects on theta-burst-induced LTP, slices were bathed with 5 nM Reelin or Mock conditioned media for 10 minutes prior to LTP induction. For analysis, the last 10 minutes of recording were averaged and compared.

**Mouse Behavior**

Wild type (5 males, 6 females) and Dab1 cKO mice (5 males, 6 females) at 3-6 months of age were subjected to behavioral studies. No sexual dimorphism of phenotype was observed. *Associative fear conditioning.* Fear conditioning was used to assess hippocampus function and associative learning and memory. Mice were placed in a 25x25 cm sound attenuation chamber with a wire grid floor and allowed to explore the context for 3 min. They then received a conditioned stimulus (CS, 90 db tone) for 30 sec. At the end of the 30 sec, mice received a mild foot shock (0.5 mA: unconditioned stimulus (US)) that co-terminated with the tone. After 1.5 min, the mice received a second CS/US
pairing, after which monitoring continued for 1.5 min. For the context test, 24 hours following CS/US pairing mice were placed back into the chamber and allowed to explore for 3 min. Mice were then subjected to the cued test, which required placing mice in a novel context (new noise, smells, textured surfaces, etc.) for a 3 min exploratory period followed by a 3 min exposure to the CS. An investigator blind to genotype monitored the mice and scored freezing at 5 sec intervals throughout the testing session. *Hidden platform watermaze.* A 1.2 m diameter pool was filled with white opaque water and a 10 cm diameter white platform was submerged just below the water surface. Large extra-maze cues were positioned around the room. During a single trial, mice were placed in the pool and allowed to swim to the escape platform for a maximum of 60 sec. Mice were given 4 trials per day for 4 days. Latency to escape, distance traveled and swim speed were measured by video tracking software (ANY-Maze, Stoelting, Illinois). At 24 and 72 hours following training day 4, the platform was removed and swim patterns were monitored for 60 sec during a probe trial. *Open-field test.* General activity and anxiety were measured by the open field test. Mice were placed in a 40 x 40 cm acrylic chamber under normal lighting conditions and allowed to explore for 30 minutes. Video tracking software monitored movement, immobility, and distance traveled (ANY-Maze, Stoelting). Anxiety was measured by comparing the amount of time spent in the center versus wall quadrants of the open field. Data was binned into 5 minute intervals to evaluate time-dependent differences in locomotor activity.
Statistical Analysis

Data in bar graphs are shown as the mean ± S.E.M., and analyzed by Student's t test, one-sample t test, or ANOVA as indicated in the results. The Bonferroni post hoc test was utilized for both one and two-way ANOVA tests. Statistical significance was determined when p<0.05.

Results

Expression Pattern of Dab1 and Other Components of the Reelin Pathway in the Postnatal Forebrain.

Reelin signaling through ApoER2/VLDLR and Dab1 is critical for several aspects of brain development, including neuronal positioning (D'Arcangelo et al., 1995; Sheldon et al., 1997; Trommsdorff et al., 1999), dendritic morphogenesis (Niu et al., 2004), and the formation and maturation of excitatory synapses (Liu et al., 2001; Groc et al., 2007; Qiu and Weeber, 2007; Niu et al., 2008; Ventruti et al., 2011). In the adult brain, Reelin signaling has been implicated in hippocampal synaptic plasticity, and learning and memory (Weeber et al., 2002; Beffert et al., 2005b; Qiu et al., 2006b). To further investigate the adult function of Reelin signaling, we first examined how the expression of Reelin and its signaling molecules transitions from a developmental to an adult pattern. We collected brain samples ranging from postnatal day (P) 7 to adulthood (P56), and
conducted Western blot analysis of Reelin, ApoER2, and Dab1 in the cerebral cortex, hippocampus and cerebellum. All three proteins were readily detected in the developing and adult structures examined (Fig. 1A). The expression of Reelin and its proteolytic fragments was highest around P7 in forebrain structures, and declined rapidly with age. In the cerebellum, Reelin expression peaked around P14, but remained elevated even during adulthood. The shifted pattern of Reelin expression in the cerebellum parallels the delayed development of this structure compared to the forebrain. A similar expression pattern was observed for ApoER2. However, the expression pattern of Dab1 appeared to be delayed compared to Reelin, and remained sustained throughout adulthood in all structures. When normalized to P7 levels, Dab1 levels in the hippocampus and cerebral cortex were highest at P14 (214 ± 3.5% and 118 ± 6.8%, respectively) and lowest at P56 (74.9 ± 3.1% and 39.9 ± 11.8%, respectively). In the cerebellum, Dab1 expression was relatively stable from P7 to P28 (85.2 ± 4.7% compared to P7 levels), but declined to significantly lower levels by P56 (58.6 ± 5.3% compared to P7 levels).

To further examine Dab1 expression in the adult forebrain, we conducted immunofluorescence staining using the previously described Dab1 B3 antibody (Howell et al., 1997). We first verified the specificity of the antibody by staining brain tissue sections obtained from adult Dab1-deficient scrambler (Scm) mutant mice and wild type (WT) littermates. Dab1 was detected prominently throughout the forebrain of WT mice, but not Scm mutants (Fig. 1B). In the WT hippocampus, the Dab1 signal appeared to be widespread and diffuse, and
particularly intense in cell bodies of the pyramidal layer, and in the neuropil of the stratum lacunsum moleculare (s.l.m.) (Fig. 1, C and D). In the neocortex, the Dab1 signal was high in the cell bodies and apical dendrites of many pyramidal neurons, particularly the large pyramidal neurons of layer V, and in the neuropil of the marginal zone (layer I). Double-labeling with Reelin antibodies indicated that this protein is highly expressed in a few isolated cells, which are located predominantly in the stratum oriens (s.o.) and s.l.m. of the hippocampus, and in all cellular layers of the neocortex (Fig. 1, C and D). These findings are consistent with previous reports, which demonstrated that Reelin is expressed by a subset of GABAergic interneurons and residual Cajal-Retzius cells in the postnatal forebrain (Alcantara et al., 1998a). When images of Dab1 and Reelin staining are overlaid, it is apparent that these two proteins are expressed mostly in non-overlapping cell populations, as previously reported in the prenatal brain (Rice et al., 1998). Occasionally, however, Dab1 was co-expressed in the cell body of a small fraction of Reelin-positive cells in both, the cortex and hippocampus (Fig. 1C, yellow arrows). Diffuse Reelin and Dab1 signals co-localized extensively in the s.l.m. of area CA1 (Fig. 1C), a region where the distal apical dendrites of Dab1-expressing pyramidal cells come in close proximity to Reelin-secreting Cajal-Retzius cells. Very faint and punctate Reelin immunoreactivity was also observed on Dab1-positive pyramidal cells in cortical layers II/III and V, and to a lesser extent in the hippocampus (Fig. 1D, right panels). It is likely that this signal represents surface-bound and/or internalized Reelin, as has been suggested previously in cortical cultures (Campo et al.,
Collectively, these data demonstrate that Reelin- and Dab1-expressing cells remain intimately juxtaposed in the adult forebrain.

**Synaptic Localization of Dab1 in the Adult Forebrain**

Because we observed Dab1 expression in the dendrites as well as the soma of pyramidal cells, we next determined if this protein localizes to synaptic structures in the adult forebrain. Crude synaptoneurosomes (SNS) were isolated from the cerebral cortex and hippocampus of 1 month-old mice, and analyzed by Western blotting using Dab1 monoclonal antibodies. Samples obtained from previously described constitutive Dab1 knockout (KO) mice (Howell et al., 1997) were used to confirm the specificity of the antibody. The data indicate that Dab1 is present at similar levels in the homogenate and SNS fractions of both, the cortex and hippocampus. Dab1 SNS levels were 94.5 ± 8.5% of homogenate levels in the cortex, and 120.8 ± 14.2% of homogenate in the hippocampus (p>0.05) (Fig. 2A), suggesting that a considerable amount of the protein is associated with synapses. To control for the quality of our SNS fractions, we reprobed the blots using antibodies directed against well-known synaptic protein markers. As expected, the postsynaptic protein NR1 and presynaptic protein synapsin IIa were enriched in SNS fractions of both the cortex and hippocampus, whereas actin levels were similar among samples (Fig. 2A).

To distinguish between pre- and postsynaptic localization of Dab1, further fractionation of synaptic and perisynaptic proteins was performed as described
previously (Dumanis et al., 2011). Dab1 was readily detected in the purified synaptic membrane fraction (LP1) at levels comparable to the initial, crude synaptoneurosome fraction (P2) (95.4 ± 3.08% LP1 normalized to P2, \( p > 0.05 \); Fig. 2B). To allow better visualization of Dab1 and Reelin, total protein levels loaded for the presynaptic vesicle (SV, also known as the LP2 fraction) and the postsynaptic density (PSD) fractions were four times higher than P2 and LP1 fractions. Quantification revealed similar levels of presynaptic and postsynaptic Dab1 (5.93 ± 0.85% SV and 5.5 ± 1.18% PSD normalized to P2 levels; \( p > 0.05 \)). A similar synaptic localization pattern was observed when the same blots were reprobed with Reelin antibodies (Fig. 2B). However, only the full-length (a) and the intermediate isoform of Reelin (b) were detected in synaptic fractions, possibly due to the higher solubility of the fully cleaved N terminal fragment (c), which was readily detectable in homogenate samples (Fig. 1A) and soluble fractions (not shown). To control for the quality of pre- and postsynaptic fractions, we reprobed blots with synaptophysin and PSD-95 antibodies. As expected, PSD-95 was detected in the PSD and not in the SV fraction, whereas synaptophysin was detected in the SV and not in the PSD fraction (Fig. 2B).

To further examine Dab1 localization at the synapse, we performed triple immunofluorescence labeling of Dab1, PSD-95, and synaptophysin in adult hippocampal sections and focused on the stratum radiatum (s.r.) of area CA1, where apical dendrites of pyramidal cells receive excitatory inputs from Schaffer collaterals. In this region, Dab1, PSD-95 and synaptophysin were detected in a punctate pattern along dendritic segments (Fig 2, C and D). Although most Dab1
positive puncta did not co-label with both PSD-95 and synaptophysin (Fig. 2,D and F), some did, suggesting Dab1 localization to at least some putative excitatory synapses (Fig. 2, D and E, yellow circle). Frequent apposition of Dab1- and synaptophysin-positive puncta was also noted in PSD-95-negative clusters (Fig. 2D white circle, and 2F), suggesting the possible additional localization of Dab1 to inhibitory synaptic structures.

**Generation of Conditional Knockout Mice with Excitatory Neuron-Specific Loss of Dab1 in the Adult Forebrain**

Complete loss of Reelin or Dab1 during development leads to dramatically disrupted neuronal positioning (D'Arcangelo et al., 1995; Sheldon et al., 1997; Trommsdorff et al., 1999), abnormal growth of cellular processes (Del Rio et al., 1997; Niu et al., 2004), and altered formation of excitatory synaptic structures (Niu et al., 2008), hindering the study of this molecular pathway in the adult brain. To overcome this limitation, we generated a novel conditional knockout mouse in which the Dab1 gene is specifically deleted in the adult forebrain. Mice carrying floxed Dab1 alleles (Dab1\textsuperscript{floxflox}) (Franco et al., 2011) were crossed with CamKIIα-Cre (T-29) transgenic driver mice (Tsien et al., 1996). This driver line has previously been extensively characterized, and reported to exhibit Cre expression almost exclusively in forebrain structures, starting around postnatal day 19 (Tsien et al., 1996; Dragatsis and Zeitlin, 2000). Cre-positive, homozygous mutant mice Dab1\textsuperscript{floxflox} (here referred to as cKO) were born at a
normal ratio, were fertile and appeared healthy and indistinguishable from Cre-negative control littermates (here referred to as WT).

To confirm the specificity of Cre expression, we introduced the ROSA26-tdTomato reporter gene into the genetic background of Dab1 cKO mice. The Dab1\(^{\text{flox/flox}}\) progeny that was Cre+ and tdTomato+ displayed an intense red fluorescence signal that was predominantly localized to forebrain structures, including the cerebral cortex and hippocampus, at 1 month (not shown) and 2 months of age (Fig. 3A). The reporter gene was expressed throughout the neocortex at these ages. However, in the hippocampus it was mostly restricted to area CA1 and to the dentate gyrus, and excluded from area CA3 (Fig. 3A).

These findings are consistent with previous reports (Tsien et al., 1996), and confirm that the CamKII\(\alpha\)-Cre driver line induces genetic recombination in the expected brain regions of cKO mice at adult ages.

To verify the loss of Dab1 protein expression in conditional mutant mice, we dissected the cerebral cortex, hippocampus, and cerebellum from adult cKO and WT mice, and performed Western blot analysis. As expected, Dab1 levels were significantly reduced in the cerebral cortex (33.4 ± 7.7% of WT) and hippocampus (48.1 ± 6.7% of WT) of 2 month-old cKO mice (\(p<0.001\), Student's \(t\) test), but not in the cerebellum (Fig. 3B). Similar results were obtained using 6 month-old mice (data not shown). We also performed immunostaining of brain sections obtained from 2-3 month-old WT and cKO mice using the Dab1 B3 antibody. Consistent with data shown in Fig. 1, Dab1 was detected throughout the neocortex and in all subfields of the hippocampus in WT mice (Fig. 3C).
However, in the cKO forebrain, Dab1 expression appeared dramatically reduced, with residual expression being restricted to a subpopulation of cells mostly located in deep cortical layers and in hippocampal area CA3 (Fig. 3C). To further examine the cell-specificity of Dab1 loss, we conducted double immunofluorescence experiments using the GABAergic interneuron marker glutamic acid decarboxylase 67 (GAD67) (Fig. 3, D-G). In the WT hippocampus (Fig. 3, D and E), Dab1 labeled mostly GAD67-negative cell bodies (presumed excitatory neurons); however, co-labeling was also observed in some interneurons located mainly in s.o. and s.l.m.. Upon closer magnification (Fig. 3E), Dab1 and GAD67 were found to co-label the soma of GABAergic interneurons positioned in s.o. (white arrow), as well as putative perisomatic inhibitory synapses in the stratum pyramidale (s.p.; yellow arrow). This expression pattern was even more obvious in sections derived from cKO mice.

Here, Dab1 was readily observed in the soma of GAD67-positive interneurons located in s.o. and s.l.m. (Fig. 3, F and G, white arrows), and in perisomatic inhibitory synapses in s.p. (Fig. 3G, yellow arrow). These data demonstrates that, in addition to excitatory neurons, Dab1 is also expressed by GABAergic basket cells, which are responsible for perisomatic inhibition of pyramidal neurons in hippocampal area CA1 (Karson et al., 2009). A large population of immature granule cells residing at the interface of the hilar region and granule cell layer was also found to express Dab1 in cKO sections (not shown). Taken together, our data indicate that Dab1 loss in cKO mice is restricted to excitatory neurons of the adult forebrain, consistent with previous
findings that the CamKIIα promoter is selectively activated in most excitatory neurons of the adult forebrain (Tsien et al., 1996; Sik et al., 1998; Dragatsis and Zeitlin, 2000).

**Analysis of Brain Anatomy and Excitatory Synaptic Structure in Dab1 cKO Mice**

To examine the consequence of Dab1 loss in excitatory neurons of the adult forebrain, we stained brain sections of 2 month-old cKO and WT control mice with thionin. All cortical brain regions appeared normal at low magnification (Fig. 4A), and cellular layers of the neocortex and hippocampus appeared intact (Fig. 4B). Contrary to previous findings resulting from the injection of Reelin interfering antibodies in the adult dentate gyrus (Heinrich et al., 2006), no granule cell dispersion was observed in adult cKO mice. Similarly, no neuroanatomical defects were observed in cKO mice at older ages (6-11 months, not shown). These findings are consistent with the established role of Reelin-Dab1 signaling in the development of cellular layers, since this process is largely complete before the time of onset of Cre expression in cKO mice (around 1 month of age). Furthermore, they demonstrate that Reelin-Dab1 signaling is not required to maintain the integrity of cellular layers in the adult forebrain.

To determine whether neuronal orientation, dendrite arborization and spine formation are affected in Dab1 cKO mice, we performed Golgi staining of 2 month-old cKO and WT brains. The gross morphology of excitatory hippocampal
pyramidal neurons in area CA1 (Fig. 4, C and D) as well as cortical neurons (not shown), which exhibit almost complete loss of Dab1 in cKO mice by this age, appeared normal. To further analyze the branching pattern of apical dendrites in hippocampal area CA1, we traced primary and secondary branches of individual pyramidal neurons in the s.r., and measured the cumulative number of secondary branches at increasing distances from the soma. The data indicate that the branching pattern of cKO neurons is similar to that of WT neurons ($p>0.05$) (Fig. 4D). We also analyzed soma and primary apical dendrite orientation of traced WT and cKO pyramidal neurons, as previously described (O'Dell et al., 2012). The orientation of both the soma and apical dendrite was found to be unaffected by the loss of Dab1 in cKO neurons ($p>0.05$) (Fig. 4D). These findings demonstrate that Reelin-Dab1 signaling is not required to maintain the morphology or the orientation of dendritic trees in the adult forebrain. Next, we collected high-magnification images of Golgi-stained secondary apical dendrites of pyramidal neurons in area CA1 to visualize spines (Fig. 4C). Mature spines with a mushroom-like head were readily observed in both genotypes, and represented the great majority of imaged spines. Therefore, we did not attempt to classify them into subcategories, but measured their density and head size. Our analysis reveals that dendritic spine density was not altered in Dab1 cKO mice (Fig. 4E) ($p>0.05$, Student's $t$ test). However, the cross-sectional area was significantly smaller in cKO mice (Fig. 4F) ($p < 0.0003$, Student's $t$ test). The spine area measurements were $0.39 \pm 0.0173 \, \mu m^2$ in WT and $0.30 \pm 0.0153 \, \mu m^2$ in Dab1 cKO mice ($n=50$ neurons per genotype). Based on previous reports that
the geometry of dendritic spines is a key determinant of their glutamate
sensitivity (Matsuzaki et al., 2001; Matsuzaki et al., 2004; Hayashi and
Majewska, 2005), these findings suggest that loss of Dab1 might affect
glutamatergic synaptic transmission and synaptic plasticity in adult cKO mice.

We have previously reported that Reelin haploinsufficiency during
development results in alterations of the biochemical and physiological properties
of adult hippocampal excitatory synapses (Qiu and Weeber, 2007; Ventruti et al.,
2011). To address whether Reelin signaling loss in the adult forebrain disrupts
the molecular composition of synapses, we prepared protein homogenate and
 crude SNS fractions from WT and Dab1 cKO mice at 2 months of age, and
analyzed them by Western blotting (Fig. 4G). As expected, the levels of Dab1
were significantly decreased in homogenate and SNS fractions derived from cKO
mice (50.2 ± 9.6% of WT in homogenate, 48.8 ± 7.8% of WT in SNS). However,
we found no significant changes in the levels of postsynaptic protein markers
such as PSD-95, NR2A, NR2B, and NR1 (Fig. 4G) (PSD-95, 89.5 ± 2.3% of WT
in homogenate, 92.4 ± 4.2% of WT in SNS; NR2A, 110.4 ± 10.1% of WT in
homogenate, 96.1 ± 4.1% of WT in SNS; NR2B, 99.4 ± 4.8% of WT in
homogenate, 92.7 ± 5.4% of WT in SNS; NR1, 113.4 ± 12.2% of WT in
homogenate, 96.9 ± 7.8% of WT in SNS). The levels of presynaptic protein
markers such as synapsin IIa and synaptophysin were similar in homogenate
fractions, but they were marginally decreased in the SNS fractions of cKO mice
(Synapsin IIa, 104.3 ± 4.0% of WT in homogenate, 86.0 ± 2.4% of WT in SNS,
\( p=0.071 \) in SNS; Synaptophysin, 98.6 ± 3.7% of WT in homogenate, 87.0 ± 1.5%
of WT in SNS, $p=0.054$ in SNS) (Fig. 4G and data not shown). Together, these results indicate that Dab1 is not required for dendritic spine formation, maintenance or composition in the adult hippocampus, but plays a role in maintaining normal spine size.

**Basal Akt and ERK1/2 Signaling Abnormalities in the Adult Dab1 cKO Forebrain**

Given the spine anomalies we observed in Dab1 cKO mice, we next sought to elucidate potential molecular underpinnings of these changes. A multitude of intracellular signaling pathways have been implicated in regulating dendritic spine formation and maturation, including the PI3K and downstream protein kinase B (PKB/Akt) (Cuesto et al., 2011; Majumdar et al., 2011), as well as mitogen-activated protein kinases (MAPKs), including ERK1 and ERK2 (Goldin and Segal, 2003; El Gaamouch et al., 2012). To determine whether loss of Dab1 in the adult forebrain affects these intracellular signaling pathways, we performed Western blot analysis of WT and cKO cortical and hippocampal lysates. At a young adult age (2 months), we detected no differences in the levels of activated, phosphorylated Akt (Ser473) (p-Akt) and activated, phosphorylated ERK1 and ERK2 (Thr202/Tyr204) (p-ERK1/2) (Fig. 5A). Intriguingly, however, we found a significant reduction in the basal levels of p-Akt and p-ERK1/2 in both the cortex and hippocampus of mature adult (4 month-old) Dab1 cKO mice compared to WT littermates (Fig. 5B). Specifically, the levels of p-Akt were $55.7 \pm$
5.3% of WT in the cortex ($p<0.01$, one-sample $t$ test), and 54.0 ± 3.1% of WT in the hippocampus of Dab1 cKO mice ($p<0.001$, one-sample $t$ test). Similarly, the levels of p-ERK2 were 57.3 ± 9.7% of WT in the cortex, and 69.1 ± 7.7% of WT in the hippocampus of the Dab1 cKO mice ($p<0.05$ for both, one-sample $t$ test). In support of these findings, the phosphorylation of downstream Akt substrates, including mTOR (Ser2448) and GSK3β (Ser9), was also markedly reduced in the forebrain of 4 month-old Dab1 cKO mice (data not shown), whereas the levels of total kinase were not affected. These results demonstrate that chronic loss of Dab1 in excitatory neurons leads to deficits in the basal activity of PI3K/Akt and ERK1/2 signaling pathways in the mature adult forebrain.

**Activity-Dependent ERK1/2 Signaling in Dab1 Mutant Cultures**

Since spine abnormalities were present in cKO mice at 2 months of age, prior to the appearance of detectable deficits in basal signaling, we wondered whether altered kinetics or reduced levels of ERK1/2 signaling in response to neuronal activity could underlie structural spine defects. Intracellular signaling is critical for conveying information from the site of postsynaptic activation to the nucleus, which in turn enables chromatin remodeling and changes in gene expression that permit long-term synaptic plasticity (Wiegert and Bading, 2011). In particular, the activation of ERK1/2 is necessary for the expression and maintenance of long-term potentiation (LTP) (Tsokas et al., 2007; Gobert et al., 2008; Maharana et al., 2013). To determine whether Dab1 is required for activity-
dependent stimulation of ERK1/2 signaling, we analyzed activity-dependent ERK1/2 signaling in adult hippocampal slices *ex vivo*. Transverse hippocampal slices were prepared from WT and Dab1 cKO mice at 2 months of age, a timepoint when differences in basal ERK1/2 activation were not yet evident (Fig. 5). The slices were allowed to recover for 2 hours, and then were stimulated for 10 minutes with 25 mM of the potassium channel blocker tetraethylammonium-chloride (TEA-Cl) to induce robust chemical LTP (cLTP) in hippocampal area CA1, as described previously (Hanse and Gustafsson, 1994). Hippocampal area CA1 was dissected for protein isolation from non-treated slices and from slices that received TEA-Cl at 5 and 45 minutes following treatment. At 5 minutes post-TEA-Cl treatment, a rapid and robust stimulation of ERK1 phosphorylation (p-ERK1/2 Thr202/Tyr204) was observed in WT (395 ± 46.2%; \( p < 0.05 \), unpaired *t* test) and Dab1 cKO slices (336 ± 42.7%; \( p < 0.05 \), unpaired *t* test) (Fig. 6A). Although the relative changes in the ratio of p-ERK1/ERK in Dab1 cKO were slightly lower than in WT slices, comparison between these groups did not reach statistical significance. The levels of p-ERK1 returned to baseline similarly in both WT (103 ± 11.4%) and Dab1 cKO (81.9 ± 6.70%) slices at 45 minutes post-TEA-Cl treatment (Fig. 6B; quantification not shown). On the other hand, significant differences were observed in the dynamics of ERK2 phosphorylation in WT and Dab1 cKO slices following cLTP induction. At 5 minutes following TEA-Cl treatment, WT slices exhibited a 234 ± 13.1% increase in relative p-ERK2 levels (\( p < 0.001 \), unpaired *t* test) versus a 187 ± 10.2% increase in Dab1 cKO slices (\( p < 0.05 \), unpaired *t* test). The magnitude of ERK2 activation was significantly
reduced in the Dab1 cKO compared to WT slices \( (p<0.01, \text{two-way ANOVA with Bonferonni’s post hoc test}) \) (Fig. 6A). At 45 minutes following treatment (Fig. 6B), WT slices still showed a 126 ± 5.2% increase in relative p-ERK2 levels \( (p<0.05, \text{unpaired t test}) \), whereas Dab1 cKO slices returned to baseline levels \( (97.8 ± 3.3\%; p>0.05, \text{unpaired t test}) \). Thus, the sustained activation of ERK2 at 45 minutes after cLTP induction was blunted in Dab1 cKO compared to WT slices \( (p<0.05, \text{two-way ANOVA with Bonferonni’s post hoc test}) \). These data suggest that Dab1 serves a critical function in regulating the kinetics of activity-dependent MAPK activation in the adult brain, as its deficiency hinders the sustained activation of ERK2 following synaptic potentiation.

**Physiological Abnormalities in Dab1 cKO Hippocampal Slices**

Application of Reelin to hippocampal slices enhances LTP at Schaffer collateral synapses through binding both ApoER2 and VLDLR, and promoting activation of NMDARs (Weeber et al., 2002; Beffert et al., 2005b). We have thus far presented evidence of biochemical and structural changes in Dab1 cKO, which is the first conclusive evidence that Dab1 continues to regulate synaptic function in the adult brain. In line with previous studies, we hypothesized that impaired activity-dependent kinase regulation and the inability to convey Reelin signaling may culminate in perturbed synaptic plasticity in Dab1 cKO mice. To test this hypothesis, we used acute hippocampal slices obtained from adult WT and Dab1 cKO mice (3-6 months old) to examine synaptic transmission, short-
term plasticity, long-term plasticity, and Reelin responsiveness at the well-defined Schaffer collateral synapses.

We first evaluated overall synaptic transmission by comparing the amplitude of the evoked fiber volley to the slope of the field excitatory postsynaptic potential (fEPSP) at increasing stimulus intensities (Fig. 7A). Comparison of the linear-fitted plot revealed no differences between WT (slope = 2.176 ± 0.26) and Dab1 cKO slices (slope = 1.929 ± 0.316), suggesting that overall synaptic transmission was normal. Short-term synaptic plasticity was evaluated by the amount of paired-pulse facilitation (PPF) with interpulse intervals (IPI) ranging from 20-300 ms (Fig. 7B). Significant reductions in PPF were found at the 40 ms interval (148 ± 7.02% WT, 134 ± 3.16% cKO; \(p<0.05\), unpaired \(t\) test), but not at other intervals.

We have previously reported deficits in LTP induced by either two trains of 100-Hz stimulation (HFS) or theta-burst stimulation (TBS-LTP) in ApoER2 knockout mice (Weeber et al., 2002). TBS-LTP consists of five trains of four pulse bursts at 200-Hz, repeated 6 times with an interburst interval of 10 s. Using this protocol, we found a significant reduction in LTP induction (i.e. first ten minutes averaged) in the Dab1 cKO (130 ± 7.19%) compared to WT controls (164 ± 8.81%; \(p<0.05\), unpaired \(t\) test) (Fig. 7C). Moreover, the maintenance of LTP (i.e. final 10 minutes averaged) in the Dab1 cKO (124 ± 10.3%) was significantly reduced compared to wild type controls (152 ± 6.9%; \(p<0.05\), unpaired \(t\) test) (Fig. 7C). Using HFS, we found reduced LTP in the Dab1 cKO (115 ± 10.8%) compared to WT slices (156 ± 111.5%; \(p<0.05\), unpaired \(t\) test).
when the final 20 minutes of recording were averaged (Fig. 7D). Although the induction of LTP (first 10 minutes) appeared to be reduced in the cKO (118 \pm 14.6\%) compared to WT (154 \pm 17.3), this effect was not statistically significant (\(p=0.176\)).

Previous work by our group and others has established that Reelin potently enhances TBS-LTP in a manner that depends on two critical domains of ApoER2: the NPxY motif, which binds Dab1 (Beffert et al., 2006) and the alternatively-spliced exon19 (Beffert et al., 2005b), which binds PSD-95 and JIP1/2. To determine if Dab1 is required for the enhancement of LTP by Reelin, we treated WT and Dab1 cKO slices with mock or Reelin conditioned media 10 minutes prior to inducing TBS-LTP. Confirming previous studies, Reelin treatment enhanced the induction of LTP (first 10 minutes) in WT mice (205 \pm 10.1\% with Reelin versus 153 \pm 7.17\% with mock; \(p<0.05\), unpaired \(t\) test) (Fig. 7E). The enhancement of LTP by Reelin was maintained even through the final 10 minutes of recording (177 \pm 14.8\% with Reelin versus 141 \pm 6.83\% with mock; \(p<0.05\), unpaired \(t\) test). Conversely, Reelin-conditioned medium had no effect on TBS-LTP in Dab1 cKO when compared to mock-treated slices at any time during recording (Fig. 7F). These data demonstrate that Dab1 affects synaptic plasticity and it is absolutely required for the modulation of hippocampal LTP by Reelin.
Behavioral Abnormalities in Adult Dab1 cKO Mice

Reelin haploinsufficiency in reeler heterozygous mice (Qiu et al., 2006b) or the loss of individual Reelin receptors ApoER2 or VLDLR (Weeber et al., 2002) leads to impairments in hippocampal-dependent associative learning. These previous studies utilized mouse models that have reduced, but not ablated, Reelin signaling. Homozygous reeler mutant or double receptor knock out mice could not be used for behavioral studies because of neurodevelopmental confounds, including neuronal ectopia (Trommsdorff et al., 1999), delayed dendrite maturation (Niu et al., 2004) and impaired synaptic development (Niu et al., 2008; Ventruti et al., 2011). To determine whether Dab1 (and thus Reelin signaling) is required for cognitive function we conducted a behavioral characterization of adult cKO and WT littermates at 3-6 months of age. We first performed a standard open-field test with a 30 min duration and found no differences in overall locomotor activity measured by distance traveled (Fig. 8A) or percent time spent in the center of the open-field, a measure of thigmotactic behavior or anxiety (Fig. 8B). A battery of other behavioral tests was implemented in order to ensure that observed deficits were not due to underlying physical or behavioral deficits. No differences were detected in measures of activity or anxiety in the elevated plus maze, motor coordination or learning in the rotarod test, or short-term memory in the y-maze test of spontaneous alternations (data not shown). To test for associative learning, we performed a two-trial fear conditioning protocol on WT and Dab1 cKO mice by pairing an aversive stimulus
(mild footshock) with an acoustic tone [conditioned stimulus (CS); white noise] in a novel context. The fear response was determined by measuring the frequency at which normal motor behavior was disrupted by freezing, a period of watchful immobility. The extent of freezing to the acoustic tone and shock during training was not significantly different between animal groups (Fig. 8C). Long-term associative memory was assessed by measuring the freezing response to the original context (context test), or to the tone in a novel context (cued test) at 24 hours post-conditioning. Our data show that Dab1 cKO mice (35.6 ± 4.07%) froze significantly less than wild type controls (49.0 ± 4.55%) in the context test (Fig. 8E; \( p < 0.05 \)), but not in the cued test (Fig. 8D). Observed deficits in contextual learning are likely due to impaired signaling mechanisms that are required for normal synaptic plasticity and long-term hippocampal-dependent associative learning.

Previous studies have identified mild impairments in spatial learning in mice deficient in ApoER2 knockout mice (Beffert et al., 2006) or mice expressing an ApoER2 isoform that lacks exon19 (Beffert et al., 2005b), but not in the heterozygous reeler model (Qiu et al., 2006b). To determine if Dab1 plays a role in this form of learning, WT and Dab1 cKO mice were trained in the hidden platform water maze task. Specifically, mice were trained to find a submerged platform in a circular pool filled with opaque water using distal visual cues positioned outside the pool. Training took place over the course of 4 days with 4 trials per day and an approximate inter-trial interval of 1 hour. No differences were seen in latency to find the platform across training (Fig. 8F). Spatial
memory was assessed using a 24-hour probe test in which the platform was removed and mice were allowed to employ spatial search strategies to locate the platform’s original location. No significant differences were seen in the distance traveled (or swim speed (not shown). Both WT and Dab1 cKO mice were similar in that they crossed over the location of the target platform significant more times than other platform areas (Fig. 8G). However, a probe trial conducted 72 hours after training revealed significant differences between WT and cKO mice. At this time, WT mice showed a marked preference for the target platform versus other platform areas ($p<0.05$), whereas Dab1 cKO did not (Fig. 8H). This defect was not due to differences in total distance traveled (Fig. 8I, $p>0.05$) or swim speed (not shown). The observation that cKO had normal spatial memory at 24 hours, but not at 72 hours after training, suggests that Dab1 is required for proper long-term memory consolidation.

Discussion

A multitude of studies have established the essential role of the Reelin-Dab1 signaling pathway in brain development. Using a novel genetic model, in this study we conclusively demonstrate that Reelin-Dab1 signaling is also required for adult brain function. We show that loss of Dab1 in excitatory neurons of the adult forebrain leads to a reduction in dendritic spine size, suppression of Akt and ERK signaling pathways, loss of hippocampal LTP, and ultimately deficits in hippocampal-dependent learning and memory without affecting cellular
layering or dendrite morphology. Our findings may prove significant for understanding and treating neuropsychiatric and neuropathological disorders associated with reduced Reelin signaling, including schizophrenia (Guidotti et al., 2000; Verbrugghe et al., 2012), autism (Fatemi et al., 2005; Ashley-Koch et al., 2007), and Alzheimer's disease (Chin et al., 2007; Herring et al., 2012).

In agreement with previous reports (Alcantara et al., 1998b; Trommsdorff et al., 1999), we observed a dramatic down-regulation of Reelin and its signaling machinery, including ApoER2 and Dab1, that paralleled the completion of postnatal synaptic development and the transition into mature neuronal circuitry (Fig. 1). In the adult forebrain, scattered Reelin-expressing GABAergic interneurons were mostly juxtaposed to Dab1-positive excitatory neurons. However, our conditional knock out strategy also revealed that Dab1 is expressed by a subset of GABAergic interneurons (Fig. 3), suggesting that Reelin signaling onto interneurons may be important for the proper function of neural networks in the adult hippocampus. In particular, the observed enrichment of Dab1 at perisomatic synapses in the pyramidal layer suggests that Dab1 is expressed by GABAergic basket cells, which are critical regulators of hippocampal network oscillations (Klausberger, 2009). Considering that hippocampal pyramidal neurons of adult reeler heterozygous mice have reduced spontaneous inhibitory postsynaptic potentials (Qiu et al., 2006b), but normal excitatory postsynaptic potentials, these data raise the possibility that the inhibitory synapse is an important site of Reelin activity that has been understudied so far and warrants further investigation. Consistent with this view,
our immunofluorescence data indicate that Dab1 co-localizes frequently with the 

presynaptic marker synaptophysin, but less frequently with the excitatory 

postsynaptic marker PSD-95 in the s.r. of area CA1 (Fig. 2). Biochemical 

fractionation data confirmed that Dab1 is expressed both pre- and 

postsynaptically, even though it does not appear to be enriched at the synapse 

(Fig. 2). Dab1 appears to be distributed in many cellular compartments, including 
the soma, proximal and distal apical dendrites of hippocampal excitatory and 
inhibitory neurons. Overall, the pattern of Dab1 expression in the adult forebrain 
is consistent with the proposed role of Reelin signaling in synaptic function. 

The dramatic loss of Dab1 in the adult forebrain of our conditional 
knockout mice did not result in any detectable changes in gross brain 
morphology, neuronal lamination, neuronal orientation or dendritic arborization 
(Figs. 3 and 4). We further show that Dab1 is not required for dendritic spine 
formation, maintenance or molecular composition in the adult hippocampus, but 
is required for mature spine morphology (Fig. 4). We have previously reported 
that reduced Reelin signaling in young adult heterozygous reeler or Dab1 
knockout mice leads to a reduction in dendritic spine density (Niu et al., 2008). 

Based on these findings, we predict that there is a postnatal window during which 
Reelin signaling promotes spinogenesis. Indeed, a marked down-regulation of 
Reelin and ApoER2 was observed from P7-P21, a stage corresponding to a 
critical period of maturation for hippocampal excitatory synapses (Bogen et al., 
2009). Despite unaltered spine density, we observed a reduction in the cross-
sectional area of dendritic spines in the adult Dab1 cKO hippocampus. This
finding is consistent with the complementary discovery that Reelin overexpression in the adult hippocampus induces dendritic spine hypertrophy (Pujadas et al., 2010). This morphological phenotype may result from a chronic reduction in synaptic activity, which is known to promote the enlargement of dendritic spines through AMPAR insertion (Fortin et al., 2010; Hill and Zito, 2013). Consistent with this view, our previous work demonstrated that Reelin facilitates the insertion of AMPAR in hippocampal neurons via Dab1-dependent activation of PI3K/Akt (Qiu et al., 2006a). The reduction in spine size may also result from a persistent deficit in synaptic plasticity, which normally leads to a large postsynaptic increase in calcium that facilitates actin branching and polymerization, generating a protrusive force that facilitates spine enlargement (Fukazawa et al., 2003). Indeed, Reelin signaling has been shown to promote the activation of NMDARs through tyrosine phosphorylation of the NR2A and NR2B cytoplasmic tails, leading to increased calcium influx (Chen et al., 2005).

Combined, these mechanisms could explain the observed changes in spine morphology in the Dab1 cKO mice, however, potential direct effects of Reelin on the actin cytoskeleton, as seen during development (Kruger et al., 2010), cannot be presently excluded.

While investigating potential signaling mechanisms underlying the spine morphology phenotype of Dab1 cKO mice, we noted basal reductions in the activation of Akt and ERK1/2 pathways (Fig. 5). Both these signal transduction pathways were previously shown to be activated by acute Reelin treatment of dissociated neurons. Specifically, Reelin was first shown to induce Akt
phosphorylation and to promote the interaction between Dab1 and the PI3K regulatory subunit p85α (Beffert et al., 2002; Ballif et al., 2003; Bock et al., 2003). Moreover, Reelin was shown to stimulate the activation of ERK1/2 in cortical neurons in a manner that depends on SFK and PI3K/Akt activation (Simo et al., 2007). While the activation of Akt and downstream signaling proteins has been confirmed in several studies (Ballif et al., 2003; Jossin and Goffinet, 2007; Ventruti et al., 2011), the stimulation of ERK1/2 phosphorylation has not yet been replicated, possibly due to differences in the composition of the Reelin conditioned medium (Ballif et al., 2003). Given our present findings that ERK1/2 phosphorylation is altered in Dab1 cKO mice, the involvement of ERK1/2 in downstream Reelin signaling warrants further investigation.

In the adult brain, both the PI3K/Akt and ERK pathways perform indispensable roles in regulating neuronal survival, synaptic plasticity, and learning and memory (Curtis and Finkbeiner, 1999; Orban et al., 1999; Sweatt, 2004). Our observed reduction in the basal activation of both Akt and ERK1/2 in Dab1 cKO mice in mature, but not young adult cKO mice, suggests a cumulative defect that results from chronic deficiency in Reelin signaling. Alternatively, it could be due to a sustained impairment in synaptic function or plasticity that is uniquely due to Dab1 loss at the synapse. Interestingly, we found that the loss of Dab1 in the adult hippocampus altered the kinetics of ERK1/2 activation following synaptic potentiation (Fig. 6). These findings implicate Dab1 as an important modulator of intracellular signaling underlying synaptic plasticity in the adult hippocampus.
The present study demonstrates that Dab1 plays an important role in adult synaptic physiology. We observed reduced paired-pulse facilitation in Dab1 cKO hippocampal slices (Fig. 7), which is consistent with previous studies utilizing Reelin or ApoER2 mutants (Beffert et al., 2006; Qiu et al., 2006b), and suggests that Reelin signaling regulates presynaptic mechanisms controlling neurotransmitter release and short-term synaptic plasticity. Indeed, specific deficits in neurotransmission have been noted in reeler mice (Qiu et al., 2006b; Hellwig et al., 2011). However, since we did not observe extensive deletion of Dab1 in area CA3 (Fig. 3), it is possible that altered postsynaptic signaling may drive the observed presynaptic impairments. Dab1 cKO mice also exhibited impairments in long-term synaptic plasticity, which is consistent with reported results in heterozygous reeler mice (Qiu et al., 2006b) and ApoER2/VLDLR mutants (Weeber et al., 2002; Beffert et al., 2005b). Unlike these previous studies, our present findings reveal physiological defects resulting from the complete loss of Reelin signaling in the adult hippocampus. Loss of Dab1 led to a profound reduction in LTP induced by high-frequency stimulation and theta-burst stimulation, and lack of LTP enhancement by Reelin. Whether the effects of Dab1 loss result exclusively from the absence of Reelin signaling, or from the disruption of other Reelin-independent signaling mechanisms in which Dab1 participates, remains to be determined.

Finally, here we demonstrated that Dab1 is required for normal hippocampal-dependent associative learning. Specifically, we observed selective hippocampal-dependent fear associative learning deficits in Dab1 cKO mice,
which is consistent with previous studies of heterozygous *reeler* mice (Qiu et al., 2006b) and ApoER2 mutants (Weeber et al., 2002; Beffert et al., 2005b; Beffert et al., 2006). Observed deficits in spatial memory in the hidden platform water maze at 72 hours, but not 24 hours, also suggests that Reelin signaling may play a role in long-term memory consolidation. In summary, our data provide the first definitive evidence that Reelin signaling through Dab1 continues to play an important role in the adult brain by regulating synaptic function and associative learning. These results will serve as a foundation upon which more detailed biochemical and physiological experiments will be designed to understand how Reelin signaling regulates synaptic function in the adult brain and how its disruption may contribute to cognitive disorders.

**References**


Figure 3.1. Dab1 and Reelin Expression in the Postnatal Forebrain. A. Western blot analysis of Reelin, ApoER2, and Dab1 in the cortex, hippocampus, and cerebellum at postnatal days (P) 7, 14, 21, 28, and 56. Blots shown are representative of data obtained from 3-4 mice per age. Reelin was detected as a full-length isoform of approximately 450 kDa (a) and two N-terminal fragments, of 370 kDa (b) and 180 kDa (c). ApoER2 was detected as three major isoforms (a-c). Dab1 was detected as a single band of approximately 90 kDa. The blots were reprobed with GAPDH and actin antibodies as loading controls. B, Immunofluorescence labeling of Dab1 in the postnatal hippocampus. Dab1 signal (green) was present in WT sections, but not in the Dab1 mutant scrambler (Scm), confirming antibody specificity. Scale bar, 100 µm. C-D, Double immunofluorescence labeling of hippocampal area CA1 and the neocortex (Cx) with Dab1 (green) and Reelin (red) antibodies. Sections were obtained from 2-3 month old WT mice. Larger panels show Dab1 and Reelin staining in mostly distinct cell populations (white arrows), with the exception of few cells that
showed colocalization (yellow arrow). (D) Smaller panels show Dab1 labeling at higher magnification (right, white arrows). Scale bars: 100 µm (large panels in B and C), 20 µm (small panels in D, left), and 5 µm and 10 µm (small panels in D, right, CA1 and Cx, respectively). s.o., stratum oriens; s.p., stratum pyramidale; s.r., stratum radiatum; s.l.m., stratum lacunosum moleculare.
**Figure 3.2. Synaptic Localization of Dab1 in the Adult Forebrain.**

**A,** Western blot analysis of Dab1 in the homogenate (Hom) and crude synaptoneurosome (SNS) fractions of the wild type (WT) and Dab1 knockout (KO) cerebral cortex and hippocampus. The data (quantified from n=4 WT mice) indicate that Dab1 is present at similar levels in homogenate and SNS fractions obtained from both, the cortex and the hippocampus (p>0.05). The blot was reprobed with antibodies against the postsynaptic NMDAR subunit NR1, presynaptic synapsin IIa, and actin as a loading control.

**B,** Western blot analysis of Reelin and Dab1 in purified synaptic fractions. Reelin and Dab1 were present in crude synaptoneuroosomes (P2), synaptic membranes (LP1), and, to a lesser extent, in presynaptic vesicles (SV) and postsynaptic density (PSD) fractions. The blot was reprobed with PSD-95 and synaptophysin antibodies to confirm the purity of the fractions.

**C, D,** Triple immunofluorescence labeling of the adult hippocampus with antibodies against Dab1 (green), PSD-95 (red) and synaptophysin (blue). The inset yellow box in C is further magnified in panels D to show dendrites projecting through the stratum radiatum of hippocampal area CA1. A triple labeled, putative excitatory synapse is circled in yellow and magnified in E, whereas a Dab1- and synaptophysin-positive (but PSD-95-negative) punctum (white circle) is magnified in F. Scale bars: 50 µm (C) and 5 µm (D).
Figure 3.3. Adult Forebrain Excitatory Neuron-Specific Loss of Dab1 Expression in cKO Mice. A, A representative brain section obtained from a 2 month-old cKO mouse also expressing the tdTomato reporter gene. The low magnification panel on the left shows predominant reporter gene expression in the forebrain, whereas the higher magnification panel on the right shows elevated activity in the cerebral cortex, hippocampal area CA1 and dentate gyrus. Scale bars: 2 mm (large panel) and 200 µm (small panel). B, Western blot analysis of Dab1 in brain regions of 2 month-old wild type (WT) and Dab1 conditional knockout (cKO) mice. Blots were reprobed with antibodies against ribosomal protein S6 as a loading control. The data were quantified from n=4-5 mice/genotype. Dab1 levels were significantly reduced in the cortex and hippocampus, but not in the cerebellum, of cKO mice compared to WT mice. Bar graphs show the mean ± SEM; ***p<0.001. C, Immunoperoxidase staining of
Dab1 in 2 month-old WT and cKO brains revealed widespread loss of Dab1 expression throughout the cortex and hippocampus. Scale bar, 200 µm. **D-E**, Double immunofluorescence of adult WT hippocampal sections stained with Dab1 (green) and GAD67 (red) antibodies. Magnified panels in E show that Dab1 is expressed mostly in the cell bodies of excitatory neurons, which are surrounded by inhibitory terminals (yellow arrows). Occasionally, co-labeling of interneuron cell bodies was noted (white arrows). **F-G**, Double immunofluorescence of similar cKO sections reveals the specific loss of Dab1 in excitatory, pyramidal neurons, which unMASKs the residual expression of Dab1 in the interneuron cell bodies (white arrows) and terminals (yellow arrows). Scale bars, 50 µm (D, F) and 25 µm (E, G). **s.o.**, stratum oriens; **s.p.**, stratum pyramidale; **s.r.**, stratum radiatum; **s.l.m.**, stratum lacunosum moleculare.
Figure 3.4. Anatomy, Dendrite Morphology and Spine Analysis in the Dab1 cKO Hippocampus. A,B, Sagittal sections of 2 month-old wild type (WT) and Dab1 cKO mice were stained with thionin. All main regions appear normal in whole brain images (A). Magnified images of the cortex and hippocampus also
show no obvious defects in cellular layers (B). Scale bars: A, 2 mm; B, 200 µm. C, Images of Golgi-stained hippocampus from 2 month-old WT and cKO mice. Insets in the larger panels were further magnified to show individual pyramidal neurons and their dendrites in area CA1 (middle panels). Representative z-stack images of secondary apical dendrites bearing spines are also shown (right panels). Scale bars: 300 µm (left panels), 100 µm (middle panels), 2 µm (right panels). D, Tracing and analysis of dendrite branching and neuronal orientation. Examples of traced WT and cKO pyramidal neurons in area CA1 are shown. 26 WT and 17 cKO neurons from 4-5 mice per genotype were used for branching analysis; 40 WT and 34 cKO neurons from 4-5 mice per genotype were used for neuronal orientation analysis. No significant change in secondary dendrite branching or orientation of apical dendrites and cell body is apparent. E,F, Quantification of spine density (E) and area (F). There was no significant difference in spine density between WT and cKO neurons. However, spine area was significantly smaller in Dab1 cKO compared to WT mice (***p<0.0001). G, Western blot analysis of Dab1 and synaptic protein markers in the homogenate and crude synaptoneurosome (SNS) fractions of wild type (WT) and Dab1 knockout (KO) hippocampus. The blots were probed sequentially or in parallel with antibodies against Dab1, postsynaptic PSD-95, NMDAR subunits NR1 and NR2A, and presynaptic Synapsin Ila. Actin was used as a loading control. Although Dab1 levels were decreased in samples obtained from cKO mice, the levels of all analyzed synaptic proteins appear similar between genotypes (data from 5-6 mice per genotype).
Figure 3.5. Basal Akt and ERK1/2 Signaling in Adult Dab1 cKO Mice.

A-B, Western blot analysis of the phosphorylation status of Akt and ERK1/2 in adult wild type (WT) and Dab1 cKO cortex (CX) and hippocampus (HC). The data were quantified as the ratio of phospho-Akt (p-Akt) over total Akt, and phospho-ERK2 (p-ERK2) over total ERK2. There were no significant differences in the levels of p-Akt and p-ERK1/2 between genotypes at 2 months of age (A). However, at 4 months of age (B) the levels of p-Akt and p-ERK2 were significantly reduced in both the cortex and hippocampus of Dab1 cKO compared to WT mice (n=4 mice per genotype). Bar graphs show the mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
Figure 3.6. Alterations of ERK2 Activation following Synaptic Potentiation in Dab1 cKO. A, Cortical neurons of wild type (WT) or constitutive Dab1 knockout (KO) were cultured for 5 days in vitro and depolarized with 20 µM potassium chloride (KCl) for 20 minutes. The phosphorylation status of Akt and Erk1/2 was analyzed and plotted as in Figure 5. Quantification of the data from multiple experiments (n=5 cultures per genotype) indicates that the levels of p-Akt and p-ERK2 were similarly up-regulated following depolarization in both, WT and Dab1 KO cultures. Bar graphs show the mean ± SEM; *p<0.05, ** p<0.01, ***p<0.001. B,C, Acute hippocampal slices from 2 month-old WT (6 slices, 3 animals) and conditional Dab1 cKO (5 slices, 3 animals) were exposed to 25 mM tetraethylammonium chloride (TEA-Cl) to induce chemical long-term potentiation. Western blot analysis of area CA1 revealed that TEA-Cl induced a significant up-regulation of p-ERK2 after 5 and 45 minutes of stimulation in WT slices. Similar treatments generated a significant difference in Dab1 cKO slices only after 5 minutes (B), but not 45 minutes (C). (*) indicates comparison using unpaired t-test with **p<0.01; ***p<0.001; (#) indicates comparison with two-way ANOVA using the Bonferonni’s post hoc test with # #, p<0.01; #, p<0.05).
**Figure 3.7. Altered Synaptic Function in Adult Dab1 cKO Mice.** Hippocampal slices were obtained from 3-6 month-old wild type (WT) and Dab1 cKO mice. **A,** Normal synaptic transmission was observed when comparing the slope of the field EPSP versus the fiber volley amplitude at increasing intensities for WT (●, n=11 slices, 4 mice) and cKO mice (○, n=18 slices, 4 mice). **B,** Short-term synaptic plasticity was evaluated by the amount of paired-pulse facilitation (PPF) with interpulse intervals (IPI) ranging from 20-300 ms in WT (●, 20 slices, n=5 mice) and cKO mice (○, 34 slices, n=6 mice). Significant reductions in PPF were found at the 40ms IPI (*p<0.05). **C,** Long term potentiation was induced with
theta-burst stimulation. The first and final ten minutes of recording were averaged separately, revealing both a reduction in LTP induction (*$p<0.05$) and maintenance (*$p<0.05$) in Dab1 cKO (●, 15 slices, $n=7$ mice) compared to WT controls (●, 13 slices, $n=6$ mice), respectively. 

**D.** Long-term potentiation was induced by high-frequency stimulation consisting of 2 trains at 100-Hz in WT (●, 8 slices, $n=4$ mice) and Dab1 cKO mice (○, 11 slices, $n=5$ mice). A reduction of LTP in cKO slices compared to WT was observed when the final 20 minutes of recording were averaged (*$p<0.05$), but not the first 10 minutes. 

**E.** Mock- and Reelin-conditioned medium was applied for ten minutes prior to inducing LTP with TBS (indicated by gray line). Reelin treatment (▲, 8 slices, $n=3$ mice) was found to enhance LTP relative to mock treatment (■, 15 slices, $n=5$ mice) in WT mice (*$p<0.05$). 

**F.** Reelin-conditioned medium (Δ, 9 slices, $n=3$ mice) had no effect on TBS-induced LTP in Dab1 cKO slices when compared to mock medium (□, 7 slices, $n=3$ mice). For all data representation, except panel A, measurements from individual slices were averaged per mouse.
Figure 3.8. Normal Fear Associative Learning Requires Dab1. **A-B,** Differences in locomotor activity and anxiety were evaluated using a 30-minute open-field test (OFT). The Dab1 cKO mice \((n=11)\) and WT controls \((n=11)\) exhibited similar levels of activity. Two-trial fear conditioning was used to determine associative learning at 24 hours following two conditioned stimulus-(CS) unconditioned stimulus (US) pairings. **C-D,** No differences were seen in freezing rates during training or in a novel context in the absence or presence of the CS (tone) when comparing WT and cKO mice. **E,** Wild type mice \((WT, n=11)\) exhibited a greater conditioning to the context compared to Dab1 cKO \((n=11; *p<0.05)\). **F-I,** Hidden-platform water maze was used to assess spatial learning and memory. No differences between WT and cKO were seen in latency to platform across 4 days of training \(4 \text{ trials / day}.\) A 60-second probe trial was conducted at 24 hours post-training. No significant difference was found in the number of target platform (TP) crossings by WT \((n=11)\) versus Dab1 cKO mice \((n=10).\) Both genotypes crossed into the TP significantly more times than in other pseudo-platform areas \(*p<0.05).\) At 72 hours, a probe trial revealed that WT mice crossed into the TP significantly more times than cKO and compared to other platform regions \(*p<0.05, \text{two-way ANOVA using the Bonferroni’s post hoc test).}\)
No difference was seen in distance traveled between the genotypes. OP, opposite platform; AP1, adjacent platform 1; AP2, adjacent platform 2.
Chapter 4

Interneuronal Disabled-1 is a Critical Regulator of
Hippocampal Excitatory Neurotransmission and Plasticity

Abstract

Disabled-1 (Dab1) is an intracellular adaptor protein that acts downstream of the secreted glycoprotein Reelin to promote proper neuronal migration during development. The locus of Reelin signaling in the forebrain is widely believed to be at excitatory synapses. Here we present evidence that Dab1 is also expressed by a subpopulation of GABAergic interneurons and is present at pre- and postsynaptic inhibitory sites. To study the function of Dab1 at inhibitory synapses, we generated a conditional knockout mouse in which Dab1 was deleted in Gad2-expressing GABAergic interneurons. Heterozygous and complete inhibitory conditional knockout mice (iHET and iKO) exhibited normal lamination of cortical structures and patterning of inhibitory synapses. However, iKO mice also presented with severe motor discoordination and cerebellar hypoplasia associated with impaired Purkinje cell migration. In the hippocampus of adult iHET and iKO mice, significant increases were seen in the levels of several excitatory synaptic proteins, including the NMDA receptor subunits NR1
and NR2B, while the levels of other excitatory and inhibitory synaptic proteins were normal (i.e. NR2A, GAD65, GAD67, and gephyrin). Functional alterations of glutamatergic synapses were confirmed by field recordings in hippocampal area CA1, which revealed significant increases in presynaptic activation in both iHET and iKO mice, as well as increased postsynaptic excitatory neurotransmission in iKO mice. Although presynaptic short-term plasticity was normal, impairments in theta-burst stimulated LTP were observed in iHET and iKO mice. Taken together, these data suggest that the expression of Dab1 by GABAergic interneurons is critical for normal hippocampal excitatory neurotransmission and synaptic plasticity.

**Introduction**

In the forebrain, the inhibitory neurotransmitter GABA is released by a highly diverse repertoire of interneurons with unique molecular, anatomical, and electrophysiological properties (Freund and Buzsaki, 1996; Somogyi and Klausberger, 2005). While only comprising a small percentage of total neurons in a given circuit (i.e. 10-25%), recent studies have implicated dysfunction of GABAergic interneurons in the pathoetiology of neurodevelopmental (Han et al., 2012; Wallace et al., 2012), neuropsychiatric (Fatemi et al., 2005; Lewis et al., 2012), and neurodegenerative disorders (Verret et al., 2012). Despite tremendous progress in the Reelin field, considerably little is known about the development and functional regulation of GABAergic interneurons.
The secreted glycoprotein Reelin regulates the positioning and dendritic development of principle neurons (Stanfield and Cowan, 1979; D'Arcangelo et al., 1995; Olson et al., 2006; Franco et al., 2011; Jossin and Cooper, 2011) through binding the lipoprotein receptors, apolipoprotein E receptor 2 and very-low density lipoprotein receptor (Hiesberger et al., 1999), and activating the downstream adaptor protein Dab1 (Howell et al., 1997; Sheldon et al., 1997; Rice et al., 1998). Reelin signaling may also influence the development of GABAergic interneurons (Hammond et al., 2006; Pla et al., 2006; Yabut et al., 2007).

Abnormal positioning of both projection neurons and GABAergic interneurons is observed in the forebrain of reeler (Hevner et al., 2004; Yabut et al., 2007) and scrambler mice (Pla et al., 2006), carrying null mutations in Reln and Dab1, respectively. However, medial ganglionic eminence (MGE)-derived interneurons transplanted from scrambler to wild-type mice disperse normally throughout cortical layers, suggesting that 1) interneuron migration is likely Reelin-independent, and 2) abnormal migration of interneurons in reeler and scrambler mutants is a consequence of projection neuron displacement. Nonetheless, the same study did observe ubiquitous expression of Dab1 in developing interneurons, suggesting that interneurons may respond to Reelin signaling in other ways (Pla et al., 2006).

In addition to its role of development, Reelin signaling in the postnatal brain is required for the formation and maturation of excitatory synapse (Sinagra et al., 2005; Qiu and Weeber, 2007; Niu et al., 2008; Ventruti et al., 2011) and modulating synaptic plasticity and learning and memory (Weeber et al., 2002;
Beffert et al., 2005; Beffert et al., 2006; Rogers et al., 2011). We recently reported that the absence of Dab1 expression in excitatory neurons of the postnatal forebrain unmask widespread expression of Dab1 by GABAergic interneurons (Trotter et al., 2013). Although the locus of Reelin signaling in the adult is widely-assumed to be at excitatory synapses, the possibility that it is also operative at inhibitory synapses has not been previously explored. Here we address this significant knowledge gap through the generation of a GABAergic interneuron-specific Dab1 conditional knockout mouse (iKO). We present evidence that interneuronal expression of Dab1 is not required for forebrain lamination or the patterning of GABAergic synapses, but rather, plays an important role in regulating the biochemical and physiological properties of glutamatergic synapses.

Materials and Methods

Mouse Colonies

All animals used for this study were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of South Florida. Animals of either sex were group housed in a standard 12 hour light/dark cycle and fed standard mouse chow ad libitum. Dab1<sup>flox/flox</sup> founder mice were genotyped as described previously (Franco et al., 2011). CamKIIα-Cre (B6.Cg-Tg(Camk2a-cre)T29-1Stl/J) and Gad2-Cre (Gad2<sup(TIM2 CRE)</sup>Yh/J) transgenic driver mice
were obtained from The Jackson Laboratory (Bar Harbor, ME). Mutant and transgenic mice were genotyped by PCR as suggested by the distributor. For all experiments, inhibitory conditional knockout heterozygous mice (iHET) carried one floxed \textit{Dab1} allele and at least one copy of \textit{Gad2-cre}, whereas complete inhibitory knockouts (iKO) carried two floxed \textit{Dab1} alleles and at least one copy of \textit{Gad2-cre}.

\textit{Tissue Histology}

Mice were deeply anesthetized with sodium pentobarbital (SomnaSol) or isoflurane and perfused transcardially with phosphate buffer (PB; pH 7.2) or saline solution (0.9\% NaCl), followed by 4\% paraformaldehyde (PFA) in PBS. Brains were dissected, post-fixed in 4\% PFA overnight at 4\(^\circ\)C, and cryoprotected by incubation in 30\% sucrose in PBS at 4\(^\circ\)C for 2 days. Brains were mounted onto a sliding microtome using OCT (Tissue-Tek) and sectioned either coronally (for immunohistochemistry) or sagittally (for histology) at 25 \(\mu\)m. To evaluate neuroanatomical organization, sections were stained with Cresyl violet (0.05\%, Sigma Aldrich), washed in distilled water, dehydrated with a graded alcohol series, cleared with xylenes, and then coverslipped with DPX mounting media. Stained sections were imaged using a Zeiss Axio Scope A1 microscope.
**Hippocampal Primary Culture**

Brains from P0 wild-type C57Bl6 pups were removed and dissected under a microscope in ice-cold isotonic buffer (137mM NaCl, 5.4mM KCl, 170µM Na2HPO4, 148µM K2HPO4, 5.5mM glucose, 58.4mM sucrose). Dissected tissue was pooled together in 15 mL tube and incubated with 1 mL 0.25% trypsin for 5 min at 37ºC. Trypsin was removed and 5 mL dissociation media was added (DMEM, high glucose, 10% heat inactivate FBS, 1X antimycotic/antibacterial solution). Hippocampi were dissociated by slowly pipetting through glass pipettes. After brief centrifugation, dissociation media was replaced by complete Neurobasal medium (cNB) supplemented with 2% B27 and 0.5mM Glutamax. Cells were plated on glass coverslips treated pre-coated with laminin and poly-D-lysine at a density of 25,000 cells / well. Cultures were maintained at 37ºC with half of the media per well being exchanged every 3 days with fresh cNB. On DIV21, cell media was aspirated and cells were washed once with PBS, followed by fixation in 4% paraformaldehyde for 15 minutes. Cells were washed 2X with PBS and then stained as described above.

**Immunostaining**

Fluorescence immunostaining was performed as described previously (Trotter et al., 2011). The following primary antibodies were used: anti-Dab1 B3 (1:500; provided by Dr. Brian Howell, SUNY Upstate Medical University), anti-GAD67 (1:2000; MAB5406, Millipore), anti-PSD-95 (1:1000; P78352, Neuromab), and anti-
Gephyrin (147 021, Synaptic Systems). The following secondary antibodies were used: goat anti-mouse IgG-Alexa 488, goat anti-rabbit IgG-Alexa 488, and goat anti-rabbit IgG-Alexa 546 (all used at 1:1000 and obtained from Invitrogen). Sections were mounted on positively-charged glass slides and coverslipped with ProLong Gold Antifade Reagent with DAPI (Invitrogen). Sections were imaged using the Olympus FV10i confocal microscope. For comparison between genotypes, images were taken with the same exposure settings and were adjusted similarly for brightness and contrast in Adobe Photoshop.

Protein Isolation and Western Blotting

Hippocampi from 2-3 month old male or female wild-type, iHET, and iKO mice were dissected and whole-cell lysates were isolated. All centrifugation steps were performed at 4°C. Tissue was homogenized in ice-cold RIPA buffer (50 mM Tris pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA) supplemented with Halt protease and phosphatase inhibitor cocktails (Thermo Scientific) and cleared by centrifugation at 15,000 × g for 10 minutes at 4°C. Supernatants were saved and stored at -80°C until use. Protein concentration was determined using the BCA Protein Assay (Thermo Scientific). Samples were adjusted to equal protein concentration and combined with Laemmli Sample Buffer containing 5% 2-mercaptoethanol. Samples were boiled for 5 minutes at 95°C, except when analyzing Reelin, VGAT, and VGLUT expression. Protein lysates were separated electrophoretically onto 4-15% TGX gels (Bio-rad) and transferred to
PVDF blotting membrane. Membranes were blocked in a solution containing 0.1M tris-buffered saline (TBS) with 0.1% Tween-20 and 5% nonfat milk, and then incubated overnight at 4°C with primary antibodies diluted in blocking solution. Primary antibodies include: monoclonal anti-Dab1 (a gift of Dr. André M Goffinet, Université Catholique de Louvain, Belgium), anti-NR2A (Millipore), anti-NR2B (Millipore), anti-NR1 (Ab9864R, Millipore), anti-synapsin (Abcam), anti-PSD-95 (P78352, NeuroMab), anti-synaptophysin (#5461, Cell Signaling), anti-Reelin (MAB5364, Millipore), VGLUT1 (135 303, Synaptic Systems), anti-VGAT (131 003, Synaptic Systems), anti-GAD65 (GAD-6, Developmental Studies Hybridoma Bank), anti-GAD67 (MAB5406, Millipore), anti-gephyrin (147 111, Synaptic Systems), and anti-β-actin (#4967, Cell Signaling). The next day, membranes were washed and incubated with anti-mouse IgG-HRP or anti-rabbit IgG-HRP secondary antibodies (Southern Biotech) diluted at 1:2000 in blocking solution. Proteins were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific) and autoradiography. The films were digitized and optical densities were measured using a computerized image analysis system with a high-powered scanner and the software program ImageJ (v1.43u, National Institutes of Health).

Electrophysiology

Hippocampus slices were prepared from 2-3 month-old Dab1 iHET, iKO, and wild-type mice as previously reported (Weeber et al., 2002). The brain was rapidly dissected and placed in ice-cold, oxygenated cutting solution containing
(in mM) 110 sucrose, 60 NaCl, 3 KCl, 28 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 5 glucose, 0.6 ascorbate, 7 MgCl$_2$, and 0.5 CaCl$_2$. Horizontal 350 µm sections were generated in cutting solution using a vibratome. The hippocampus was carefully dissected and transferred to room temperature cutting solution diluted 1:1 with artificial cerebral spinal fluid (ACSF). ACSF contains (in mM): 125 NaCl, 2.5 KCl, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 25 glucose, 1 MgCl$_2$, and 2 CaCl$_2$. Slices were maintained in this solution with constant 95% O$_2$/5% CO$_2$ perfusion for 10 min before being transferred to the recording chamber (Fine Science Tools, San Francisco, California, USA). Slices were recovered for at least 1 hour before recording. The recording chamber was maintained at 30° ± 0.5°C with an ACSF flow rate of 1 ml/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded in stratum radiatum of hippocampal area CA1 via glass micropipettes pulled to an approximate tip diameter of 1 µm (1–4 MΩ) and loaded with ACSF. Responses were elicited via stimulation of Schaffer collaterals arising from the CA3 region. Stimulating electrodes were made using formvar-coated nichrome wire, and used to deliver biphasic stimulus pulses (1–15 mV, 100 µsec duration, 0.05 Hz). The delivery of stimulation was controlled by pClamp 10.0 software (Axon Instruments, Forster City, California, USA), a Digidata 1322A interface (Axon Instruments), and stimulus isolator (model 2200, A-M Systems, Sequim, Washington, USA). All signals were amplified using a differential amplifier (model 1800, A-M Systems), filtered at 1 kHz and digitized at 10 kHz. Baseline stimulus intensity was set at the level that elicited 40-50% of the maximum fEPSP response as determined from the input-output curve. Input-output relationships
were determined by stimulating slices from 1-15 mV at 0.5 mV increments. Short-term plasticity was measured via paired-pulse facilitation, which was induced by stimulating slices at half-max intensity with sequential pulses spaced at 20 ms intervals from 20-300 ms. Long-term potentiation (LTP) was induced by a theta-burst protocol, which consisted of five bursts at 200 Hz separated by 200 ms, repeated six times with an inter-train interval of 10 s. For analysis, the first 10, last 10, and complete 60 minutes of recording following theta-burst stimulation were averaged and compared.

**Statistical Analysis**

All data is shown as the mean ± S.E.M. All western blotting and LTP data was analyzed using a one-way ANOVA followed by the Bonferroni’s post hoc test. Analysis of neurotransmission data required nonlinear regression analysis followed by comparison of fitted lines using the extra sum-of-squares F-test. Statistical significance was determined when $p < 0.05$. 
Results

*Dab1 is Expressed by GABAergic Interneurons in the Postnatal Hippocampus.*

We recently demonstrated that Dab1 expression by excitatory pyramidal cells of the postnatal forebrain is critical for hippocampal synaptic function and learning and memory (Trotter et al., 2013). In this paper we described a novel conditional knockout mouse in which Dab1 was selectively-ablated from excitatory neurons (ecKO), a model which incidentally revealed expression of Dab1 by GABAergic interneurons. Here we expand on these findings by further evaluating the co-expression of Dab1 with the inhibitory interneuron marker glutamic acid decarboxylase 67 (GAD67). Similar to our previous findings, Dab1 was ubiquitously expressed by pyramidal cells in hippocampal area CA1, with a particular enrichment in the soma and apical dendrites (Fig. 1A). In addition, Dab1 and GAD67 were co-expressed by interneurons residing near the interface of stratum radiatum (s.r.) and lacunosum moleculare (s.l.m; Fig. 1A, magnified panel). In area CA1 of ecKO mice, Dab1-expressing pyramidal cells were almost undetectable at 2-3 months of age. With the exception of a sparse population of Dab1-positive pyramidal cells, remaining Dab1-positive cells were mostly GAD67-positive as well (Fig.1A). In fact, few GABAergic interneurons positioned in s.r. and s.l.m. were Dab1-negative.
Since Reelin signaling is often studied at the Schaffer collateral / CA1 synapse (Weeber et al., 2002; Beffert et al., 2005; Qiu and Weeber, 2007), we also evaluated Dab1 and GAD67 co-expression in hippocampal area CA3 from which the Schaffer collaterals emanate. Similar to CA1, Dab1 and GAD67 frequently co-labeled interneurons positioned throughout s.r. and s.l.m. Unfortunately, GAD67 staining is not suitable for detection of interneurons positioned in either stratum pyramidale or lucidum, which contain extensive perisomatic inhibitory synapses. As previously reported, we did not observe significant loss of Dab1 in pyramidal cells in CA3 (Fig. 1B). Nevertheless, Dab1- and GAD67 co-labeled neurons were similarly positioned throughout s.r. and s.l.m in wild-type and ecKO mice (Fig 1B, magnified). Similar to the hippocampal formation, loss of Dab1 expression in excitatory neurons in ecKO mice, allowed better visualization of GAD67- and Dab1 co-labeled interneurons in the cortex (Figure 4.7).

Expression of Dab1 at Inhibitory Synapses

Expression of Dab1 by GABAergic interneurons suggests that Dab1 may traffic to inhibitory synapses, rendering them sensitive to Reelin signaling. To address this possibility, dissociated hippocampal neurons were prepared from P0 wild-type pups and cultured for 21 days in vitro. Dab1 was co-labeled with GAD67 and gephyrin, markers for the pre- and postsynaptic sites of inhibitory synapses, respectively (Fig. 2A-B). In culture, most GABAergic interneurons are
readily identified by intense somatic staining of GAD67, which heavily associates with the Golgi apparatus (Kanaani et al., 2010). Most GAD67-positive interneurons expressed Dab1, which was found to traffic extensively throughout their neurites (Fig. 2A). Some GAD67 clusters found on neurites, which correspond to presynaptic inhibitory terminals, were also Dab1-positive (Fig. 2A).

We previously reported that Dab1 does not localize to all PSD-95-positive excitatory synapses in hippocampal area CA1, despite tight apposition with the presynaptic protein synaptophysin (Trotter et al., 2013). This suggested that Dab1 could also traffic to other compartments of hippocampal pyramidal cells, including inhibitory postsynaptic sites. Here, we have confirmed that Dab1 localizes to some, but not all, gephyrin-positive inhibitory postsynaptic sites in the dendrites of pyramidal cells (Fig. 2B). We also confirmed trafficking of Dab1 to excitatory synapses by co-staining Dab1 with PSD-95. Similar to inhibitory postsynaptic sites, Dab1 was found to traffic only to a subset of PSD-95-positive dendritic spines positioned perpendicular to the dendritic shaft (Fig. 2C). Both PSD-95 and Dab1 co-localized extensively within the dendritic shaft, likely reflect common intracellular trafficking pathways. Taken together, these data suggest that Dab1 might mediate Reelin signaling differentially at subsets of pre- and postsynaptic inhibitory and excitatory sites.
Selective Loss of Dab1 in GABAergic Interneurons does not Affect Forebrain Development.

Specifically addressing the role of Reelin signaling in the development and function of GABAergic interneurons has been hampered by a lack of adequate mouse models. To overcome this limitation, we generated a novel interneuron-specific conditional knockout by crossing mice carrying floxed Dab1 alleles (Franco et al., 2011) with the recently reported Gad2-cre driver line (Taniguchi et al., 2011). Mice with a single floxed Dab1 allele and carrying at least one copy of Gad2-cre (iHET) were viable and had no obvious phenotypic differences from wild-type littermates. However, mice carrying two floxed Dab1 alleles and at least one copy of Gad2-cre (iKO) resembled reeler and scrambler mutants, exhibiting severe ataxia and resting tremors (data not shown). Selective loss of Dab1 in GABAergic interneurons was verified by performing co-labeling of Dab1 and GAD67. In hippocampal area CA1 of adult wild-type and iKO mice, similar expression levels of Dab1 by pyramidal cells was observed (Fig. 3A-B). Importantly, magnification of GAD67-positive cells confirmed the absence of Dab1 immunoreactivity in inhibitory interneurons of adult iKO mice.

Next we evaluated Dab1 protein levels in the hippocampus, cortex, and cerebellum of 2-3 month old wild-type (n = 6), iHET (n = 7), and iKO (n = 4) mice. Since GABAergic interneurons comprise only 10-25% of total cells in the cortex and hippocampal formation and Dab1 appears to be expressed by only a subset of interneurons, we did not anticipate significant changes in the expression of
Dab1. Indeed, no significant changes in Dab1 levels were detected in the cortex or hippocampus of iHET and iKO mice compared to wild-type controls (Fig. 3C). However, a dramatic down-regulation of Dab1 levels was observed in the cerebellum of both iHET (78.1 ± 4.11%, p < 0.001) and iKO mice (41.4 ± 2.25%, p < 0.0001) compared to wild-type levels (100 ± 4.30%). The level of Dab1 in iKO mice was also significantly lower than iHET mice (p < 0.0001) (Fig. 3C).

We next determined if the expression of Dab1 by GABAergic interneurons is required for proper lamination of brain structures. Sections from 3-week old wild-type and iKO (n = 4-5) mice were stained with Cresyl-violet, a dye which recognizes the Nissl substance, a collection of rough endoplasmic reticulum and other acidic components present in neuronal cytoplasm and nuclei. Normal layering of the cortex and hippocampal formation was observed in wild-type and iKO mice (Fig. 3D). Surprisingly, the iKO cerebellum was smaller and exhibited a complete absence of foliation, closely resembling the reeler (Mariani et al., 1977; Goffinet, 1983) and scrambler (Sweet et al., 1996; Sheldon et al., 1997) cerebellum and explaining the severe motor phenotype observed in these mice. Thus, expression of Dab1 by GABAergic interneurons is not required for the lamination of cortical structures but is essential for hindbrain development.
Patterning of Inhibitory Synaptic Terminals is Normal in the Forebrain of iKO Mice.

Abnormal migration and axonal development of GABAergic interneurons in reeler (Yabut et al., 2007) and scrambler mice has been reported to result from projection neuron displacement (Pla et al., 2006). To directly address whether or not Dab1 is required for the development of inhibitory neurons, we studied interneuron placement and synapse development in iKO mice using GAD67 immunostaining. GAD67 reliably detects a proportion of GABAergic interneuron cell bodies in the hippocampus and in some cortical layers (Esclapez et al., 1994). Using densitometric analysis, we quantified GAD67-positive synaptic terminals throughout synaptic lamina of the hippocampal formation (i.e. CA1, CA3, and DG) and cortical layers (Fig. 4) of 3 week old wild-type (n = 5) and iKO (n = 4) mice. We found no significant quantitative differences in GAD67 intensity in any region studied (data not shown). Although cell placement could not be directly measured using GAD67 immunostaining, the lack of change in overall patterning of inhibitory synapses supports the general notion that interneuron placement and their gross morphological development does not require Reelin signaling.

Unlike the forebrain, we did observe significant changes in the placement of GAD67-positive cerebellar Purkinje cells (PCs) (Fig. 4), explaining the ataxic gait and resting tremor observed in iKO mice and consistent with previous reports of PC ectopia in reeler and scrambler mutants (Goffinet, 1983; Goldowitz
et al., 1997). The preponderance of GAD67-positive PCs and other interneurons were accumulated ectopically in deep cerebellar masses (Fig. 4). A small population of PCs was able to position properly in the PC layer and elaborates small dendritic processes into the molecular layer (Fig. 4, arrow).

**Altered Expression of Synaptic Proteins in Adult iHET and iKO Mice**

Reelin signaling during development is required for the biochemical maturation of hippocampal glutamatergic synapses (Sinagra et al., 2005; Qiu and Weeber, 2007; Ventruti et al., 2011). However, earlier studies did not exclude the possibility that Reelin signaling modulates the composition of excitatory synapses indirectly through acting on GABAergic interneurons. Here we addressed whether interneuronal expression of Dab1 is required for the expression of several pre- and postsynaptic proteins of both inhibitory and excitatory synapses. First, since a proportion of Reelin-expressing GABAergic interneurons are also Dab1-positive (Trotter et al., 2013), we determined if loss of interneuronal Dab1 affected Reelin levels. No differences were found in full-length Reelin or either of its major proteolytic fragments (Fig. 5A). The quantification of hippocampal Dab1 levels was discussed already for figure 3, but is shown here for comparison.

The synaptic vesicle-associated proteins, synapsin-1 and synaptophysin, are expressed at both excitatory and inhibitory synapses (Bragina et al., 2007; Bogen et al., 2009). No differences were observed in their expression levels in the hippocampus of iHET and iKO compared to wild-type mice (Fig. 5B). We next
evaluated the levels of vesicular GABA transporter (VGAT) and vesicular glutamate transporter 1 (VGLUT1), which are exclusively expressed at inhibitory and excitatory presynaptic terminals, respectively. The levels of VGLUT1 were up-regulated in iKO (128 ± 5.07%) compared to wild-type mice (100 ± 3.37%, p < 0.05) (Fig. 5B). No differences were detected when comparing iHET (110 ± 5.53%) to iKO or wild-type levels. Moreover, VGAT protein levels were up-regulated in iKO (145 ± 1.62%) compared to iHET (106 ± 8.10%, p < 0.01) and wild-type mice (100 ± 3.92%, p < 0.01) (Fig. 5B). No difference was detected when comparing iHET to wild-type levels.

Previous studies have suggested that Reelin may regulate the expression of the Gad1 gene product, GAD67 (Liu et al., 2001; Rogers et al., 2013), the primary enzyme responsible for tonic GABA production in the brain (Erlander et al., 1991). In neither iHET nor iKO were changes observed in the expression of GAD67 (Fig. 5B), suggesting that interneuronal expression of Dab1 may not be required for the regulation of GAD67 levels by Reelin. The other major enzyme responsible for GABA production is glutamic acid decarboxylase 65 (GAD65), which is encoded by Gad2 (Martin and Rimvall, 1993). In this study, we employed a Gad2-cre driver line, which was generated using a 3' knockin of Cre in the Gad2 promoter region (Taniguchi et al., 2011), an approach that could have undesired effects on GAD65 expression. No relationship was found between GAD65 levels and the dosage of Gad2-cre (data not shown), nor were there differences in GAD65 expression in iHET and iKO mice compared to wild-type controls (Fig. 5B). These data suggest that Dab1 expression by GABAergic
interneurons does not significantly affect the overall number of inhibitory
presynaptic terminals or production of GABA, as would otherwise be indicated by
alterations in GAD67 and GAD65.

We also evaluated the expression of postsynaptic proteins of
glutamatergic synapses (i.e. NR1, NR2A, NR2B, and PSD-95) and GABAergic
synapses (i.e. gephyrin). Despite selective loss of Dab1 in GABAergic
interneurons, we saw a significant up-regulation of the obligatory NMDA receptor
subunit NR1 in both iHET (118 ± 6.33%, p < 0.05) and iKO (130 ± 1.36%, p <
0.05) compared to wild-type mice (100 ± 4.83%). Interestingly, NR2A levels were
not different between genotypes, but similar to NR1, the levels of NR2B were
increased in iHET (130 ± 5.06%, p < 0.01) and iKO (131 ± 0.498%, p < 0.05)
mice compared to wild-type levels (100 ± 7.20%). No difference was detected
when comparing NR1 and NR2B levels in iHET and iKO mice. Expression of the
glutamatergic scaffold protein PSD-95 was also increased in iKO (125 ± 5.81%, p
< 0.05) but not iHET mice (107 ± 3.67%) when compared to wild-type levels
(100 ± 4.02%). An absence of alterations in the expression of the postsynaptic
inhibitory scaffold protein gephyrin in iHET and iKO mice (Fig. 5) emphasizes
that loss of Dab1 in interneurons may be particularly deleterious for the normal
biochemical composition and function of excitatory synapses.
Increased Excitatory Neurotransmission but Impaired Synaptic Plasticity in iHET and iKO Mice.

Based on our findings of altered levels of major excitatory synaptic proteins in the hippocampus of iHET and iKO mice, we next determined whether the physiological function of the well-defined Schaffer collateral (SC) / CA1 synapse was altered in adult 2-3 month old mice. Overall synaptic transmission was evaluated by comparing the slope of the field excitatory postsynaptic potential (fEPSP) to the amplitude of the evoked fiber volley (FV) at increasing stimulus intensities in wild-type (n = 45 slices, 10 mice), iHET (n = 52 slices, 11 mice), and iKO slices (n = 29 slices, 5 mice). A significant change in the input/output relationship (I/O) was seen in both iHET and iKO mice. A nonlinear regression was performed and the line fits were compared using the extra sum-of-squares F test, revealing that the iHET I/O differed significant from both wild-type (p < 0.0001) and iKO lines (p < 0.001). Moreover, the I/O relationship of iKO slices also differed significantly from wild-type slices (p < 0.001) (Fig. 1A).

To determine whether alterations in the I/O relationship were due to changes in pre- or postsynaptic neurotransmission, we compared the FV and fEPSP between genotypes across a range of stimulus intensities (i.e. 1-15 mV). Comparison of nonlinear-fitted lines revealed that FV amplitude was increased in a gene dose-dependent fashion, with iHET having increased FV amplitude compared to wild-type slices (p < 0.0001) and iKO having increased FV amplitude compared to both iHET and wild-type slices (p < 0.0001, both) (Fig.
6B). In contrast, comparing the fEPSP line-fit between genotypes revealed increased postsynaptic neurotransmission in iKO compared to iHET and wild-type slices (p < 0.0001, both) (Fig. 6C).

Alterations in basal excitatory neurotransmission may affect the ability of synapses to undergo short-term and long-term synaptic plasticity, processes which are critical for memory formation. Despite significantly enhanced neurotransmission in both the iHET and iKO mice, we did not observe any differences in paired-pulse facilitation, a measure of presynaptic short-term plasticity (Fig. 6D). However, we did observe significant impairments in long-term potentiation (LTP), which was induced using a theta-burst stimulation protocol. Significant reductions in LTP induction were seen in iKO slices (129 ± 10.0%) compared to wild-type slices (175 ± 10.8%, p < 0.05) when the first ten minutes of recording were averaged (Fig. 6 E-F). The maintenance of LTP was impaired in both iHET (122 ± 8.71%, p < 0.05) and iKO (100 ± 7.66%) slices compared to wild-type slices (153 ± 8.39%) when the last 10 minutes of recording were averaged. Similar differences were observed when the entire recording session was averaged and compared (Fig. 6E-F). Taken together, these data reveal a critical role for Dab1-expressing GABAergic interneurons in the regulation of excitatory synaptic transmission and plasticity.
Discussion

The Reelin signaling pathway has a well-established role in regulating the positioning and dendritic morphogenesis of principle neurons during development (Stanfield and Cowan, 1979; D'Arcangelo et al., 1995; Olson et al., 2006; Franco et al., 2011; Jossin and Cooper, 2011) and more recently, has been implicated in modulating the function of excitatory synapses in the postnatal forebrain (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011). However, previous studies have largely neglected the possibility that Reelin signaling also affects the development and function of GABAergic interneurons. Here we present evidence that Dab1, an obligate effector of the Reelin signaling pathway, is also expressed by GABAergic interneurons and that it traffics to both pre- and post-synaptic inhibitory sites. Using a novel interneuron-specific Dab1 conditional knockout mouse (iKO), we demonstrate that expression of Dab1 by GABAergic interneurons is not required for the overall patterning of inhibitory synapses in the postnatal forebrain, but rather, serves to modulate the composition and physiological function of glutamatergic synapses. Our data provide compelling evidence that the inhibitory synapse is an important locus of Reelin signaling in the adult, a better understanding of which may prove crucial to treating disorders associated with Reelin signaling impairments, such as Schizophrenia and Alzheimer’s disease.

In a recent study, we reported that Dab1 was expressed by GABAergic interneurons in area CA1 of the postnatal hippocampus (Trotter et al., 2013).
Here we expanded on these findings by confirming expression of Dab1 by GABAergic interneurons throughout the hippocampal formation and cortex (Fig. 1 and supplemental Fig. 1). In the hippocampus, we noted that Dab1-expressing interneurons were readily identified at the interface of stratum radiatum and lacunosum moleculare. Although determining the specific interneuron subtypes that express Dab1 falls beyond the scope of the current study, we predict that most interneuron subtypes whose somas are positioned within stratum radiatum and stratum lacunosum moleculare will prove to be Dab1-positive. These may include, but are not limited to, the Schaffer collateral/commissural pathway-associated interneurons (Cope et al., 2002), cholecystokinin-positive basket cells (Pawelzik et al., 2002), and neurogliaform cells (Price et al., 2005). In particular, the significance of interneurons positioned within stratum lacunosum-moleculare is that they gate inputs from layer III of the entorhinal cortex that synapse onto distal apical dendrites of CA1 pyramidal cells (Capogna, 2011). Moreover, these interneurons likely gate other inputs originating from the Schaffer collaterals of hippocampal area CA3, nucleus reunions of the thalamus (Wouterlood et al., 1990), the amygdala (Pikkarainen et al., 1999), and the inferotemporal cortex (Iwai and Yukie, 1988). Owing to the diversity of interneuron cell types within this region of the hippocampus and others containing Dab1-expressing GABAergic interneurons, determining how Reelin signaling modulates interneuron function will ultimately depend upon identifying and targeting genetically-distinct, Dab1-expressing interneuron subtypes.
Our finding that Dab1 traffics to inhibitory pre- and postsynaptic compartments in mature hippocampal neurons (DIV21) challenges the notion that the locus of Reelin signaling in the adult forebrain is primarily at excitatory synapses. Indeed, we alluded to this possibility recently, because even in CA1 pyramidal cells, Dab1 only partially co-localized with the excitatory synapse scaffold protein PSD-95 (Trotter et al., 2013). Although the significance of Dab1 trafficking to presynaptic inhibitory terminals has not been tested here, we speculate that Dab1 in combination with Reelin receptors, may render a certain subset of inhibitory synapses sensitive to Reelin signaling, perhaps those undergoing synaptic plasticity. The lack of ubiquity of Dab1 at pre- and postsynaptic inhibitory sites, as well at postsynaptic excitatory sites, further suggests that Dab1 is not required for the function of all synapses within a given neuron, but may be trafficked only in specific contexts, similar to the insertion of AMPARs into silent, NMDAR-expressing synapses following potentiation (Liao et al., 1995; Gomperts et al., 1998). Moreover, co-localization of Dab1 with the postsynaptic inhibitory scaffold protein gephyrin (Fig. 2), further suggests that Dab1 may influence the function of inhibitory synapses in postsynaptic pyramidal cells, which our group and several others have already demonstrated are sensitive to Reelin signaling during synapse maturation and in the adult (Sinagra et al., 2005; Qiu et al., 2006a; Groc et al., 2007; Qiu and Weeber, 2007).

Interestingly, heterozygous reeler mice (HRM) actually have selective reductions in the amplitude and frequency of spontaneous GABA\(_{A}\)R-mediated inhibitory postsynaptic currents (Qiu et al., 2006b), suggesting that pyramidal cells of HRM
have reduced inhibitory innervation. This observation would be consistent with a role for Reelin signaling in regulating the formation and/or function of inhibitory synapses in a manner that may reflect pre- and/or postsynaptic signaling at inhibitory sites.

The iKO mice generated in this study utilized a *gad2*-cre driver line, which exhibits widespread cre recombinase activity as early as 4 days in interneurons dispersed throughout the forebrain and in Purkinje cells (PCs) of the cerebellum (Allen Atlas reference), but likely occurs much earlier during embryonic development when the *gad2* promoter is first activated (Lopez-Bendito et al., 2004). Therefore, it is not possible to completely disentangle phenotypes observed in iHET and iKO mice with regards to whether they are developmental in origin or represent ongoing functions of Dab1 in GABAergic interneurons. The lack of changes in lamination of cortical structures in iKO mice is consistent with the notion that Reelin signaling acts directly on radially-migrating principle neurons in the developing cortex (Hammond et al., 2001; Franco et al., 2011). Moreover, the lack of obvious changes in the positioning of GAD67-positive interneurons and synaptic patterning in the hippocampus and cortex is consistent with a recent study which found that the migration and final placement of GABAergic interneurons in the cortex occurs independent of Reelin signaling (Pla et al., 2006). We are presently verifying the placement of specific biochemically-defined subsets of interneurons, as GAD67 does not clearly label all interneurons in the forebrain, particularly the cortex, and may not sensitively detect abnormal placement or development of individual interneuron subtypes.
Despite normal forebrain development, iKO mice presented with an ataxic gait and resting tremors, phenotypic hallmarks of the reeler and scrambler mutants (Mariani et al., 1977; Sweet et al., 1996). Moreover, we observed that the cerebellum of iKO mice was significantly smaller than the wild-type cerebellum and lacked foliation. Purkinje cells were primarily amassed in the cerebellar cortex and few migrated outward to extend dendrites into the molecular layer (Fig. 3). In addition to GABAergic interneurons, Purkinje cells also express gad2, suggesting that perturbed cerebellar development in iKO mice may directly result from impaired Reelin signaling in Purkinje cells. However, in mice, PC migration starts at embryonic day 12 and is completed by 2-3 days prior to birth (Sotelo and Dusart, 2009). Consider then, that the expression of gad2 by PCs of the rat cerebellum does not occur until at least postnatal day 7 (Greif et al., 1991). Although the time course of gad2 expression in mouse PCs has not been previously studied, these findings suggest that gad2-driven expression of Cre may not occur in Purkinje cells until well after their migration, further suggesting that the observed cerebellar phenotype may result from deletion of Dab1 in other gad2-expressing cells that require Reelin signaling to properly orchestrate development of the cerebellar cortex. Although the specific origin of the cerebellar phenotype of iKO mice is not the focus of the present study, using a reporter mouse to establish the time-course of Cre recombination in these mice may prove useful.

Since the density and patterning of inhibitory synapses appeared grossly normal throughout the postnatal forebrain of iKO mice (Fig. 4), we next
determined if loss of Dab1 specifically in interneurons affected the expression level of excitatory and inhibitory pre- and post-synaptic proteins. We observed a significant up-regulation of both NR1 and NR2B in whole-cell hippocampal lysates from adult iHET and iKO mice (Fig. 5), which could reflect a shift in the developmental regulation of the NMDAR subunits or a change in the number of excitatory synapses. Interestingly, we did not observe changes in NR2A levels in either iHET or iKO mice, suggesting that interneuronal expression of Dab1 is required for regulating only a particular subtype of NMDAR complexes.

Since loss of Dab1 in interneurons led to specific changes in NR1/NR2B expression levels, it is important to consider that the NR2A and NR2B subunits confer the NMDAR complex with unique biophysical properties (Chen et al., 1999; Gielen et al., 2009) and differences in intracellular and synaptic trafficking (Barria and Malinow, 2002; Lavezzari et al., 2004). In general, NR2B expression is higher in the postnatal and juvenile brains whereas NR2A predominates throughout adulthood and advanced ages, a transition which is believed to underpin age-dependent constraint on memory function (Hestrin, 1992; Monyer et al., 1994; Sheng et al., 1994; Cui et al., 2013). Since the changes we observed in NR1/NR2B levels were in the adult hippocampus, our data may reflect a role of Reelin signaling onto interneurons in mediating the development of glutamatergic synapses, specifically by promoting the developmental down-regulation of NR2B-containing NMDARs. Consistent with this idea, previous studies have demonstrated that Reelin promotes the maturation of glutamatergic synapses by facilitating the developmental shift of NMDAR subunit composition (i.e. NR2B →
NR2A) (Qiu and Weeber, 2007; Campo et al., 2009). Moreover, reduced Reelin signaling during development leads to deficits in synaptic plasticity in the prefrontal cortex and altered memory function, which can be rescued by pharmacologically-blocking the NR2B subunit (Iafrati et al., 2013).

Since we observed alterations in the level of excitatory synaptic proteins, we further studied the impact of Dab1 loss in GABAergic interneurons on the function of the SC/CA1 synapse. Much to our surprise, partial loss of Dab1 in only a small subset of hippocampal interneurons was sufficient to increase presynaptic neurotransmission, possibly reflecting increased number of presynaptic afferents or intrinsic excitability. In iHET mice, increased presynaptic FV amplitude resulted in less excitatory output relative to wild-type mice, a finding similar to observations made in the HRM mouse (Qiu et al., 2006b). Interestingly, iKO mice had increased pre- and postsynaptic neurotransmission, the latter of which may represent some form of compensation. Despite increased presynaptic neurotransmission, short-term presynaptic plasticity, measured as paired-pulse facilitation, was not altered in either iHET or iKO mice.

Previous studies have found that reduced levels of Reelin signaling impair long-term potentiation (LTP) induced by theta-burst stimulation (Weeber et al., 2002; Beffert et al., 2006; Qiu et al., 2006b; Trotter et al., 2013), while increased levels enhance it (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011). To our surprise, we observed dramatic impairments in the induction of LTP in iKO mice and in LTP maintenance in both iHET and iKO mice (Fig. 6). In fact, the LTP impairment observed in iHET mice is magnitudely similar to impairments
found in HRM (Qiu et al., 2006b) and ecKO mice (Trotter et al., 2013). On the other hand, the complete loss of LTP in iKO mice after an hour is similar to LTP impairments observed in ApoER2 mutant mice (Weeber et al., 2002; Beffert et al., 2006), suggesting that ApoER2 may also be expressed by GABAergic interneurons.

Taken together, these findings emphasize that expression of Dab1 by a subpopulation of GABAergic interneurons is critical for synaptic plasticity in the adult hippocampus. These data open up several avenues of future study, including 1) determining if Dab1 renders GABAergic interneurons responsive to Reelin signaling, and 2) establishing whether observed biochemical and physiological alterations of glutamatergic synapses reflect a developmental or adult function of Dab1-expressing GABAergic interneurons. Our findings emphasize the importance of dissociating cell- and synapse-specific functions of the Reelin signaling pathway, as it may prove critical for understand and treating a multitude neuropsychiatric and neurodegenerative disorders.

References


Figure 4.1. Dab1 is Expressed by GABAergic Interneurons in the Postnatal Hippocampus. A) Co-labeling of Dab1 with the GABAergic marker GAD67 revealed expression of Dab1 by a subset of GAD67-positive interneurons in hippocampal area CA1 of 2-3 m.o. WT mice (white arrows, magnified images).
Loss of Dab1 expression specifically in pyramidal cells in excitatory conditional knockout mice (ecKO) further revealed a large subpopulation of Dab1 and GAD67 co-labeled interneurons, most prominently found along the border of stratum radiatum (s.r.) and stratum lacunosum moleculare (s.l.m.). B) Similar to CA1, a large population of Dab1-and Gad67 co-labeled interneurons were observed throughout s.r. and s.l.m. of area CA3. As expected, Dab1 expression by pyramidal cells of CA3 was preserved ecKO mice. Scale bars, 100 µm. Abbreviations: s.o., stratum oriens; s.p., stratum pyramidale; s.l., stratum lucidum.
Figure 4.2. Dab1 is Expressed at Excitatory and Inhibitory Synapses in Hippocampal Neurons (DIV21). A) Dab1 and the inhibitory pre-synaptic protein GAD67 were co-expressed by a majority of interneurons in culture. Upon further magnification (white box, right panels) Dab1 can be detected in some putative presynaptic puncta (white circle). However, not all GAD67-positive presynaptic terminals are Dab1-positive (yellow circle). B) Dab1 and the postsynaptic inhibitory synapse scaffold protein gephyrin were co-expressed by all pyramidal cells in culture. Upon closer magnification (white box, right panels), Dab1 and Gephyrin are found to co-localize at some inhibitory postsynaptic sites (white circle), but not all sites (yellow circle). C) Dab1 and the excitatory synapse scaffold protein PSD-95 were co-expressed by all pyramidal neurons in culture. Magnification of a dendritic segment (white box), reveals localization of Dab1 at a subset of PSD-95-labeled dendritic spines positioned perpendicular to the dendritic shaft (white circle). However, Dab1 was not expressed by all PSD-95-positive clusters, indicating that Dab1 is not ubiquitously-expressed at excitatory synapses. Scale bars: 40 µm (left panels) and 5 µm (right panels).
Figure 4.3. Selective Loss of Dab1 Expression in GABAergic Interneurons does not Affect Forebrain Development. A-B) Dab1 and GAD67 were co-labeled in 2-3 m.o. WT and inhibitory interneuron-specific conditional knockout mice (iKO). DAPI was included as a nuclear co-stain. In hippocampal area CA1 of WT mice, Dab1 was detected in most GAD67-positive cells along the interface of stratum radiatum (s.r.) and stratum lacunosum moleculare (s.l.m.)(white arrows). In contrast, Dab1 was not detected in GAD67-positive cells in iKO mice (yellow arrows). However, Dab1 expression in the soma and dendrites of pyramidal cells appeared normal. Scale bars 50 um (left panels) and 25 um (magnified panels, right). C) Western blot analysis of Dab1 protein levels in adult WT (n = 6), inhibitory knockout heterozygous (iHET; n = 7), and iKO mice (n = 4) revealed normal Dab1 expression levels in the hippocampus (HIPP) and cortex.
(Cx). A dramatic gene dose-dependent down-regulation of Dab1 was detected in the cerebellum of iHET and iKO mice. **, p < 0.001; ***, p < 0.0001 (comparison to WT); #, p < 0.0001 (comparison to iHET). D) Nissl-stained sections from 3-4 week old WT and iKO mice show normal lamination of cortical structures but severely perturbed cerebellar development in iKO mice, marked by reduced cerebellar size and an absence of foliation. Scale bars, 200 μm.
Figure 4.4. Patterning of Inhibitory Synaptic Terminals is Normal in the Forebrain of iKO Mice. GAD67 immunostaining in sections from 3-4 week old WT and iKO mice revealed no major differences in GABAergic interneuron placement and synaptic patterning throughout the hippocampus (Hipp) or cortex (Cx). In the WT cerebellum, Purkinje cells (PCs) are found to align in a signal layer (white arrow) and extend dendrites outward into the molecular layer. In contrast, PCs in the iKO cerebellum largely accumulate in the cerebellar cortex. Only a small population of PCs migrates outward to extend dendrites into the molecular layer (white arrow). Scale bars, 200 µm.
Figure 4.5. Altered Expression of Synaptic Proteins in Adult iHET and iKO Mice. Hippocampal homogenates from 2-3 month old WT (n = 12), iHET (n = 12), and iKO (n = 3) mice were used to measure the levels of pre- and postsynaptic proteins. A) No changes were detected in the levels of Dab1 or full-length Reelin (450 kDa) and its major proteolytic fragments (i.e. 370 kDa and 180 kDa). B) No changes were detected in the levels of the presynaptic proteins synapsin, synaptophysin, GAD65, and GAD67. However, a selective up-
regulation of VGLUT1 and VGAT was detected in iKO mice. C) No significant changes were detected in the levels of the postsynaptic NMDAR subunit NR2A or in the inhibitory scaffold protein gephyrin. The levels of the NMDAR subunits NR1 and NR2B were increased in iHET and iKO lysates compared to wild-type controls. PSD-95 levels were significantly elevated in only iKO mice. *, p < 0.05 compared to WT; #, p < 0.05 compared to iHET.
Figure 4.6. Altered Excitatory Neurotransmission and Synaptic Plasticity in Adult iHET and iKO Mice. A) Input/output was evaluated by comparing fEPSP slope to fiber volley (FV) volley amplitude, both of which were determined across a range of stimulus intensities (i.e. 1-15 mV). Comparison of non-linear fitted lines revealed that the I/O relationship was different between WT (n = 45 slices, 10 mice), iHET (52 slices, 11 mice), and iKO mice (29 slices, 5 mice). To better understand what contributed to altered I/O relationship, the fEPSP and FV were compared separately. B) FV amplitude was compared to stimulus intensities between genotypes. Comparison of non-linear fitted lines revealed significantly
different curves between WT, iHET, and iKO slices, reflecting a dose-dependent increase in presynaptic activation associated with reduced Dab1 expression by interneurons. C) Comparison of the fEPSP slope to stimulus intensity, revealed a significant increase in postsynaptic neurotransmission in iKO compared to iHET and WT mice. D) No differences were seen in short-term presynaptic plasticity measured by paired-pulse facilitation (PPF) at 20-300 ms interpulse intervals in WT (n = 16 slices, 4 mice), iHET (n = 24 slices, 5 mice), and iKO (n = 16 slices, 3 mice). E-F) LTP was induced in WT (n = 12 slices, 4 mice), iHET (n = 15 slices, 5 mice), and iKO (n = 13 slices, 5 mice) slices using a theta-burst stimulation protocol. Significant reductions were seen in the induction of LTP (first 10 minutes) in iKO compared to WT slices. Significant reductions in the maintenance of LTP (last 10 minutes) and overall LTP (total 60 minutes) were seen in both iHET and iKO slices compared to WT controls. *, p < 0.05.
**Figure 4.7. Dab1 is Expressed by GABAergic Interneurons of the Adult Cortex.** Intense immunoreactivity of GAD67 at inhibitory synapses was seen in the WT cortex, particular in layers containing projection neurons (i.e. II/III and V). Although GAD67-positive cell bodies were difficult to identify, a subpopulation of interneurons were more readily detected in cortical layer VI. Upon closer magnification (yellow box, right panels), perinuclear co-labeling is seen between GAD67 and Dab1 in putative GABAergic interneurons (white arrows). With loss of Dab1 in most excitatory neurons in the ecKO mice, some cells in deep cortical layers appeared intensely Dab1-positive. Upon closer magnification (yellow box), GAD67 and Dab1 co-label a readily visualized subpopulation of GABAergic interneurons (white arrows). However, not all Dab1-positive cells were GAD67-positive. Scale bars, 200 µm (left panels) and 25 µm (right panels).
Chapter 5

Concluding Remarks

Despite a wealth of evidence implicating altered Reelin signaling in the pathoetiology of various disease states (e.g. Alzheimer’s disease and schizophrenia), a critical review of the literature reveals that still, very little is known regarding the regulation and function of Reelin signaling, particularly following the completion of neuronal migration. Importantly, the specific mechanisms responsible for initiating and controlling the magnitude, duration, and termination of the Reelin signal, are entirely unknown. Although the projection neuron and excitatory synapse represent the two primary loci of Reelin signaling studied in the developing and adult brain, respectively, their roles as physical sites of Reelin signaling should not overshadow other potential loci, including GABAergic interneurons and their inhibitory synapses.

In this dissertation, we have addressed some important knowledge gaps in the field by demonstrating in Chapter 2, that Reelin signaling may be initiated following synaptic potentiation through tPA-mediated proteolysis. Then in Chapter 3, we provide the first conclusive evidence that Reelin signaling in adult excitatory neurons is required for hippocampal synaptic plasticity and associative learning. Finally, in Chapter 4, we focused on an entirely new locus of Reelin
signaling, the inhibitory synapse. We demonstrate that expression of Dab1 by GABAergic interneurons is critical for proper excitatory neurotransmission and synaptic plasticity, but not for the placement of interneurons and patterning of inhibitory synapses. Below, we will discuss the importance of these findings and address future studies that may shed further light on the Reelin signaling pathway and its critical role in the developing and adult brain.

Proteolysis: An Important Initiator of the Reelin Signal

In Chapter 2, we provided evidence that synaptic activity promotes tPA-dependent proteolysis of Reelin. Specifically, we found that Reelin is processed by tPA at a single site between EGF-like repeats 6 and 7. Although tPA was not involved in cleaving Reelin under basal conditions, induction of NMDAR-independent and -dependent forms of LTP led to distinct tPA-dependent changes in Reelin expression and processing. In this section, we will discuss the implications of our findings and address future studies that may yield helpful insight of this novel mode of Reelin signaling regulation. Moreover, we will discuss some of the limitations encountered with the ex vivo slice culture model, which will be useful to consider when studying Reelin processing in general.

What is the function of Reelin proteolysis following synaptic activity? In our study, we were unable to determine if Reelin proteolysis contributed to changes in the level of Reelin signaling, owing to the limited amount of protein available from CA1 mini-slices. However, the importance of Reelin proteolysis can be
inferred from structure-function studies of individual Reelin fragments.
Specifically, full-length Reelin and fragments containing EGF-like R5-6 bind the conserved ligand-binding modules of ApoER2 and VLDLR (Yasui et al., 2010), and are sufficient to induce canonical Reelin signaling (Jossin et al., 2004). On the other hand, recent studies suggest that full-length Reelin and fragments containing N-R2 act in a non-canonical fashion through binding EphBs and initiating forward signaling (Bouche et al., 2013). Additional studies suggest that N-R2 containing forms of Reelin may also bind β1-integrins (Dulabon et al., 2000; Dong et al., 2003; Schmid et al., 2005). Thus, individual or sequential cleavage events may specify the extent to which Reelin fragments activate canonical and non-conical Reelin signaling. Based on our observation that cLTP selectively up-regulated the Reelin N-R6 fragment, we predict that this fragment itself may contribute to an increase in canonical Reelin signaling, which is known to facilitate the expression of LTP (Weeber et al., 2002; Beffert et al., 2005; Beffert et al., 2006). Since we did not observe a significant up-regulation of the N-R2 fragment, our findings also suggest that Reelin is not always sequentially processed at both major cleavage sites. To test the functional significance of Reelin processing on canonical signaling, future studies will require a novel method for detecting Dab1 tyrosine phosphorylation when only limited quantities of protein are available (e.g. Phos-tag SDS-PAGE). Other downstream signal components of the canonical (e.g. SFK activation) and non-canonical pathway (e.g. EphB phosphorylation) should also be tested.
Our finding that Reelin is directly cleaved by tPA suggests that it may act downstream of this critical extracellular protease, whose activity-dependent secretion promotes synapse formation, hippocampal LTP, and learning and memory (Pang et al., 2004; Nagappan et al., 2009). Although we have focused exclusively on tPA, it is likely that other proteases contribute to basal and activity-dependent processing of Reelin. In support of this notion, recent studies have reported that disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4 and -5) are both capable of cleaving Reelin at N- and C-terminal cleavage sites in vitro (Hisanaga et al., 2012; Krstic et al., 2012). Moreover, although MMP-9 does not cleave Reelin directly, it does activate Reelin-cleaving enzymes (Hisanaga et al., 2012; Krstic et al., 2012). Even though little is known about the function of ADAMTS-4 and -5 in the adult brain, MMP-9 is an activity-dependent protease with an essential role in regulating synaptic plasticity (Wang et al., 2008). Future studies should address the role of these enzymes in activity-dependent processing of Reelin. It is possible that different forms of synaptic plasticity (e.g. LTP or long-term depression) modulate Reelin processing via distinct proteolytic pathways.

In addition to its cleavage by tPA, we also found that plasmin was capable of cleaving Reelin at several sites and that the N-R2 region of Reelin was comparatively-resistant to cleavage. This finding suggests that tPA does not only influence Reelin processing directly, but may also indirectly regulate Reelin processing via conversion of plasminogen into plasmin. This finding is in contrast to the brain-derived neurotropic factor (BDNF), whose conversion from its pro- to...
mature form, requires a single cleavage by plasmin (Pang et al., 2004). Although
the current study did not focus on plasmin-mediated Reelin processing, the
additional cleavage sites that are sensitive to plasmin may represent a more
complex mechanism for controlling the duration and magnitude of Reelin
signaling. The involvement of plasmin may explain why forskolin treatment led to
tPA-dependent down-regulation of Reelin fragment levels, which contrasts the
selective up-regulation of Reelin N-R6 seen in TEA-treated slices.

Although we were able to detect activity-dependent processing of Reelin
in ex vivo hippocampal slices, the model system employed may not be ideal. The
assumption that Reelin associates with the ECM may mistakenly lead many to
believe that this is the preferred state of extracellular Reelin under basal
conditions. In fact, the fate of Reelin following secretion has not been rigorously
studied and we may ultimately find that Reelin more readily floats within the
interstitial space between neurons. In our hands, while evaluating the synaptic
expression of Reelin and Dab1 with subcellular fractionation (Chapter 3), we
observed that Reelin is highly-soluble and may not, in most structures, bind very
stably to the extracellular space (data not shown). This could explain why Reelin
immunostaining revealed limited localization to stratum radiatum of hippocampal
area CA1, as well as most synaptic subfields of the forebrain. The presence of
punctate Reelin on pyramidal cell bodies of the cortex and to a lesser extent,
stratum pyramidale of the hippocampus, may reflect surface-bound or
internalized Reelin of cells with active Reelin signaling. A similar punctate stain
was not detected on synaptic structures under basal conditions. Because of the

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solubility of Reelin, the immunostaining may vastly underrepresent the presence of Reelin in hippocampal synaptic subfields. An alternative explanation is that synaptic subfields, such as stratum radiatum, may not be the major site of Reelin signaling. Testing both of these possibilities will help determine whether ex vivo slices lend themselves to meaningful experimental manipulation of Reelin processing, or if in vivo models are superior for the study of activity-dependent Reelin processing.

The limitation noted in the previous paragraph may also explain why extensive experimental variation was observed when studying Reelin processing in acute hippocampal slices. The solubility of Reelin may lead to its leaching from the slice into the culture media, and observed findings could simply reflect changes in the limited Reelin that remains bound to the slice or that is stored in intracellular compartments. Indeed, CA1 itself may be too heterogeneous for the study of Reelin processing as most Reelin is contained within interneuron intracellular compartments and along the hippocampal fissure. A more focused approach would utilize an anatomical structure where significant Reelin levels are found, such as in the outer molecular layer of dentate gyrus or stratum lacunsum moleculare of CA1, where Reelin is secreted by perforant fibers. Future studies should first determine whether the abundant Reelin found along the hippocampal fissure is contained within axonal fibers or is extracellular.

Taken together, we predict that synaptic activity initiates Reelin signaling via release of activity-dependent proteases, such as tPA. Additionally, differential proteolysis of Reelin may fine-tune down-stream signaling by regulating the
relative balance of canonical and non-canonical signaling. The fact that Reelin fragments are also found under basal conditions suggests that tonic Reelin signaling may also be important. Ultimately, the identification of proteases responsible for both activity-dependent and basal Reelin processing will help integrate our understanding of downstream Reelin signaling within the larger physiological framework of synaptic processes, such as plasticity. Future studies should establish and validate model systems for elucidating mechanisms of Reelin processing and signaling in the intact adult brain, as this may yield the most interpretable results.

Synaptic Function of Dab1

It is well-established that Reelin signaling influences the formation and maturation of excitatory synapses, although the extent to which Reelin signaling contributes to the function of adult excitatory synapses is not well understood. Moreover, the possibility that Reelin signals onto other structures, such as inhibitory synapses, has not been previously addressed. Chapters 3 and 4 of this dissertation addressed these knowledge gaps through the characterization of two novel conditional knockout mice, in which Dab1 was selectively deleted in either excitatory neurons (eKO) or inhibitory interneurons (iKO). As mentioned in Chapter 1, Reelin signaling has numerous functions across the lifespan of an organism that can be bracketed into distinct ontological periods, such as neuronal positioning or synapse formation. In this section, we will summarize
key findings made using the excitatory and inhibitory cKOs, particularly as they
relate to these ontological periods. Also, we will discuss a range of experimental
questions that can now be asked by implementing conditional knockout
strategies for the study of Reelin signaling.

In agreement with a shift in the function of Reelin signaling in the postnatal
forebrain, we observed a dramatic down-regulation of Reelin and ApoER2, and to a
lesser extent, Dab1, from postnatal day 7 through 21. This window of postnatal
development spans a critical period of synapse formation, maturation, and
elimination. Since our lab is primarily interested in the function of Reelin signaling at
adult synapses, we studied the localization of Reelin and Dab1 in the mature
hippocampus and cortex. Surprisingly, we found that Dab1 was expressed by both
excitatory principle neurons and a subpopulation of GABAergic interneurons. In the
eKO mice, the expression of Dab1 by interneurons was even more apparent. Upon
closer examination, we also found that Dab1 and Reelin were co-expressed by a
subset of cells, likely corresponding to residual Cajal-Retzius cells or GABAergic
interneurons.

When evaluating the synaptic expression of Dab1 in CA1 pyramidal cells, we
found that Dab1 was expressed at both pre- and postsynaptic sites. We confirmed in
the brain and in cultured primary neurons, that Dab1 was expressed at only a subset
of excitatory postsynaptic sites. Furthermore, Dab1 was also present at some, but
not all, pre- and post-synaptic inhibitory sites. Although we did not quantify the
specific proportion of synaptic structures positive for Dab1, our finding that Dab1
was not expressed at all inhibitory and excitatory synapses, warrants further
investigation. It is conceivable that Dab1 traffics exclusively to immature, plastic synapses, rendering them sensitive to Reelin signaling. Based on these findings, future studies should address subcellular trafficking of endogenous Dab1 at different morphological and developmental classes of excitatory and inhibitory synaptic structures.

The remainder of this chapter will be broken into several sub-sections, discussing the pre- and post-synaptic function of Dab1 at inhibitory and excitatory synapses.

**Excitatory Presynaptic Locus**

Both subcellular fractionation and immunostaining experiments demonstrated that Dab1 traffics to the presynaptic site. In support of a presynaptic function of Dab1, previous studies have found that Reelin regulates neurotransmitter release by controlling the levels of the SNARE protein, SNAP-25 (Hellwig et al., 2011). Moreover, Reelin over-expressing mice exhibit dramatic increases in the density of presynaptic terminals (Pujadas et al., 2010). Finally, findings of impaired paired-pulse facilitation, a measure of presynaptic short-term plasticity, in *reeler*, HRM and ApoER2 EIG mutants (Lacor et al., 2000; Beffert et al., 2006; Qiu et al., 2006) further support this idea. Interestingly, in our ecKO mice, we found that the postnatal loss of Dab1 in area CA1 led to PPF deficits. As these mice exhibit minimal loss of Dab1 in CA3, altered postsynaptic signaling may drive observed presynaptic impairments. For example, disruption of trans-synaptic signaling molecules such as the
EphB/Ephrin complex, which have been recently found to interact with Reelin during development (Senturk et al., 2011; Bouche et al., 2013) and have established roles in regulating synaptic plasticity (Rodenas-Ruano et al., 2006), may help explain how postsynaptic loss of Dab1 could drive impaired pre-synaptic plasticity. Alternatively, PPF deficits observed in eKO mice could originate from indirect alterations in the properties of CA3 pyramidal cells due to reduced Reelin signaling in the mossy fiber and perforant pathways. Future studies that utilize viral-delivery of Cre, particularly in CA3, may more definitely address the specific function of Reelin signaling at the excitatory presynaptic site.

**Excitatory Postsynaptic Site**

Previous experiments have found that Reelin signaling is critical for the formation, maturation, and adult function of excitatory synapses. An important limitation of most of these studies is that they utilized mouse models in which Reelin signaling was either partially (or completely) impaired during development, or employed supra-physiological gain-of-function approaches. To study the extent to which Reelin signaling affects mature excitatory synapses, we generated the eKO mice, which exhibited loss of Dab1 in hippocampal area CA1 starting around postnatal day 19. We observed no significant changes in the placement and dendritic morphology of CA1 pyramidal cells, as well as in dendritic spine density or the levels of major components of glutamatergic synapses. This finding suggests that the established function of Reelin in regulating dendritogenesis and the
formation of dendritic spines occurs during earlier, ontologically-distinct windows of Reelin signaling.

Interestingly, we did find that the size of dendritic spines was reduced on the apical dendrites of CA1 pyramidal cells, implying that Reelin may be required for the formation of large, mature dendritic spines in the adult hippocampus. In support of this idea, Reelin-overexpressing mice have profound increases in dendritic spine size (Pujadas et al., 2010). Since we evaluated spine density and morphology only at 2 months of age, it is possible that Dab1 loss during later stages of postnatal synaptic development (P20-P30) may be responsible for this effect. Alternatively, Dab1 may play a critical day-to-day function in activity-dependent stabilization and enlargement of dendritic spines. Considering that we saw restricted Dab1 expression to a subset of excitatory synaptic sites, comparing morphological subtypes of spines in which Dab1 traffics to those that are altered in eKO mice, may be particularly revealing. Also, inducible expression or viral delivery of Cre at earlier and later postnatal days will be useful for determining the specific windows within which Dab1 mediates spine formation and morphogenesis.

Potentially explaining observed alterations in the morphology of dendritic spines, we also found that loss of Dab1 led to a gradual decrease in the basal activation of Akt, a kinase critical for downstream Reelin signaling (Beffert et al., 2002; Jossin and Goffinet, 2007). To our surprise, basal activation of the mitogen-activated kinases, ERK1 and ERK2, was also decreased. These findings suggest that either tonic Reelin signaling contributes substantially to the regulation of Akt and MAPK pathways, or that Dab1, as an intracellular adaptor protein, engages in
Reelin-independent signaling processes that contribute to kinase regulation. We also found that the activation of ERK1/2 by synaptic potentiation was blunted in ecKO mice at 2 months of age, prior to basal decreases in ERK1/2 activation. This finding is interesting in light of our data demonstrating that synaptic potentiation promotes the proteolysis of Reelin, which may initiate downstream Reelin signaling. However, a role of Reelin in MAPK regulation has been studied previously and yield conflicting results (Ballif et al., 2003; Simo et al., 2007). Future studies should address whether other signaling components previously identified as important for Reelin signaling during development, are also impaired in ecKO mice, both tonically or in response to Reelin treatment.

Our finding of impaired induction of LTP in eKO mice is consistent with an important role for Dab1 in regulating hippocampal synaptic plasticity. Moreover, the inability of Reelin to enhance LTP is in complete agreement with published studies (Weeber et al., 2002). At the age that we conducted hippocampal field recordings, Dab1 is almost entirely absent from CA1 pyramidal cells, which exhibit marked reductions in spine volume and activity-dependent ERK1/2 activation. Considering these basal differences, as well as reduced PPF, the impairments of LTP may simply result from underlying biochemical and structural deficits that limit the expression of LTP. Although these data definitively establish that Dab1 is a critical regulator of synaptic plasticity, it is still difficult to establish that this is due in part to an active and ongoing role of Dab1 and Reelin signaling in the molecular processes that give rise to LTP. An alternative explanation is that tonic Reelin signaling, driven by day-to-day experiences and hormonal influences, accounts for observed
differences in LTP. Future studies should establish whether Reelin signaling is actually activated by synaptic plasticity and its role, if any, in the fundamental biochemical, structural, and physiological changes that enable the induction and maintenance of long-term synaptic plasticity. This information is critical, as it will likely define the temporal window within which Reelin signaling impairments associated with various neurological disorders, contribute to disruptions in synaptic function and cognition.

Another important point to consider in the future is that LTP at the Schaffer collateral / CA1 synapse has been the primary model for studying Reelin signaling thus far. Considering that Reelin levels seem to be low in stratum radiatum compared to other hippocampal regions (s.p. and s.l.m. of CA1), future studies may gain more information by determining how generalizable observations made at the SC/CA1 synapse are. Moreover, field recordings provide very gross measures of overall connectivity and changes in plasticity in a given circuit. The use of whole-cell patch recordings should be a high priority, as they will provide more definitive measures of how loss of Dab1 impairs distinct cell-autonomous and non-autonomous measures of spontaneous and evoked synaptic activity and plasticity. Moreover, rescue experiments utilizing viral delivery of wild-type or mutant Dab1 constructs, will be helpful for verifying mechanisms of Reelin signaling at synapses.

In agreement with published studies, we also verified that Reelin signaling in the adult brain is essential for associative learning and maintenance of long-term spatial memories. Importantly, our findings are not confounded by many of the earlier developmental problems that might have contributed to altered learning and
memory in other Reelin signaling mutants (Weeber et al., 2002; Beffert et al., 2005; Beffert et al., 2006; Qiu et al., 2006). However, it is difficult to conclude based on the current findings whether or not Reelin signaling is actively involved in the formation or maintenance of memories, per se. Future studies should establish whether learning itself can drive changes in Reelin signaling. In eventuality, the development of a spatiotemporal model of Reelin signaling in the adult brain will be required to validate the relevance of mechanisms and functions of Reelin signaling garnered in other experimental models (e.g. cortical development or dissociated neurons).

**Inhibitory Pre- and Postsynaptic Locus**

Our finding that Dab1 is expressed by GABAergic interneurons and traffics to inhibitory synapses will likely have profound implications on how we view Reelin signaling in the developing and adult brains. GABAergic interneurons are highly heterogeneous, comprising at least 22 cell types that differ in their biochemical, physiological, and anatomical properties. They are responsible for many aspects of network function, including fine-tuning of excitatory transmission through feed-back and feed-forward inhibition and preventing over-excitation (Le Magueresse and Monyer, 2013).

Both the placement and morphological development of interneurons is severely perturbed in *reeler* and *scrambler* mice (Hevner et al., 2004; Pla et al., 2006; Yabut et al., 2007). A recent study, however, demonstrated that interneuron misplacement in these mice is a function of abnormal positioning of projection
neurons, and concluded that Reelin signaling does not influence the positioning of either early- or late-born interneurons (Pla et al., 2006). Although this study did not address other aspects of interneuron development, it did report that all GABAergic interneurons studied were Dab1-positive. Consistent with this finding, we also found that a substantial portion of GABAergic interneurons are Dab1-positive in the mature brain, and that the absence of Dab1 in developing GABAergic interneurons did not grossly affect their placement and patterning of inhibitory synapses. However, our measures of interneuron placement and synaptic patterning were crude, and loss of Dab1 in interneurons could give rise to abnormal development of specific subtypes of interneurons. To address this limitation, we are presently studying the placement of interneurons using biochemical markers (e.g. parvalbumin, calbindin, etc.) that recognize distinct interneuron subtypes. However, most biochemical markers for interneurons do not report morphology adequately. To overcome this, future studies should also employ Cre-sensitive fluorescent reporters that allow detection of fine neural processes. In parallel, the identification of the specific interneuron subtypes expressing Dab1, will allow more specific and detailed interrogation of the role of Reelin-dependent, or independent, signaling in GABAergic interneurons.

It is widely presumed that Reelin signaling influences the formation and maturation of glutamatergic synapses by directly signaling upon them, although this has not been directly measured. Our data challenges this idea, as even partial loss of Dab1 in GABAergic interneurons, led to specific up-regulations of the NR1 and NR2B NMDAR subunits, but not NR2A. This change in the expression of NR2B, in particular, implies that the developmental switch in NMDAR subunit composition
from NR2B $\rightarrow$ NR2A may be perturbed by loss of Reelin signaling in interneurons. Indeed, several studies have found that loss of Reelin signaling in cultured neurons and in the brain leads to impaired maturation of NMDARs (Sinagra et al., 2005; Groc et al., 2007; Qiu and Weeber, 2007; Iafrati et al., 2013). Interestingly, a similar maturation of NMDAR subunit composition has also been reported to occur in a subset of interneurons (Matta et al., 2013). However, because interneurons comprise a small proportion of cells in the hippocampus (< 10%), it is doubtful that observed biochemical changes originate exclusively from this cell type. Future studies should employ whole-cell recordings to determine how loss of Dab1 in interneurons affects the maturation of NMDARs in both pyramidal cells and interneurons.

The sensitivity of excitatory neurotransmission to reduced Dab1 expression in GABAergic interneurons is highlighted by our findings of enhanced evoked presynaptic activity in iHET and iKO mice, as well as increased postsynaptic EPSP in the iKO mice. The increase in presynaptic neurotransmission could be caused by disinhibition of CA3 pyramidal cells, or an increase in the number or excitability of the Schaffer collaterals. Even more surprising was that partial loss of Dab1 in interneurons led to impairments in LTP. The extent to which these findings reflect developmental abnormalities or ongoing functions of interneuronal Dab1 may be addressed by generating interneuron-specific inducible conditional knockouts. Another important question that should be addressed is whether observed biochemical and physiological changes reflect Reelin-dependent or –independent functions of Dab1.
Conclusion

A requisite to understanding how Reelin signaling impairments contribute to diseases such as Schizophrenia, is acquiring a detailed account of the specific loci of Reelin signaling in the brain. The partitioning of developmental and adult processes into distinct ontological phases will enable a focused dissection of mechanisms and functions of Reelin signaling that may contribute to neurological disorders with exclusively developmental, adult, or mixed etiologies. Based on data presented in this dissertation, we predict that Reelin signaling utilizes distinct mechanisms to influence several important sites in the brain, including excitatory and inhibitory pre- and post-synaptic structures.

References


Appendix 1

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