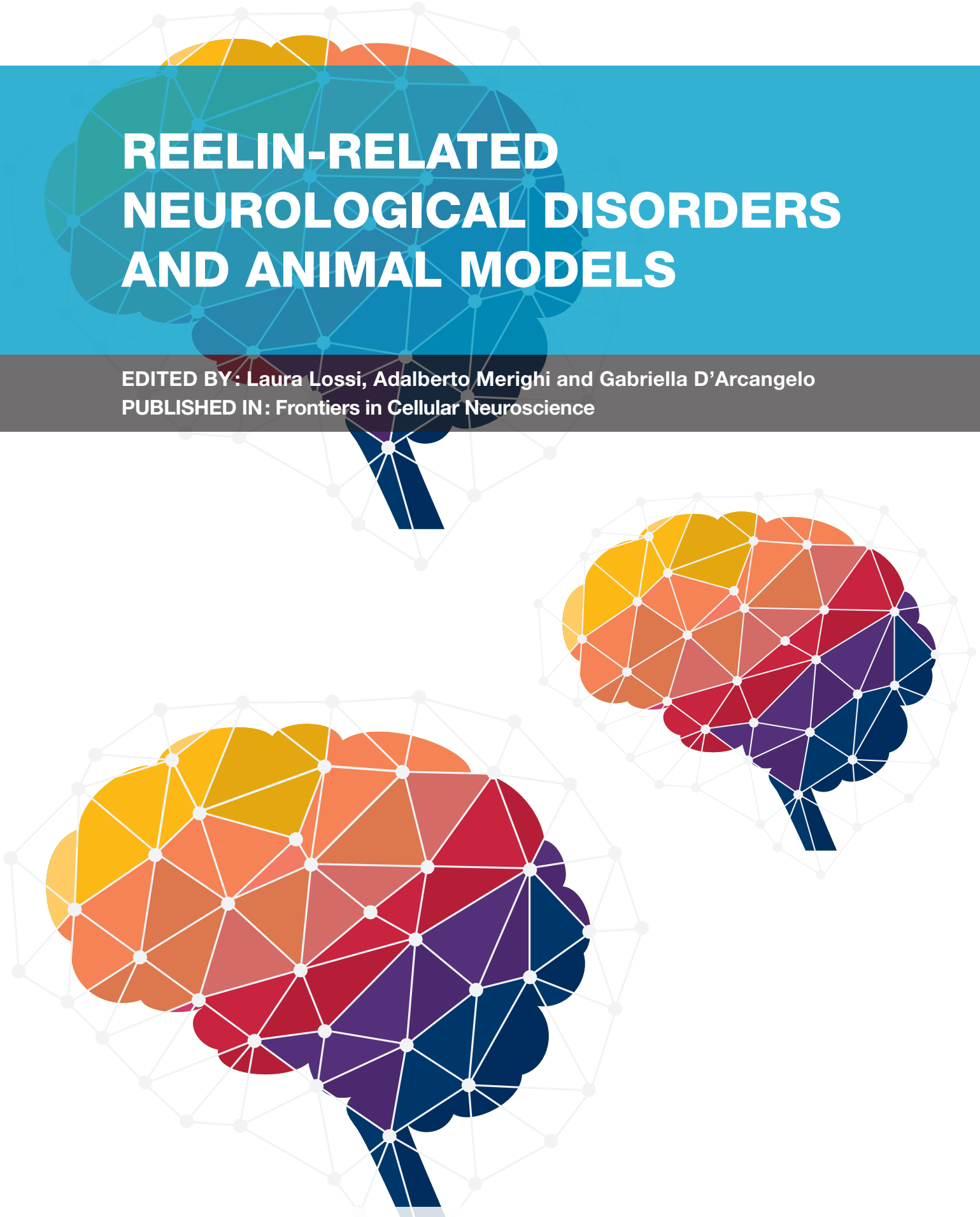
REELIN-RELATED NEUROLOGICAL DISORDERS AND ANIMAL MODELS

EDITED BY: Laura Lossi, Adalberto Merighi and Gabriella D'Arcangelo
PUBLISHED IN: Frontiers in Cellular Neuroscience



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ISSN 1664-8714
ISBN 978-2-88945-111-1
DOI 10.3389/978-2-88945-111-1

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REELIN-RELATED NEUROLOGICAL DISORDERS AND ANIMAL MODELS

Topic Editors:
Laura Lossi, University of Turin, Italy
Adalberto Merighi, University of Turin, Italy
Gabriella D'Arcangelo, Rutgers, the State University of New Jersey, USA

The Reeler mutation was so named because of the alterations in gait that characterize homozygous mice. Several decades after the description of the Reeler phenotype, the mutated protein was discovered and named Reelin (Reln). Reln controls a number of fundamental steps in embryonic and postnatal brain development. A prominent embryonic function is the control of radial neuronal migration. As a consequence, homozygous Reeler mutants show disrupted cell layering in cortical brain structures. Reln also promotes postnatal neuronal maturation. Heterozygous mutants exhibit defects in dendrite extension and synapse formation, correlating with behavioral and cognitive deficits that are detectable at adult ages.

The Reln-encoding gene is highly conserved between mice and humans. In humans, homozygous RELN mutations cause lissencephaly with cerebellar hypoplasia, a severe neuronal migration disorder that is reminiscent of the Reeler phenotype. In addition, RELN deficiency or dysfunction is also correlated with psychiatric and cognitive disorders, such as schizophrenia, bipolar disorder and autism, as well as some forms of epilepsy and Alzheimer's disease. Despite the wealth of anatomical studies of the Reeler mouse brain, and the molecular dissection of Reln signaling mechanisms, the consequences of Reln deficiency on the development and function of the human brain are not yet completely understood. This Research Topic includes reviews that summarize our current knowledge of the molecular aspects of Reln function, original articles that advance our understanding of its expression and function in different brain regions, and reviews that critically assess the potential role of Reln in human psychiatric and cognitive disorders.

Citation: Lossi, L., Merighi, A., D'Arcangelo, G., eds. (2017). Reelin-Related Neurological Disorders and Animal Models. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-111-1

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Reelin and Neuropsychiatric Disorders

frontiers in Cellular Neuroscience

EDITORIAL
published: 05 January 2017
doi: 10.3389/fncel.2016.00299

Editorial: Reelin-Related Neurological Disorders and Animal Models

Gabriella D'Arcangelo[1]*, *Laura Lossi*[2] *and Adalberto Merighi*[2]

[1] Department of Cell Biology and Neuroscience, Rutgers, The State University of New Jersey, Piscataway, NJ, USA,
[2] Laboratory of Neuroscience, Department of Veterinary Sciences, University of Turin, Turin, Italy

Keywords: brain, development, neuron, reeler, autism, schizophrenia

Editorial on the Research Topic

Reelin-Related Neurological Disorders and Animal Models

Homozygous, loss-of-function mutations in the mouse *Reelin* gene cause a severe neurological phenotype known as *reeler*. These mutant mice exhibit marked cerebellar hypoplasia, dyslamination of cortical and hippocampal cellular layers, and malposition of specific neuronal populations in the brain stem and spinal cord. Similarly, homozygous mutations in the very conserved human *REELIN* gene cause a severe neurodevelopmental disorder known as lissencephaly with cerebellar hypoplasia (LCH). These structural defects underscore the essential role that Reelin plays in the control of neuronal migration in the prenatal and early postnatal brain. However, the Reelin protein also affects synapse formation and function in the postnatal and adult brain, and this function is reflected in the manifestation of behavioral and cognitive deficits in heterozygous *reeler* mice that express reduced levels of Reelin and do not exhibit overt brain structural defects. A reduction in REELIN expression is also found in human patients affected by neuropsychiatric disorders, including autism, schizophrenia, and depression. How does a deficit in REELIN expression contribute to these diseases? In order to understand the potential role of Reelin dysfunction in neuropsychiatric disorders we need to gain a deeper understanding of the molecular mechanisms by which this protein controls all aspects of brain development and function. This Research Topic is a collection of reviews that summarize and interpret many recent findings in the Reelin field. The Topic also includes original research articles that provide novel information on Reelin expression and function in different regions of the central nervous system.

Reelin is a large glycoprotein that is secreted by different neuronal populations at different stages of brain development. Shortly after its discovery, it became clear that Reelin is subject to proteolytic cleavage after secretion, resulting in the generation of multiple extracellular protein fragments. The functional significance of this process was not well understood. In recent years, several studies have shed some light on this event, identifying specific cleavage sites, and addressing the consequence of proteolytic cleavage for Reelin biological function. Ranaivoson et al. review structural aspects of Reelin as a ligand, discussing its proteolytic fragments, and the binding of uncleaved and cleaved products to known cell surface receptors. Lussier et al. further review Reelin proteolytic cleavage and its potential role in modulating synapse function in the normal adult brain or in neurodegeneration. Two reviews then discuss in depth molecular aspects of Reelin signal transduction that are initiated by distinct Reelin protein isoforms. Lee and D'Arcangelo discuss how different Reelin ligands trigger different signaling pathways, and thus control different functions such as neuronal migration, maturation, and synaptic activity. Bock and May further review in depth canonical and non-canonical signaling pathways, emphasizing the role of different pathways in the control of the neuronal cytoskeleton. Together, these reviews provide a detailed

Edited and reviewed by:
Egidio D'Angelo,
University of Pavia, Italy

***Correspondence:**
Gabriella D'Arcangelo
darcangelo@dls.rutgers.edu

Received: *21 November 2016*
Accepted: *19 December 2016*
Published: *05 January 2017*

Citation:
D'Arcangelo G, Lossi L and Merighi A (2017) Editorial: Reelin-Related Neurological Disorders and Animal Models. Front. Cell. Neurosci. 10:299. doi: 10.3389/fncel.2016.00299

and comprehensive summary of the current state of the field's mechanistic understanding of Reelin signaling activities.

In the prenatal forebrain Reelin is expressed mainly by Cajal-Retzius cells in the marginal zone to control the radial migration of excitatory neurons, whereas a subpopulations of interneurons mainly express this protein in the postnatal forebrain likely to modulate synaptic function. In the cerebellum, Reelin is mainly expressed by granule cells and granule cell precursors, controlling the radial migration of Purkinje cells and possibly affecting cerebellar circuit function. In addition, less-characterized cell types also express Reelin in other regions of the brain and spinal cord, affecting positioning and connectivity of several neuronal target populations. A series of original research articles in this Topic reveal novel aspects of Reelin expression and function in the central nervous system. Carceller et al. describes the phenotypical identity of unique Reelin-expressing cells in the piriform cortex. Bosch et al. investigated the effects of *in vivo* Reelin overexpression on the development of synaptic structures in the adult hippocampus. They demonstrate striking effects on the morphology of presynaptic as well as postsynaptic structures. Furthermore, they show that Reelin overexpression affects the trafficking of NMDA receptor subunits and associated proteins from synaptic toward extrasynaptic and cytosolic sites, thus modulating glutamatergic neurotransmission. Granule cell dispersion in the dentate gyrus is frequently associated with temporal lobe epilepsy in human patients. Using organotypic slice cultures Orcinha et al. re-examined the previously noted correlation between Reelin loss and granule cell dispersion after seizures. They show that the central fragment of Reelin rescues the abnormal migration of dentate cells, establishing a causal relationship between Reelin loss and granule cell dispersion in epilepsy. Cocito et al. focused on the cerebellum, and documented alterations in neuronal proliferation and apoptosis in the homozygous *reeler* mouse lacking Reelin. Finally, Vaswani and Blaess review findings related to the expression pattern and role of Reelin in the ventral brain stem and spinal cord. They describe specific neuronal populations that express either Reelin or components of the canonical signaling pathway, and review data in support of the notion that Reelin affects specifically the final stages of migration of its cell targets. However, unlike the neocortex, Reelin appears to regulate both tangential (midbrain and spinal cord) and radial migration (hindbrain). Future work will be needed to clarify the mechanism underlying these activities, and to determine whether, as in the forebrain, Reelin affects the maturation or synaptic function of brain stem and spinal cord target neurons.

The association and possible involvement of Reelin dysfunction in neuropsychiatric disorders is addressed by several excellent reviews. Lammert and Howell discuss human REELIN heterozygous mutations in autism spectrum disorder. They describe the several *de novo* mutations identified so far in autistic patients, their location and their potential effect on Reelin protein structure and brain development. However, the author caution that, given the lack of an autistic phenotype in heterozygous *reeler* mice or in human subjects carrying *REELIN* gene deletion, second-hit mutations, or environmental insults may be necessary to develop the disease. Guidotti et al. delve into the association between human REELIN dysfunction and schizophrenia, and review the experimental evidence from post-mortem human tissue and animal models pointing to epigenetic mechanisms that cause reduced *REELIN* gene expression in GABAergic corticolimbic neurons. The studies so far suggest that this deficit may disrupt synaptic connectivity and predispose human subjects not only to schizophrenia, but also to bipolar disorder. Caruncho et al. focus on the role of Reelin in the pathogenesis of depression, and discuss data from an animal model of the disorder, produced by repeated corticosteroid injections. This treatment causes the loss of Reelin-positive cell in the subgranular zone of the dentate gyrus, where adult neurogenesis takes place. Finally, Ishii et al. provide a thorough summary and critical discussion of the human genetic and experimental animal evidence relating Reelin dysfunction to neuropsychiatric disorders, and emphasize how knowledge of the Reelin signaling mechanisms could potentially be translated into therapeutic intervention for neuropsychiatric disorders.

Overall, the data presented in this Topic provide strong support for the idea that Reelin plays and essential role of in brain development and in adult brain function, and compel us to further investigate this protein's function using multidisciplinary approaches, including structural, cellular, anatomical, physiological, and genetic approaches, in the hope that the newly acquired knowledge will help us to develop much-needed forms of pharmacological intervention for the treatment of neuropsychiatric disorders.

AUTHOR CONTRIBUTIONS

GD wrote the editorial. LL and AM read and approved the text.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

MINI REVIEW
published: 27 May 2016
doi: 10.3389/fncel.2016.00137

Structural Insights into Reelin Function: Present and Future

Fanomezana M. Ranaivoson[1,2], *Sventja von Daake*[1,2] *and Davide Comoletti*[1,2,3]*

[1] Child Health Institute of New Jersey, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ, USA, [2] Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ, USA, [3] Department of Pediatrics, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ, USA

Reelin is a neuronal glycoprotein secreted by the Cajal-Retzius cells in marginal regions of the cerebral cortex and the hippocampus where it plays important roles in the control of neuronal migration and the formation of cellular layers during brain development. This 3461 residue-long protein is composed of a signal peptide, an F-spondin-like domain, eight Reelin repeats (RR1–8), and a positively charged sequence at the C-terminus. Biochemical data indicate that the central region of Reelin binds to the low-density lipoprotein receptors apolipoprotein E receptor 2 (ApoER2) and the very-low-density lipoprotein receptor (VLDLR), leading to the phosphorylation of the intracellular adaptor protein Dab1. After secretion, Reelin is rapidly degraded in three major fragments, but the functional significance of this degradation is poorly understood. Probably due to its large mass and the complexity of its architecture, the high-resolution, three-dimensional structure of Reelin has never been determined. However, the crystal structures of some of the RRs have been solved, providing important insights into their fold and the interaction with the ApoER2 receptor. This review discusses the current findings on the structure of Reelin and its binding to the ApoER2 and VLDLR receptors, and we discuss some areas where proteomics and structural biology can help understanding Reelin function in brain development and human health.

Keywords: Reelin, ApoER2, VLDLR, brain development, cortical layers, structure-function

OPEN ACCESS

Edited by:
Laura Lossi,
University of Turin, Italy

Reviewed by:
Kazunori Nakajima,
Keio University School of Medicine, Japan
Filippo Sean Giorgi,
University Hospital of Pisa, Italy

***Correspondence:**
Davide Comoletti
comoleda@rwjms.rutgers.edu

Received: *01 March 2016*
Accepted: *10 May 2016*
Published: *27 May 2016*

Citation:
Ranaivoson FM, von Daake S and Comoletti D (2016) Structural Insights into Reelin Function: Present and Future. Front. Cell. Neurosci. 10:137. doi: 10.3389/fncel.2016.00137

INTRODUCTION

Disruptions of the autosomal recessive *Reelin* gene were identified two decades ago to be responsible for the *reeler* phenotype in mice strains originated from Edinburgh and Orleans (D'Arcangelo et al., 1995; Hirotsune et al., 1995). The Edinburgh homozygous mutant *reeler* mouse displays a complete loss of transcription of the gene (D'Arcangelo et al., 1995) whereas the Orleans strain expresses a Reelin protein that lacks a C-terminal portion (Hirotsune et al., 1995; D'Arcangelo et al., 1997; de Bergeyck et al., 1997). Despite the different genomic abnormalities, both strains are characterized by specific neurological phenotypes including tremors, ataxia, cerebellar hypoplasia and malformation of cellular layers throughout the brain (Falconer, 1951; Angevine and Sidman, 1961; Caviness and Rakic, 1978; Pinto-Lord et al., 1982; Rakic and Caviness, 1995; Lambert de Rouvroit and Goffinet, 1998, and others). The involvement of Reelin in layer formation in brain cortical structures was extensively investigated (for an excellent review, see D'Arcangelo, 2014). It is now well established that during embryonic brain development,

Reelin has a crucial role in controlling the radial migration of neurons, allowing them to reach their appropriate positions in laminated structures such as the cerebral cortex, the hippocampus or the cerebellum (Lambert de Rouvroit and Goffinet, 1998). Control of neuronal migration and layer formation is achieved by expression and secretion of Reelin by specific sub-types of cells, namely by Cajal-Retzius cells in marginal regions of the cerebral cortex and the hippocampus (D'Arcangelo et al., 1995; Ogawa et al., 1995; Del Río et al., 1997; Nakajima et al., 1997; Schiffmann et al., 1997; Alcantara et al., 1998), or by granule cell precursors localized in the external granule layer of the embryonic cerebellum (D'Arcangelo et al., 1995; Miyata et al., 1996; Alcantara et al., 1998). Additionally, small level of *Reelin* expression have been detected in deeper layers of the cerebral cortex (Yoshida et al., 2006; Uchida et al., 2009; Hirota et al., 2015). It is thought that secreted full length Reelin directs the migration of neurons in contact with these regions, whereas proteolytic fragments (see below), which diffuse towards deeper cortical layers, may target local neurons and initiate their polarization and their radial migration (Utsunomiya-Tate et al., 2000; Kubo et al., 2002; Jossin et al., 2007). Reelin was also shown to influence neurite formation in early postnatal brain (Del Río et al., 1997; Olson et al., 2006; Matsuki et al., 2010; Nichols and Olson, 2010) and to impact synapse formation and function in late postnatal and adult brain (Borrell et al., 1999; Liu et al., 2001; Rice et al., 2001; Qiu et al., 2006; Iafrati et al., 2014). At least some of these effects come into play through the association of Reelin with two well-known receptors of the low density lipoprotein receptor (LDLR) superfamily: the apolipoprotein E receptor 2 (ApoER2) and the very-low-density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999; Benhayon et al., 2003).

Following the initial observation that *REELIN* mRNA levels are reduced in patients with schizophrenia (Impagnatiello et al., 1998), several investigators reported a deficiency in Reelin expression in different groups of psychiatric subjects, including those with bipolar disorder (Knuesel, 2010). The reduction in Reelin expression occurs most likely through epigenetic mechanisms that affect promoter methylation (Veldic et al., 2004; Grayson et al., 2006) although evidence for genetic association between schizophrenia and *REELIN* polymorphisms also exists in patient subpopulations (Goldberger et al., 2005; Wedenoja et al., 2008). Furthermore, Reelin has been reported to suppress schizophreniform symptoms in mice (Ishii et al., 2015). In addition to schizophrenia, reduced expression and *REELIN* polymorphisms have been reported in some groups of autistic patients (De Rubeis et al., 2014).

This review discusses the architecture and three-dimensional structure of Reelin, highlighting the elements that are involved in the interaction with ApoER2 and VLDLR. We will discuss the proteolysis of Reelin and we will suggest areas of exploration that, in our opinion, will provide valuable information of the structural and functional mechanisms underlying the biological function of Reelin.

STRUCTURE OF REELIN

Reelin is a ∼440 kDa secreted glycoprotein (∼386 kDa with ∼18 putative N-linked glycosylation sites) expressed from a large genomic section (∼450 kb spanned by 65 exons) located on chromosome 5 in mouse and 7 in humans. Mouse and human Reelin have ∼94.2% and ∼87.2% sequence identity at the amino acid and DNA levels, respectively (DeSilva et al., 1997). From the N- to the C-termini, Reelin is composed of a Reeler domain similar to the N-terminal domain of F-spondin, followed by a unique region named "H" (∼300 amino acids), subdivided in three subdomains termed X, Y and Z (Ichihara et al., 2001) and eight Reelin-specific repeats followed by a highly basic region. Each Reelin repeat (RR) is ∼350–390 amino-acid residues long, divided into two homologous sub-repeats (A and B) separated by an epidermal growth factor (EGF) domain of about 35 amino-acid residues (**Figure 1**). The eight RRs are structurally similar, with an average sequence identity in the range of 83.5–87.9% when comparing the same RR of Reelin homologs from 167 species. Interestingly, the amino acid identity of the eight RR in the same molecule is in the range of 36.4–39.7% (Manoharan et al., 2015), suggesting that albeit the identity is sufficient to maintain similar tertiary structure, these domains are likely to be functionally different (e.g., interact with different receptors).

Although the overall structure of Reelin has never been reported, individual structures of selected RRs were solved by macromolecular crystallography. The crystal structure of RR3 (Nogi et al., 2006, PDB ID: 2DDU) revealed that each sub-repeat of Reelin is composed by 11 β-strands organized in two antiparallel β-sheets that are arranged in parallel, forming a jelly-roll folding similar to the carbohydrate-binding module (CBM) of other unrelated proteins, and to the "galactose-binding domain-like" superfamily. One of the features of a RR is a compact "horseshoe-like" conformation, where sub-repeats A and B make extensive interfacial contacts, burying ∼1600 $Å^2$ of solvent-accessible area, including a loop protruding from the concave side of sub-repeat A (Nogi et al., 2006). This loop bulges from the middle of the fifth strand (strand "H") of the β-sheet at the concave side and is found in all aligned Reelin sub-repeats A. The EGF-like domain that separates the two sub-repeats in the primary structure lies on the lateral side of the tertiary arrangement where it interacts with both sub-repeats A and B. A stretch of ∼14 amino-acids termed "bacterial neuraminidase repeat" (BNR) or "Asp-box" is found in all Reelin sub-repeats in close proximity to the EGF-like domain. This motif (X-X-S/T-X-D/N-X-G-X-X-W, where X can be any amino acid) has been found in a few other mammalian proteins such as Sialidase1–3 proteins (Gaskell et al., 1995) and forms a β-hairpin that seems to have a structural rather than a functional purpose (Copley et al., 2001).

The β-sheets of the sub-repeats are curved, creating a concave and a convex side of the jelly-roll shape where a Ca^{2+} ion is bound at the convex side of RR3B, but also in all four sub-repeats in the RR5–6 Reelin fragment (Yasui et al., 2007, PDB ID: 2E26). The importance of this bound Ca^{2+} is further emphasized by the finding of a similar calcium-binding site in

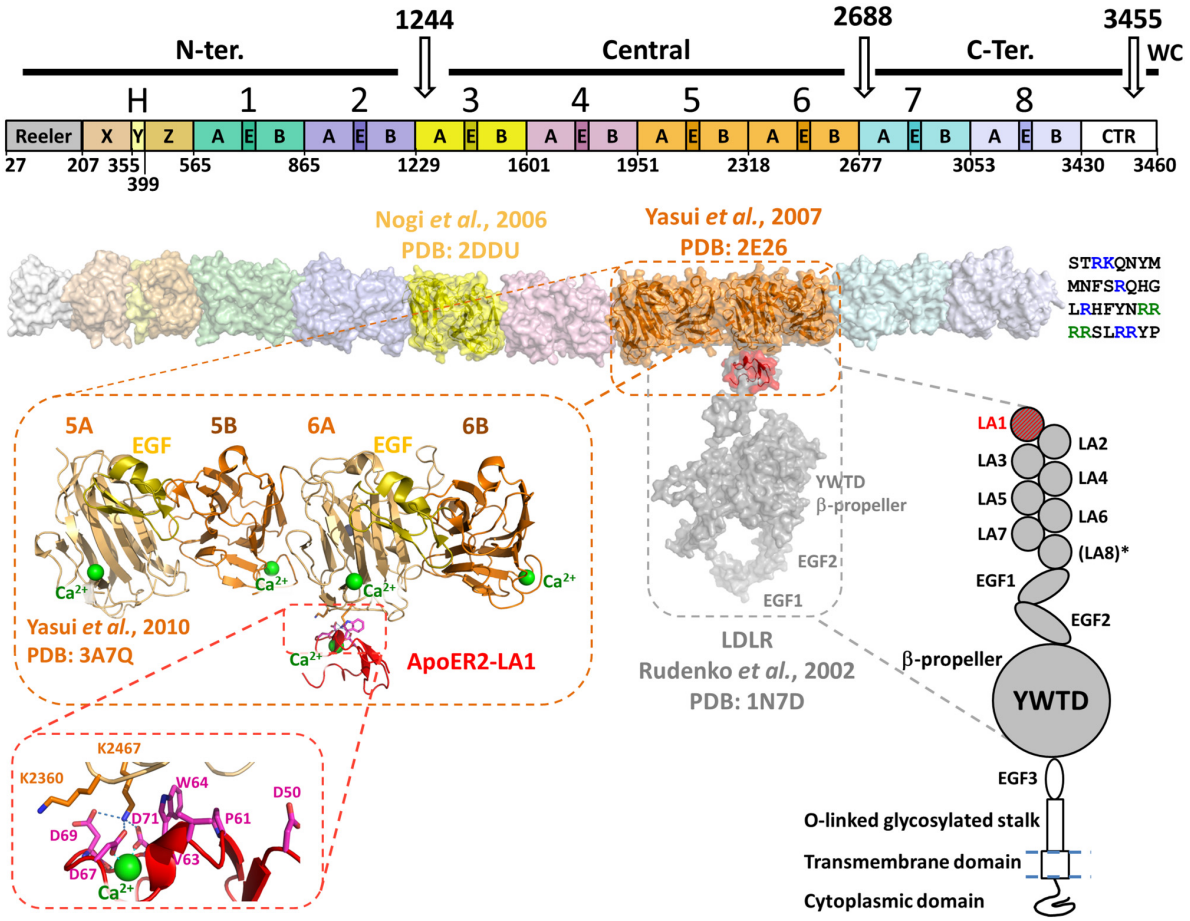

FIGURE 1 | Domain organization and structural model of Reelin in association with apolipoprotein E receptor 2 (ApoER2) or very-low-density lipoprotein receptor (VLDLR). Top diagram shows the domain organization of Reelin. Three *in vivo* proteolysis sites (white arrows) are represented on the top of the diagram, highlighting the N-terminal (N-ter.), Central, C-terminal (C-ter.) and the WC cleavage products. The Reelin repeats are numbered (1–8) and their sub-domain composition (subrepeats A and B separated by an epidermal growth factor (EGF)-like (E) domain) is marked. On the left side of RR1 the Reeler domain and the domains X, Y and Z (fragment "H") are marked. The white box on the right side (C-terminally RR8) represents the conserved fragment of the CTR. The boundaries of each domain and RR are indicated on the bottom of the diagram and are identified by numbers corresponding to the mouse Reelin sequence (Uniprot : Q60841) and are adapted from Ichihara et al. (2001). Below the bar diagram, a three-dimensional model of Reelin (surface representation) was assembled using the established crystal structures of RR3 (yellow, PDB: 2DDU) and RR5–6 (orange, PDB: 2E26) and homology-models of each other RR and the X, Y and Z domains. The template used for the Reeler domain model was the N-terminal domain of F-spondin (PDB: 3COO). The homology models were built with the Swiss-model server[1] and the RR models were positioned relatively to each other to best reproduce the inter-repeat interface observed in the crystal structure of RR5–6. The CTR is represented at the C-terminus of the Reelin model as a single-letter amino acid sequence. The basic residues are highlighted in blue, and the WC cleavage recognition sequence (RRRR) is green. Schematic representation of the LDLR-like receptor is shown on the lower right side of the figure. The domain organization of ApoER2 and VLDLR is similar to the architecture of LDLR, with the exception of an extra LDLR class A (LA) module not found in the latter (LA8*). Both ApoER2 and VLDLR receptors contain eight LA modules, three EGF-like domains, a unique β-propeller formed by YWTD (or LDLR class B) repeats separating EGF2 and EGF3. They also contain an O-linked glycosylation portion (stalk) upstream a single-pass transmembrane domain and a C-terminal cytoplasmic domain. No structure of large fragments of ApoER2 or VLDLR are currently available, thus the structure of the equivalent fragment of LDLR (PDB: 1N7D) was used to illustrate the binding with Reelin on the three-dimensional model (Rudenko et al., 2002). LDLR LA1 was overlaid with ApoER2 LA1 (red) in complex with RR5–6 (PDB: 3A7Q). The overall three-dimensional model uses the same color-code as the domain organization diagrams, for both partners. The crystal structure of RR5–6 in complex with ApoER2 LA1 is shown in more detail in the zoomed-in inset to highlight the folding of this Reelin fragment, and the location of the interfacing residue with ApoER2. The Reelin sub-repeats and EGF-like domain are distinguished by their color: A in light orange, B in dark orange and EGF in yellow. The Ca^{2+} ions characteristic to Reelin sub-repeats and the receptor LA module are represented as green spheres. A detailed view of the major interfacing residues is illustrated in the inset, showing the critical interaction pattern established by Reelin Lys2467 with ApoER2 Asp69, Asp71 and Asp67.

other structurally related proteins, like in the CBM domains (Nogi et al., 2006) or the Cleaved Adhesin domain family (Ganuelas et al., 2013).

Together with the crystal structure of RR3, single particle electron tomography was used to image the four-repeats-containing Reelin fragment RR3–6 (Nogi et al., 2006). In addition to the compactness of each individual RR, repeats RR3 through RR6 appeared to be in close

[1]http://swissmodel.expasy.org

contact with each other, leading to a roughly straight particle. This particle was solved as a flattened rod-shaped three-dimensional map, with a maximum dimension of approximately four times the longest dimension of a single repeat. The structural continuity between RRs was analyzed in further detail with the crystal structure of RR5–6 (Yasui et al., 2007). The two repeats were related to each other by an almost perfect translational symmetry, and the inter-repeat interface buried solvent-accessible area of ∼1500 Å^2, mainly composed by hydrophobic contacts, suggesting a stable intramolecular conformation of the two repeats.

IN VIVO PROTEOLYSIS OF REELIN

In vivo, proteolysis of the mature protein occurs, over time, at two positions, within RR3 but close to the interface with RR2, and between RR6 and RR7. By antibody mapping, the fragmentation pattern of Reelin predicts the generation of three major proteolytic fragments (the so-called N-terminal, central and C-terminal fragments). The N-terminal cleavage site has been determined to occur between Pro-1244 and Ala-1245 within RR3 (Koie et al., 2014). Consistently, Reelin-P1244D mutant became protease resistant and had a longer biological effect compared to wild type (WT) Reelin (Koie et al., 2014). The C-terminal cleavage site of WT Reelin, between RR6 and RR7 was also recently identified between Ala2688 and Asp2689 (^{2685}RSPA/DAG^{2691}) (Sato et al., 2016). A Reelin mutant, in which Asp2689 is replaced by Lys appears to be resistant to C-terminal cleavage when incubated with culture supernatant of cerebellar granular neurons. Finally, bioinformatics analysis of the C-terminal Reelin sequence indicates the presence of a Furin recognition proteolysis site [R-X-(R/K)-R] between residues 3452 and 3455 (^{3452}RRRR^{3455}/SLRRYP^{3461}). The presence of this cleavage site and the release of the last six amino acids were confirmed experimentally and named WC (Within the C-Terminal Region [CTR]) site (Kohno et al., 2015). Although the exact function of this cleavage is currently unknown, sequence conservation among mammals suggests a critical function in the mammalian brain. Taken together, these data indicate that cleavage of Reelin may play a critical role in regulating the duration and range of Reelin signaling in the developing brain.

THE REELIN RECEPTORS

Reelin binds with similar affinity to cell surface receptors VLDLR and ApoER2, two members of the LDLR family. These receptors are differentially expressed in different organs, tissues, and cell types (Trommsdorff et al., 1999). Reelin binding to ApoER2/VLDLR receptors activates intracellular Src family kinases (SFKs), which in turn phosphorylate the adaptor protein Disabled-1 (Dab1) at specific tyrosine residues (Arnaud et al., 2003; Suetsugu et al., 2004). Although binding to Reelin triggers common signal transduction mechanisms, they mediate diverse biological functions (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Benhayon et al., 2003).

Structurally, both receptors are composed of an extracellular domain of ∼800 amino acids, followed by a single transmembrane and an intracellular domain (**Figure 1**). The architecture of both receptors is very close to the prototypical LDLR (Rudenko et al., 2002), with seven or eight "LDLR class A" (LA) domains followed by three EGF modules and a unique YWDT β-propeller domain inserted between EGF2 and EGF3 (**Figure 1**).

A variety of protein-protein interaction assays such as protein pull-down, surface plasmon resonance and isothermal titration calorimetry assays were used to evaluate and quantify the interaction of Reelin to either receptor (Andersen et al., 2003; Yasui et al., 2007, 2010). These experiments determined that RR3–6 contained the necessary binding elements to associate with ApoER2/VLDLR receptors (Jossin et al., 2004) and that the minimal binding cassette seems to be the RR5–6 fragment. On the receptor side, it was shown that the LA-containing regions of ApoER2 and VLDLR were necessary to bind RR5–6 (D'Arcangelo et al., 1997; Koch et al., 2002; Andersen et al., 2003). In particular, the first LA module (LA1) was necessary for the Reelin interaction providing a dissociation constant (K_D) in the nanomolar range. Interestingly, according to the reported K_D values, supplemental LA domains appeared to modulate the affinity towards RR5–6. Between the two receptors, ApoER2-LA1 appeared to have higher affinity to RR5–6 than VLDLR-LA1 (Andersen et al., 2003; Yasui et al., 2010).

The crystal structure of the [RR5–6:ApoER2-LA1] complex revealed that the LA1 module interacts essentially with RR6A, close to the RR5–6 inter-Repeat interface (Yasui et al., 2010). Therefore, the observed essential role of RR5 in solution for the receptor association is likely to be indirect. Additionally, the high affinity interaction contrasts with the small interface area (696 Å^2) observed in the crystal structure, suggesting that other portions of Reelin or the receptor may contribute to the interaction. Indeed, a multivalent binding mode is usually observed for the known ligand interactions involving LA modules in the LDLR family (Blacklow, 2007). For example the Receptor-Associated Protein (RAP), an endoplasmic reticulum escort protein for LDLR-like proteins, is capable of binding LA pairs rather than single LA modules (Andersen et al., 2000, 2001; Fisher et al., 2006).

A Ca^{2+} ion, characteristically bound to LA modules, is found in the ApoER2-LA1 structure, providing important structural basis for the observed Ca^{2+}-dependency of the Reelin-receptor association (D'Arcangelo et al., 1999; Andersen et al., 2003). However, this ion-dependency may also result from the Reelin-bound Ca^{2+} described above (Nogi et al., 2006). Overall, the interaction between RR5–6 and the ApoER-LA1 is in accord with the canonical mode of interaction between LA modules and their protein partners as described by Blacklow (2007). This interaction involves a lysine (equivalent to RR5–6 Lys2467) or an arginine in interaction with three LA aspartate

residues that are involved in the Ca^{2+}-coordination, e.g., Asp67, Asp69 and Asp71 in ApoER2-LA1. Additionally, an aromatic side chain (equivalent to LA1 Trp64) stacks the aliphatic groups of the lysine side chain for a proper positioning for the interaction with the aspartates. Interestingly, the two structures of RR5–6 alone (Yasui et al., 2007) or in complex with the LA1 module of ApoER2 (Yasui et al., 2010) were almost perfectly superimposable, indicating that the binding of the receptor did not induce major structural changes within the repeats and sub-repeats of the RR5–6 fragment (**Figure 1**).

The VLDLR and ApoER2 receptors appear to mediate at least some of the known functions of Reelin in the central nervous system; however, other receptors may be responsible for additional function in the brain or in peripheral organs. In fact, Reelin was also found to associate with EphB2 and EphB3 by co-immunoprecipitation of both endogenous proteins from brain lysates (Sentürk et al., 2011). Unlike lipoprotein receptors, EphB2 appears to bind the N-terminal region of Reelin. This region was also reported to bind other putative Reelin receptors such as the Cadherin-related neuronal receptor-1 or CNR1 and integrin α3β1 (Senzaki et al., 1999; Dulabon et al., 2000). However, the binding to these receptors have not been further confirmed and characterized.

FUTURE PERSPECTIVES

A great deal of information is currently available on the structure and function of Reelin in the developing brain. Nevertheless, many questions remain unanswered. For example, Reelin/VLDLR or ApoER2 interaction occurs in the central region of Reelin but the N-terminal portion of Reelin also influences ApoER2 and VLDLR function. This is indicated by the inhibition of Reelin activity by the CR-50 antibody, which binds to an epitope located between residues 251 and 407 of Reelin (Del Río et al., 1997; D'Arcangelo et al., 1997, 1999; Miyata et al., 1997), presumably through inhibition of Reelin multimerization (Utsunomiya-Tate et al., 2000; Kubo et al., 2002). Interestingly, residue Cys2101, located in the central fragment of Reelin was recently shown to be involved in Reelin dimerization (Yasui et al., 2011), indicating that the multimerization of Reelin is complex. Moreover, the Reelin poly-basic 32-residue long CTR (**Figure 1**) appears to be functionally significant for the ApoER2 or VLDLR signaling pathway (Nakano et al., 2007; Kohno et al., 2009) and for the structure and positioning of neurons in the developing and the postnatal cerebral cortex (Kohno et al., 2015). This fragment is extremely conserved in mammals, birds and reptiles (Nakano et al., 2007) and seems to be important for the efficient secretion of Reelin (D'Arcangelo et al., 1997; de Bergeyck et al., 1997) by maintaining the structural integrity of RR8 (Kohno et al., 2009). Together, these studies highlight the importance of other parts of Reelin that, whilst not directly involved in the receptor interaction, contribute to its function. At the molecular and structural levels, further analyses are clearly needed to fully understand the implication of these parts of Reelin, as well as of the other RRs that have not been characterized so far. Furthermore, emerging investigations indicate that alternative cell surface receptors, such as the EphB2 and EphB3, likely interact with other parts of Reelin. Kohno et al. (2015) observed that the fragment RR7–8 that does not bind to the LDLR-like receptors is able to bind to neuronal cell membrane as long as it includes an intact CTR.

Final Considerations

It will be interesting to characterize the full-length Reelin structurally, alone, and bound to its canonical receptors. However, the large size, extensive N-linked glycosylation, multimerization, and potential intra-molecular flexibility, make this study difficult to complete using protein crystallography. A combination of lower resolution approaches, including small angle X-ray scattering (Rubio-Marrero et al., 2016) and cryo electron microscopy (EM; Zhou, 2008) are likely to be the methods of choice. Another fascinating element is the significance of the proteolysis of Reelin. The degradation pattern seems to be well established and it is currently thought to negatively modulate the function of Reelin. However, it is also possible that cleavage into various RR segments enable these smaller fragments to diffuse to more distant brain layers and to bind and activate currently unrecognized cell surface receptors. In either case, the dynamic regulation of these events is not well understood and it should be an area of intense research. A precise profiling of Reelin degradation over time will be necessary to understand Reelin function in health and disease. Outstanding work has been done to determine the N- and C-terminal cleavage sites, as well as the newly recognized WC site. However, it is likely that, *in vivo*, Reelin is further degraded into smaller fragments that can have currently unrecognized activities.

Finally, it was recently reported that full-length Reelin, but not its central fragment, is capable of activating Erk1/2 signaling, leading to increased p90RSK phosphorylation and the induction of immediate-early gene expression. Remarkably, because Erk1/2 activation is not mediated by the canonical signal transduction pathway, a non-canonical pathway that works during brain development must exist (Lee et al., 2014).

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing of the manuscript. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by NIMH RO1-MH092906, CAUT14APL028, and BRAIN EAGER MCB-1450895 to DC. We would like to apologize to the authors of many excellent articles that, for space considerations, were not included in this review.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 29 March 2016
doi: 10.3389/fncel.2016.00075

Reelin Proteolysis Affects Signaling Related to Normal Synapse Function and Neurodegeneration

*April L. Lussier[1], Edwin J. Weeber[1] and G. William Rebeck[2]**

[1] Department of Molecular Pharmacology and Physiology, USF Health Byrd Alzheimer's Disease Institute, University of South Florida, Tampa, FL, USA, [2] Department of Neuroscience, Georgetown University, Washington, DC, USA

Reelin is a neurodevelopmental protein important in adult synaptic plasticity and learning and memory. Recent evidence points to the importance for Reelin proteolysis in normal signaling and in cognitive function. Support for the dysfunction of Reelin proteolysis in neurodegeneration and cognitive dysfunction comes from postmortem analysis of Alzheimer's diseases (AD) tissues including cerebral spinal fluid (CSF), showing that levels of Reelin fragments are altered in AD compared to control. Potential key proteases involved in Reelin proteolysis have recently been defined, identifying processes that could be altered in neurodegeneration. Introduction of full-length Reelin and its proteolytic fragments into several mouse models of neurodegeneration and neuropsychiatric disorders quickly promote learning and memory. These findings support a role for Reelin in learning and memory and suggest further understanding of these processes are important to harness the potential of this pathway in treating cognitive symptoms in neuropsychiatric and neurodegenerative diseases.

Keywords: Reelin, Alzheimer's disease, neurodegeneration, proteolysis, learning and memory

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****Correspondence:***
G. William Rebeck
Bill.Rebeck@georgetown.edu

Received: *15 January 2016*
Accepted: *11 March 2016*
Published: *29 March 2016*

Citation:
Lussier AL, Weeber EJ and Rebeck GW (2016) Reelin Proteolysis Affects Signaling Related to Normal Synapse Function and Neurodegeneration. Front. Cell. Neurosci. 10:75. doi: 10.3389/fncel.2016.00075

Neurodegenerative diseases are characterized by the progressive loss of synapses and neurons, accounting for cognitive deterioration. One molecular pathway that is well characterized in playing a role in adult synaptic plasticity and learning and memory is the Reelin signaling pathway (Weeber et al., 2002; Chen et al., 2005; Qiu et al., 2006a,b; Qiu and Weeber, 2007; Rogers et al., 2011). Reelin is also involved in a number of neurodegenerative and neuropsychiatric disorders presenting with cognitive deficits, including schizophrenia (Guidotti et al., 2000; Chen et al., 2002; Fatemi, 2005; Torrey et al., 2005), bipolar disorder (Fatemi et al., 2000; Torrey et al., 2005), depression (Knable et al., 2004; Lussier et al., 2009, 2011, 2013a,b; Fenton et al., 2015), epilepsy (Fournier et al., 2010; Haas and Frotscher, 2010; Dutta et al., 2011) and autism (Fatemi et al., 2005). Furthermore, Reelin signal transduction pathways appear to be particularly vulnerable in Alzheimer's disease (AD), potentially contributing to its pathogenesis (Hoe et al., 2006; Hoareau et al., 2008). Thus, a better understanding of Reelin signaling could be useful in developing therapies against synaptic and neuronal loss in a number of conditions.

REELIN IN DEVELOPMENT

Reelin is an extracellular matrix protein important in brain development during embryogenesis (for detailed reviews, see Lambert de Rouvroit et al., 1999; Rice and Curran, 2001; Tissir and Goffinet, 2003). During development Reelin is expressed by Cajal–Retzius cells

in the hippocampus and cortex and granule cells in the cerebellum (Ogawa et al., 1995; Del Río et al., 1997; Frotscher, 1998; Hirota et al., 2015). In the adult brain GABAergic interneurons in the cortex and hippocampus secrete Reelin (Alcantara et al., 1998; Pesold et al., 1998). Much of what we know about the Reelin signaling pathway in development comes from mouse models that have knock-down or overexpression of critical proteins in the pathway: Reelin, lipoprotein receptors, and Disabled-1 (Dab1; Howell et al., 1997b; Hiesberger et al., 1999; Trommsdorff et al., 1999; Beffert et al., 2002; Drakew et al., 2002; Weeber et al., 2002; Qiu et al., 2006a; Pujadas et al., 2010, 2014; Teixeira et al., 2011; Trotter et al., 2013; Lane-Donovan et al., 2015).

REELIN SIGNALING PATHWAY

Once Reelin is secreted by GABAergic interneurons into the extracellular space it binds to the lipoprotein receptors, very-low-density lipoprotein receptor (VLDLR) and Apolipoprotein receptor 2 (ApoER2; D'Arcangelo et al., 1999; Weeber et al., 2002; Herz and Chen, 2006; **Figure 1**). Ligand interactions lead to receptor dimerization and tyrosine phosphorylation of the downstream intracellular adaptor protein Dab1 (Howell et al., 1997a; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Strasser et al., 2004; Herz and Chen, 2006; Trotter et al., 2013, 2014; Divekar et al., 2014). Dab1 phosphorylation activates Src family tyrosine kinases (SFK), such as Fyn, which phosphorylates N-methyl-D-aspartate (NMDA) receptors allowing increases in Ca^{2+} influx (Chen et al., 2005). Enhancement in Ca^{2+} influx allows for maturation of NMDA receptors from the NR2B to NR2A receptor subtype, increased membrane α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor insertion, and can contribute to the induction and enhancement of long-term potentiation (LTP; Weeber et al., 2002; Beffert et al., 2005; Chen et al., 2005; Herz and Chen, 2006; Qiu et al., 2006b; Qiu and Weeber, 2007). In addition, Dab1-induced phosphorylation also can activate Phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB/Akt) which then causes Glycogen synthase kinase 3 beta (GSK3β) inhibition (Beffert et al., 2002), in turn suppressing tau hyperphosphorylation (Ohkubo et al., 2003).

As Reelin positive cells are found in highest numbers in the CA1 stratum lacunosum and hilus, they are in prime locations to influence learning and memory, and neurogenesis, respectively. Indeed, Reelin has been shown to enhance synaptic plasticity and learning and memory (Weeber et al., 2002; Herz and Chen, 2006; Rogers and Weeber, 2008), as well as alter migration of adult born neurons (Zhao et al., 2007; Pujadas et al., 2010; Teixeira et al., 2012). In the hippocampus, extracellular Reelin accumulates in the stratum lacunosum (Pesold et al., 1999; Lussier et al., 2009) which makes it in a prime location to influence synaptic activity in the CA1 (Weeber et al., 2002; Herz and Chen, 2006;

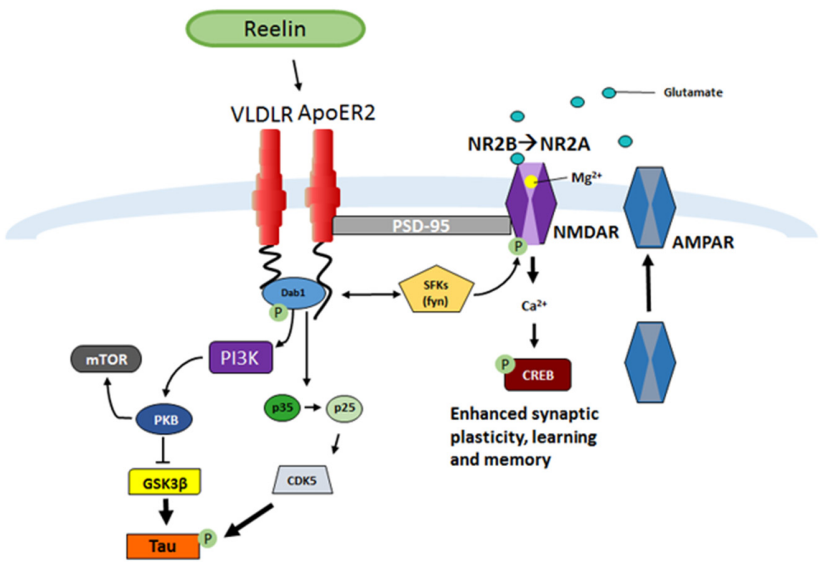

FIGURE 1 | Reelin signaling pathway in adult synaptic plasticity. Reelin binds to the lipoprotein receptors apolipoprotein receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) which causes receptor clustering and the src family tyrosine kinases (SFK) tyrosine phosphorylation of the intracellular adaptor protein Disabled-1 (Dab1), which results in the phosphorylation of N-methyl-D-aspartate receptor (NMDAR; D'Arcangelo et al., 1997, 1999; Weeber et al., 2002; Niu et al., 2004; Beffert et al., 2005; Chen et al., 2005; Qiu et al., 2006b; Qiu and Weeber, 2007; Burrell et al., 2014; Divekar et al., 2014). A subsequent increase in calcium influx leads to depolarization of the post-synaptic membrane and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) insertion (Weeber et al., 2002; Qiu et al., 2006b; Qiu and Weeber, 2007). A consequence of the increase in Ca^{2+} influx and depolarization of the cell is increased CREB phosphorylation and protein synthesis, which ultimately results in increased synaptic plasticity and learning and memory (Niu et al., 2008; Rogers et al., 2011, 2013). Another result of Dab1 phosphorylation is activation of phosphatidylinositol-3-kinase (PI3K), protein kinase B (PKB/Akt), and modulation of Glycogen synthase kinase 3 beta (GSK3β), which inhibits Tau phosphorylation (Beffert et al., 2002). Phosphorylation of Dab1 also regulates the conversion of p35 to p25 and results in activation of CDK5, also responsible for Tau phosphorylation (Beffert et al., 2004).

Rogers and Weeber, 2008). Endogenous cleavage of Reelin in these regions may be used to regulate Reelin's effects on these processes.

REELIN PROCESSING

Reelin signaling may not be driven by the simple production and release of Reelin from interneurons, as with neuropeptides or small molecule transmitters, but it may be regulated by the directed proteolysis of sequestered, full length, extracellular Reelin. Reelin has been shown to have two main sites of cleavage, between EGF-like repeats 2–3 (R2–3) and repeats 6–7 (R6–7; Jossin et al., 2004; **Figure 2**). These cleavage sites result in five major fragments that can be found in the adult and developing brain (Jossin et al., 2007; Krstic et al., 2012; Trotter et al., 2014). The middle R3–6 fragment interacts with the VLDLR and ApoER2 and is considered the fragment that is involved in initiating the downstream signaling of the Reelin cascade (Jossin et al., 2004). Our laboratory (Trotter et al., 2014) and others (Nagy et al., 2006; Nogi et al., 2006; Nakano et al., 2007; Hisanaga et al., 2012; Krstic et al., 2012) have attempted to identify Reelin-cleaving enzymes, such as the serine protease tissue plasminogen activator (tPA), matrix metalloproteinases (MMP), and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and the functional role of this proteolytic processing.

We have recently identified one mechanism for the normal processing of extracellular Reelin, through the effects of the serine protease tPA in the brain (Trotter et al., 2014). The activity-dependent proteolysis of Reelin between R6 and R7 in wild-type mice was not seen in tPA KO mice, supporting

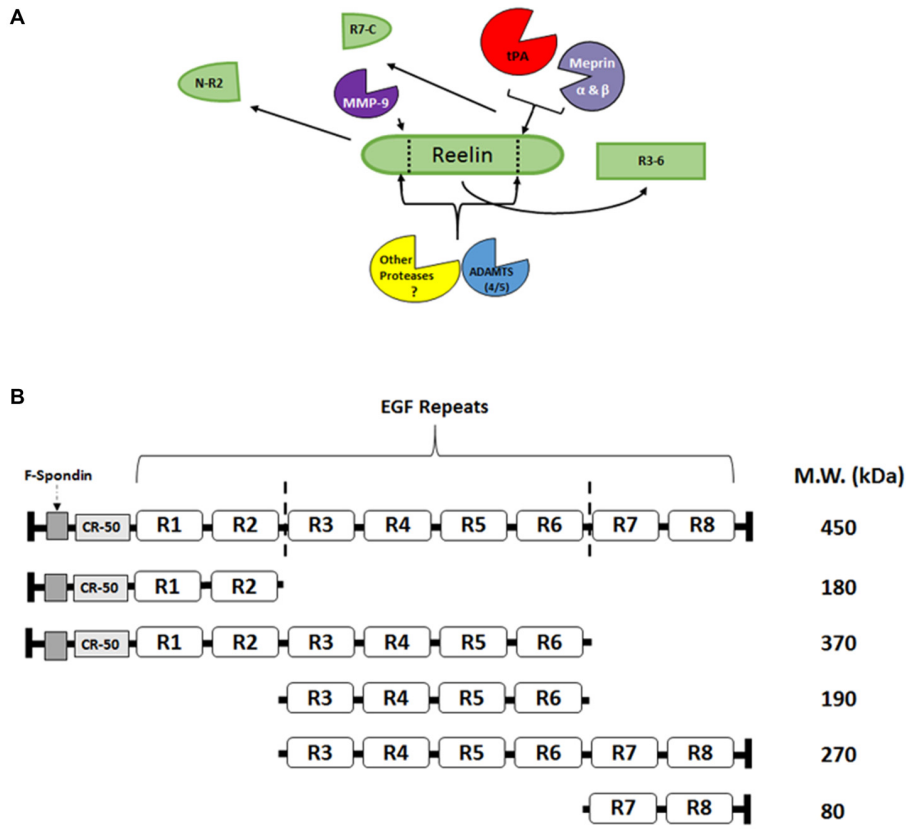

FIGURE 2 | Reelin proteolysis in the adult brain. (A) Full length Reelin is released into the extracellular space by GABAergic interneurons in the adult brain. This full length Reelin is enzymatically cleaved between epidermal growth factor (EGF) repeats 2–3 (R2-R3) and 6–7 (R6-R7; indicated by dotted lines; **A,B**), by a number of different enzymes. For example, tissue plasminogen activator (tPA), Meprin α and β have been shown to cleave Reelin between R6 and R7 (Kohno et al., 2009; Krstic et al., 2012; Trotter et al., 2014; Sato et al., 2016), while matrix metalloproteinases (MMP)-9 cleaves Reelin between R2 and R3 (Krstic et al., 2012). The ADAMTS 4 and 5 have been shown to cleave Reelin at both sites (Hisanaga et al., 2012; Krstic et al., 2012). Other, yet to be identified proteases, are also potentially involved in Reelin processing. **(B)** Full length Reelin (450 kDa) is cleaved by a number of enzymes which result in the production of five fragments that range from 370–80 kDa. The R3-R6 fragment [included in the full length Reelin (450 kDa), 370 kDa, 190 kDa, and 270 kDa fragments] has been shown to bind to the lipoprotein receptors, ApoER2 and VLDLR (Jossin et al., 2004). The N-R2 fragment (180 kDa) has been shown to bind to alpha$_3$beta$_1$-integrins (Dulabon et al., 2000) and neuronal migration has been shown to be disrupted *in vivo* by the CR-50 antibody (Nakajima et al., 1997). The C-terminal region (R7-C; 80 kDa) has been shown to be involved the secretion of Reelin, as well as its proper folding (de Bergeyck et al., 1997; Jossin et al., 2004), and for downstream signaling efficacy (Nakano et al., 2007).

a role of this protease in NMDAR-independent LTP induced cleavage of Reelin (Trotter et al., 2014). In cell culture, Reelin cleavage between R6–7 by tPA was blocked by serpin E1 inhibitor (Krstic et al., 2012). Our cell-free conditions in which we incubated tPA with Reelin for 45 min also produced increased the N-R6 fragment (370 kDa), which was blocked with Plasminogen activator inhibitor (PAI-1; serpin E1) and diisoporpyl fluorophosphates (a serine protease inhibitor), but not blocked by Aprotinin or CR-50 (an antibody that binds in the N-terminal region of Reelin; D'Arcangelo et al., 1997; Trotter et al., 2014). Similarly, metalloproteases meprin α and β cleave Reelin between the R6 and R7 repeats (Sato et al., 2016). However, neither tPA knock-out mice (Trotter et al., 2014) nor meprin β knock-out mice (Sato et al., 2016) demonstrate differences in basal levels of full length Reelin or Reelin fragments, suggesting that combinations of proteases are involved in constitutive Reelin levels and proteolysis. Furthermore, Reelin proteolysis may be important in activity-dependent or pathological conditions.

Much of what is known about signaling abilities of specific Reelin domains comes from research on the canonical Reelin-lipoprotein-Dab1 pathway (**Figure 1**). In support of the importance of the middle R3–6 fragment in lipoprotein receptor binding, cleavage within the R3 repeat has recently been shown to decrease Dab1 phosphorylation (Kohno et al., 2009; Koie et al., 2014). However, the other fragments have also been suggested to be vital for normal Reelin signaling. For example, the N-R2 fragment has been shown to bind to alpha$_3$beta$_1$-integrins (Dulabon et al., 2000) and the CR-50 antibody can disrupt *in vivo* neuronal migration (Nakajima et al., 1997). The C-terminal region (R7-C) has been suggested to be involved in Reelin secretion, folding (de Bergeyck et al., 1997; Jossin et al., 2004), and signaling efficacy (Nakano et al., 2007), although no known receptors have been identified for R7-C binding. Recently, Kohno et al. (2015), have shown that the C-terminal region is critical in postnatal cerebral cortex development but not in embryonic stages. Further research is needed to fully elucidate the importance of these specific fragments in normal and pathological conditions.

REELIN AND NEUROPSYCHIATRIC/ NEURODEGENERATIVE DISORDERS

Support for the role of Reelin proteolysis in human disease has been found in both neuropsychiatric and neurodegenerative disorders. For example, the N-R2 fragment is increased in AD and frontotemporal dementia patients when compared to non-demented patients (Sáez-Valero et al., 2003; Botella-López et al., 2006). In patients with confirmed diagnosis for depression and bipolar disorder, the N-R2 fragment is found to be decreased in blood samples, while for schizophrenia patients the N-R6 fragment is increased (Fatemi et al., 2001). Reelin may also play a role in seizure control: epilepsy models have altered Reelin processing (Tinnes et al., 2011, 2013; Kaneko et al., 2016), which may be MMP-dependent. These differences in Reelin fragment levels point to an importance in Reelin levels and proteolytic dysfunction in disease states.

REELIN AND AD PATHOETIOLOGY

In AD, loss of synapses and neurons is accompanied neuropathologically by amyloid deposits composed of the Amyloid beta (Aβ) peptide, and neurofibrillary tangles composed of modified versions of the tau protein (Trojanowski and Lee, 2002; Schellenberg and Montine, 2012; Sheng et al., 2012). Exogenous Aβ application and endogenous Aβ aggregates block various forms of synaptic plasticity and inhibit memory formation and retrieval (Klyubin et al., 2005; Selkoe, 2008; Talantova et al., 2013). Hyperphosphorylated forms of tau are also associated with the disruption of synaptic plasticity, learning and memory (Trojanowski and Lee, 2002; Santacruz et al., 2005; Lasagna-Reeves et al., 2011, 2012; Shipton et al., 2011). Altered Reelin signaling has been linked to AD through analyses of human brain samples (Herring et al., 2012; Notter and Knuesel, 2013), and animal models connecting Reelin to the processes of amyloid accumulation (Chin et al., 2007; Kocherhans et al., 2010; Pujadas et al., 2014) and to tau phosphorylation (Ohkubo et al., 2003; Herz and Chen, 2006; Kocherhans et al., 2010; Cuchillo-Ibáñez et al., 2013). In addition, Reelin signaling has been associated with human AD synaptic dysfunction in a non-targeted transcriptomic approach (Karim et al., 2014), and the Reelin gene was associated with AD pathological findings in elderly controls in a non-targeted genomic approach (Kramer et al., 2011). Finally, two of the strongest genetic risk factors for AD, Apolipoprotein E (APOE) and clusterin (APOJ), encode proteins that bind to the Reelin receptors (Reddy et al., 2011; Tapia-González et al., 2011).

These lines of research have led to the investigation of possible mechanisms for how Reelin could specifically affect AD. Reelin may modify amyloid levels by directly interacting with amyloid precursor protein (APP; Hoe et al., 2009) or altering APP metabolism to decrease the generation Aβ (Rice et al., 2013; Pujadas et al., 2014). Reelin also causes GSK3β inhibition (Beffert et al., 2002), which suppresses tau hyperphosphorylation (Ohkubo et al., 2003). In mouse models of AD, overexpressing Reelin prevented AD pathological changes (Pujadas et al., 2014), and lowering levels of Reelin accelerated Aβ deposition and the synaptic dysfunction caused by the presence of amyloid (Kocherhans et al., 2010; Lane-Donovan et al., 2015). In addition to these effects on the neuropathologic accumulations in AD brain, several lines of evidence suggest that Reelin and Aβ have antagonistic effects on neuronal survival and signaling. These findings include reduction of Reelin and Reelin signaling in an AD mouse model (Mota et al., 2014), electrophysiological measures of Reelin and Aβ effects on hippocampal brain slices (Durakoglugil et al., 2009), and behavioral studies in AD mouse models with altered levels of Reelin (Pujadas et al., 2010; Lane-Donovan et al., 2015).

REELIN AS A THERAPEUTIC TARGET

As mentioned above, different Reelin fragments are altered in neuropsychiatric and degenerative diseases. These alterations

may be an indication of disruption in Reelin processing and may be useful in identifying biomarkers for disease states. Reelin has been shown to be sequestered by Aβ plaques in an age-dependent manner (Knuesel et al., 2009; Doehner and Knuesel, 2010; Kocherhans et al., 2010; Stranahan et al., 2011). Removal of Reelin from the synapse can alter many Reelin-dependent functions, causing abnormal cellular migration, dendritic morphology atrophy and deficits in synaptic plasticity (Herz and Chen, 2006; Rogers and Weeber, 2008; Bu, 2009). Given the progressive memory decline seen in AD patients, it is possible that the sequestering of Reelin by the amyloid plaques can alter its normal regulation via cleavage mechanisms and its normal enhancement of learning and memory. It is interesting to note that crossing a transgenic mouse that overexpresses Reelin with an AD mouse model protects from amyloid plaque formation and rescues learning and memory deficits when compared to the AD mice (Pujadas et al., 2014), while decreasing Reelin in AD models accelerates plaque formation and increases tau hyperphosphorylation (Kocherhans et al., 2010). Interestingly, a novel inducible Reelin knockout mouse line has revealed that adult knockdown of Reelin expression results in no discernable differences in normal learning and memory and actually enhances late LTP (Lane-Donovan et al., 2015). When these Reelin knockdown mice were crossed with Tg2576 AD mice they did not cause an increase Aβ pathology; however, these mice showed poorer learning in the hidden platform water maze and deficits in the 24 h probe test when compared to controls (Lane-Donovan et al., 2015). These results support the importance of Reelin signaling in normal cognitive function and shows that a loss of Reelin signaling in an AD mouse model increases cognitive dysfunction.

Introduction of exogenous Reelin into the brain can have surprisingly profound effects on synaptic plasticity and cognition. Hippocampal injection(s) of Reelin and its fragments has demonstrated significant improvements in models of Reelin deficiency (Rogers et al., 2013), Angelman syndrome (Hethorn et al., 2015), and schizophrenia (Ishii et al., 2015). Remarkably, exogenous Reelin also enhanced learning and memory as well as increased synaptic plasticity in wild-type mice (Rogers et al., 2011). Thus, therapeutic approaches to promoting Reelin signaling could be useful in protecting synapse function and survival in a range of disorders. This work would require a better understanding of which domains of Reelin are necessary for the regulated Reelin signaling, and assays for examining whether new Reelin-based therapies promote receptor clustering, intracellular signaling, synapse stabilization, and neuronal protection. Although more work is required to fully understand the function of each of these Reelin fragments, the current research points to a therapeutic potential for altering specific Reelin fragments in treating neuronal dysfunction and cognitive deficits in neurodegenerative and neuropsychiatric disorders.

AUTHOR CONTRIBUTIONS

ALL wrote the review and generated the figures. EJW and GWR edited the content and structure of the review. Each author contributed ideas of topics for inclusion.

ACKNOWLEDGMENTS

This work was supported by R01 AG035379-05 for EJW and GWR.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

MINI REVIEW
published: 09 May 2016
doi: 10.3389/fncel.2016.00122

New Insights into Reelin-Mediated Signaling Pathways

Gum Hwa Lee[1]* *and Gabriella D'Arcangelo*[2]

[1] College of Pharmacy, Chosun University, Gwangju, South Korea, [2] Department of Cell Biology and Neuroscience, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

Reelin, a multifunctional extracellular protein that is important for mammalian brain development and function, is secreted by different cell types in the prenatal or postnatal brain. The spatiotemporal regulation of Reelin expression and distribution during development relates to its multifaceted function in the brain. Prenatally Reelin controls neuronal radial migration and proper positioning in cortical layers, whereas postnatally Reelin promotes neuronal maturation, synaptic formation and plasticity. The molecular mechanisms underlying the distinct biological functions of Reelin during and after brain development involve unique and overlapping signaling pathways that are activated following Reelin binding to its cell surface receptors. Distinct Reelin ligand isoforms, such as the full-length protein or fragments generated by proteolytic cleavage differentially affect the activity of downstream signaling pathways. In this review, we discuss recent advances in our understanding of the signaling transduction pathways activated by Reelin that regulate different aspects of brain development and function. A core signaling machinery, including ApoER2/VLDLR receptors, Src/Fyn kinases, and the adaptor protein Dab1, participates in all known aspects of Reelin biology. However, distinct downstream mechanisms, such as the Crk/Rap1 pathway and cell adhesion molecules, play crucial roles in the control of neuronal migration, whereas the PI3K/Akt/mTOR pathway appears to be more important for dendrite and spine development. Finally, the NMDA receptor (NMDAR) and an unidentified receptor contribute to the activation of the MEK/Erk1/2 pathway leading to the upregulation of genes involved in synaptic plasticity and learning. This knowledge may provide new insight into neurodevelopmental or neurodegenerative disorders that are associated with Reelin dysfunction.

Keywords: brain development, neuronal migration, dendrites, synaptogenesis, signal transduction

OPEN ACCESS

Edited by:
Hansen Wang,
University of Toronto, Canada

Reviewed by:
Kazunori Nakajima,
Keio University School of Medicine, Japan
Lavinia Alberi,
University of Fribourg, Switzerland
Eric C. Olson,
SUNY Upstate Medical University, USA

***Correspondence:**
Gum Hwa Lee
gumhwalee@chosun.ac.kr

Received: *04 March 2016*
Accepted: *27 April 2016*
Published: *09 May 2016*

Citation:
Lee GH and D'Arcangelo G (2016) New Insights into Reelin-Mediated Signaling Pathways. Front. Cell. Neurosci. 10:122. doi: 10.3389/fncel.2016.00122

INTRODUCTION

Reelin is an extracellular glycoprotein that controls diverse aspects of mammalian brain development and function (D'Arcangelo, 2014). The most prominent activity of Reelin is the control of neuronal migration and cellular layer formation in the developing brain. This is evident from anatomical studies of *reeler* mutant mice that lack Reelin expression (Lambert de Rouvroit and Goffinet, 1998). These mutants exhibit a neurological phenotype characterized by ataxia and a typical "reeling" gate. Anatomically, their brains exhibit widespread neuronal lamination defects due to the failure of radially-migrating neurons to reach their destination in the developing forebrain, and cerebellar hypoplasia, which is likely due to the failure of Purkinje

cells to form a cellular layer (Goffinet, 1983; Miyata et al., 1997). Similar phenotypes are observed in human patients carrying *REELIN* homozygous mutations, resulting in lissencephaly with cerebellar hypoplasia (Hong et al., 2000).

In addition to controlling neuronal migration in the prenatal brain, Reelin plays important roles in the postnatal and adult brain, promoting the maturation of dendrites, synaptogenesis, synaptic transmission and plasticity, thus modulating the formation and function of synaptic circuits. This view is supported not only by animal studies involving heterozygous *reeler* mice, which model some behavioral dysfunction similar to schizophrenia (Costa et al., 2002), but also by recent human genetic studies identifying heterozygous *REELIN* mutations in lateral temporal epilepsy (Dazzo et al., 2015), and pointing to *REELIN* as a risk factor in autism (De Rubeis et al., 2014). Furthermore, accumulating evidence that Reelin signaling antagonizes the toxic effects of β-amyloid at the synapse, underscores the potential relevance of this "developmental" factor for neurodegenerative disorders (Durakoglugil et al., 2009; Krstic et al., 2012; Pujadas et al., 2014).

To foster a better understanding of the mechanisms of development and disease, in this review we focus on recent advances in our knowledge of the signaling transduction pathways that regulate the different biological activities of Reelin in the brain.

REELIN EXPRESSION AND CLEAVAGE

The spatiotemporal regulation of Reelin expression underlies its multifaceted roles in brain development. During the embryonic development of forebrain structures Cajal-Retzius cells secrete high levels of Reelin in the marginal zone, thus regulating neuronal migration and cellular layer formation (D'Arcangelo et al., 1995; Ogawa et al., 1995). These cells begin to die shortly after birth and disappear from the neocortex once neuronal migration is completed. In the hippocampus, however, residual Cajal-Retzius continue to secrete Reelin at early postnatal days, affecting aspects of development such as axonal or dendrite branching and maturation (Del Río et al., 1997; Niu et al., 2004; Kupferman et al., 2014). As postnatal development continues, the expression of Reelin becomes predominantly localized to a subset of GABAergic interneurons that are positioned throughout cortical and hippocampal cell layers (Alcántara et al., 1998; Pesold et al., 1998). Albeit at reduced levels, these interneurons continue to express Reelin in the juvenile and adult forebrain. The significance of this late postnatal and adult pattern of expression is likely related to the modulation of synaptic activity and plasticity (Weeber et al., 2002; Beffert et al., 2005; Pujadas et al., 2010; Trotter et al., 2013).

The mouse full-length Reelin protein is approximately 385 kDa and is 95.2% identical to the human protein (D'Arcangelo et al., 1995). The main body of the protein is composed of eight unique repeats (R), each centered around an epidermal growth factor (EGF)-like cysteine pattern that is typical of extracellular proteins (**Figure 1**). At the N terminus there is a signal peptide and a small region of similarity with F-spondin, whereas at the C terminus there is a small carboxy-terminal region (CTR) that is positively charged. The presence of the signal peptide indicated that Reelin is an extracellular protein. Indeed, it is readily detected in the culture medium of Reelin-expressing cells (D'Arcangelo et al., 1997). Secretion is essential for function, and mutations that interfere with secretion cause a *reeler* phenotype identical to that resulting from null mutations (D'Arcangelo et al., 1997; de Bergeyck et al., 1997). After secretion, full-length Reelin is cleaved by metalloproteases at two specific sites, generating three large fragments, an N-terminal (Nt = N-R2), a central (C = R3-R6), and a C-terminal (Ct = R6-CTR) fragment (**Figure 1**). The C fragment alone is sufficient to activate intracellular signaling and to induce layer formation in cortical slice cultures (Jossin et al., 2004; Yasui et al., 2007). However, the full-length protein is more potent than the C fragment, presumably due to the presence of the Nt region, which promotes aggregation, and the CTR, which promotes proper folding (Utsunomiya-Tate et al., 2000; Kubo et al., 2002; Nakano et al., 2007; Kohno et al., 2015). Recent studies identified the cleavage sites that produce the three major Reelin

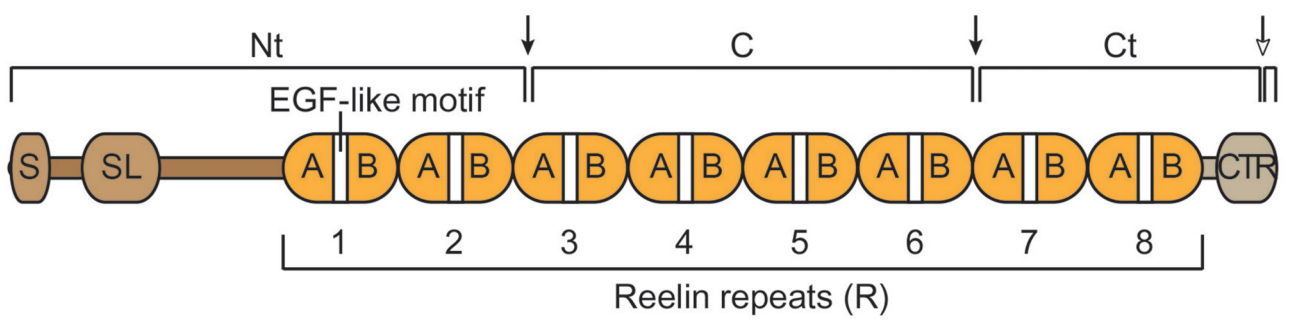

FIGURE 1 | Schematic structure of the Reelin protein and its cleavage fragments. Reelin contains a signal peptide (S), an F-spondin-like domain (SL), eight consecutive Reelin repeats (R) each harboring an epidermal growth factor (EGF)-like motif that separates two subdomains (A and B), and a positively charged carboxy-terminal region (CTR). The full-length protein is cleaved by extracellular metalloproteases at specific sites (arrows), an N-terminal (Nt) site within R3 and a C-terminal site between R6 and R7. These two cleavage events generate three large fragments, an N-terminal (Nt), a central (C) and a C terminal (Ct) fragment. An additional cleavage event (empty head arrow) occurs within the CTR (WC) and generates a small carboxy-terminal peptide.

fragments (Koie et al., 2014; Sato et al., 2016) and demonstrated that the Nt cleavage affects the duration and the range of Reelin signaling activity in the developing cortex (Koie et al., 2014). Further studies are needed to identify proteases that carry out these processing events *in vivo*. In addition, recent studies further identified another cleavage site within the CTR (WC). Cleavage at this site releases a six amino acid carboxy-terminal peptide, reducing signaling activity and hindering dendrite development in the postnatal neocortex (Kohno et al., 2015).

Taken together, the evidence so far indicates that Reelin processing downregulates the activity of the full-length protein; however cleavage events also produce diffusible fragments that potentially stimulate signaling activity away from the site of secretion (Jossin et al., 2007).

REELIN RECEPTORS

The best-characterized Reelin receptors are the apolipoprotein E receptor 2 (ApoER2, also called LRP8) and the very low-density lipoprotein receptor (VLDLR). These proteins belong to the low-density lipoprotein receptor (LDLR) family. They have partial functional redundancy and play an essential role in Reelin-mediated neuronal migration based on the observation that double knockout mice display a *reeler*-like phenotype (Trommsdorff et al., 1999). ApoER2 and VLDLR bind Reelin with high affinity and internalize the ligand in endocytic vesicles, leading to the activation of downstream signaling molecules (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Strasser et al., 2004; Yasui et al., 2010). After the signal is transduced, some receptor molecules recycle to the membrane whereas others are targeted for lysosomal degradation (Hong et al., 2010). A Reelin domain contained within the C fragment and including the Lys2467 residue is essential for ApoER2/VLDLR interaction, signal transduction and cortical layer formation (Jossin et al., 2004; Yasui et al., 2007). Despite functional overlap, ApoER2 and VLDLR play distinct roles in neuronal migration due, in part, to their different expression pattern. In the developing neocortex VLDLR is expressed almost exclusively in apical processes of migrating neurons at the top of the cortical plate where it mediates a mode of migration known as somal or terminal translocation, whereas ApoER2 is also expressed in the intermediate zone where it likely promotes the transition from multipolar to bipolar morphology and early stages of radial migration (Hirota et al., 2015). Other reported differences between the two receptors include their ability to internalize Reelin at different rate and in distinct lipid compartments, thus likely differentially affecting signal transduction machineries (Duit et al., 2010).

Other transmembrane proteins that have been proposed to function as Reelin receptors include β1-containing integrins, which were first reported to bind Reelin *in vitro* (Dulabon et al., 2000). However, genetic knock out studies later demonstrated that β1 integrins are required for radial glia scaffold formation rather than for neuronal migration *per se* (Belvindrah et al., 2007). Even though their function is not essential, possibly due to redundancy with other cell adhesion molecules, *in utero* electroporation studies suggest that β1 integrins contribute to corticogenesis as downstream effectors. Reelin signaling was shown to alter integrin-dependent cell adhesion by downregulating α3 integrin levels in the cortical plate (Sanada et al., 2004), and by activating integrin α5β1, thus promoting the anchoring of leading processes to the fibronectin-rich marginal zone (Sekine et al., 2012). It should be noted that in this model integrins do not bind Reelin directly and therefore do not function as receptors. Recently, another study suggested a direct interaction between Reelin and EphB tyrosine kinase receptors. The Nt region of Reelin was reported to bind EphB and activate forward signaling in neurons (Bouché et al., 2013). However, EphB-deficient mice display only a very mild migration phenotype, suggesting that they do not play a major role during prenatal brain development. Their involvement in postnatal functions of Reelin remains to be elucidated.

Taken together, genetic and biochemical data so far support the notion that ApoER2 and VLDLR are the major Reelin receptors in the developing brain.

REELIN SIGNAL TRANSDUCTION IN THE CONTROL OF NEURONAL MIGRATION

Disabled-1 (Dab1) is an intracellular adaptor protein that is essential for Reelin signal transduction. This protein binds the cytoplasmic tail of lipoprotein receptors, including ApoER2 and VLDLR (Trommsdorff et al., 1999) and upon Reelin binding, becomes phosphorylated on tyrosine residues by Src-family kinases (SFKs) Fyn and Src (Howell et al., 1999a; **Figure 2A**). These kinases are themselves upregulated in a Dab1-dependent way via a positive feedback mechanism (Arnaud et al., 2003; Bock and Herz, 2003). Dab1 phosphorylation is required for neuronal migration, as demonstrated by the observation that phospho-mutant Dab1 mice (Howell et al., 2000), double *Fyn/Src* knockout mice (Kuo et al., 2005), as well as spontaneous or genetically engineered *Dab1* knockout mice (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997; Yoneshima et al., 1997; Kojima et al., 2000) all show similar *reeler*-like phenotypes. Dab1 signaling is rapidly downregulated by a mechanism that involves the ubiquitination of phospho-Dab1 by the E3 ubiquitin ligase component Cullin 5, and its degradation by the proteasome system (Feng et al., 2007).

Genetic studies demonstrated that Dab1, and thus Reelin signaling, is specifically required for a specific mode of radial migration termed somal or terminal translocation, but not for glial-guided locomotion (Franco et al., 2011). The molecular mechanism of translocation involves the recruitment of Crk adaptor proteins, which bind phospho-Dab1 and cause the activation of the GTP exchange factor (GEF) C3G, and the subsequent activation of the Rap1 GTPase (Franco et al., 2011; Jossin and Cooper, 2011; **Figure 2A**). Consistently, double *Crk*/*CrkL* mutant mice display a *reeler*-like cortical phenotype (Park and Curran, 2008). The Crk/C3G/Rap1 pathway ultimately promotes the interaction between migrating neurons and Reelin-

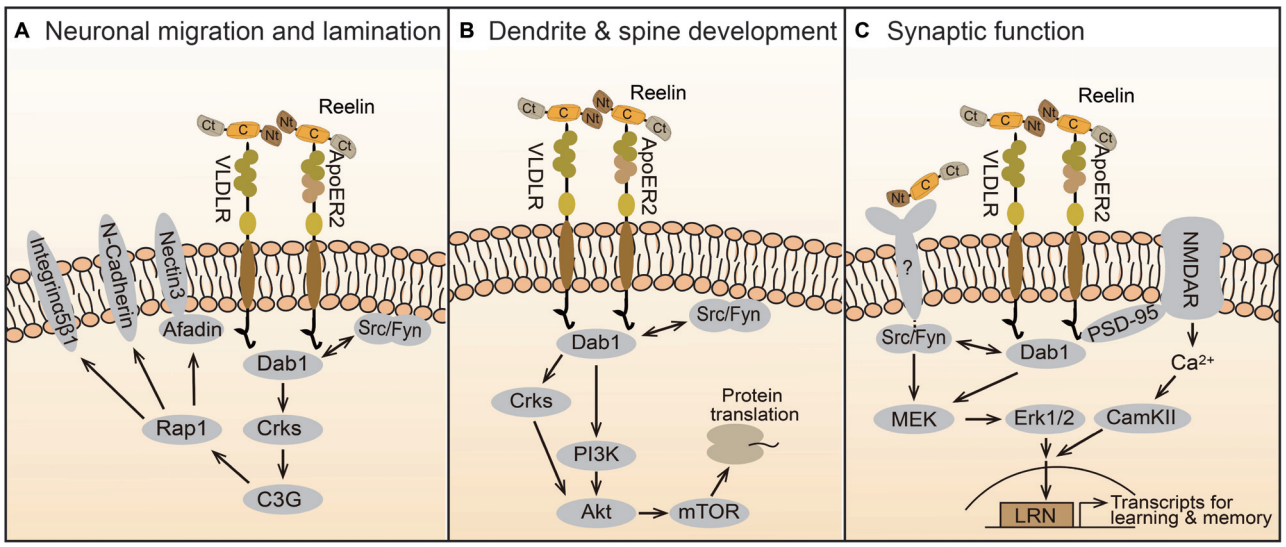

FIGURE 2 | Reelin signaling mechanisms in brain development and function. Reelin is secreted as a full-length protein that contains three large cleavable domains, an Nt, a C, and a Ct domain. The central domain binds to ApoER2 and VLDLR receptors, which internalize the ligand and transduce the Reelin signal by activating Src/Fyn kinases that phosphorylate the adaptor protein Dab1. Downstream of this canonical pathway, distinct signaling cascades regulate specific biologic activities at different times during brain development. **(A)** Prenatally, Reelin controls neuronal migration and cortical layer formation through the Crk/C3G/Rap1 pathway. This signaling cascade regulates the function of cell adhesion molecules, including nectin3, N-Cadherin, and Integrin α5β1, which facilitate somal translocation and cellular layer formation. **(B)** During early postnatal development, the Crk adaptor proteins and the PI3K-Akt-mTOR pathway contribute to Reelin activity by promoting protein translation, dendrite outgrowth and spine development. **(C)** In the late postnatal and adult brain Reelin affects synaptic function and plasticity. This activity is mediated in part by ApoER2, which interacts with the NMDAR through PSD-95, causing Ca^{2+} influx and the activation of CamKII. An unknown receptor also mediates the activation of the MEK-Erk1/2 pathway by Src/Fyn kinases. Together these signaling pathways promote synaptic activity and plasticity through the induction of immediate-early genes involved in learning and memory such as those containing LRN enhancers.

producing Cajal-Retzius cells through adhesion molecules such as nectins 1/3 and N-Cadherin, enabling neuronal translocation and inside-out layer formation (Gil-Sanz et al., 2013; **Figure 2A**). Given the enrichment of ApoER2 and VLDLR in the apical processes of migrating neurons near the marginal zone, both these receptors are likely to mediate the signal transduction that promotes translocation (Hirota et al., 2015). In addition, Reelin-Dab1 signaling through Rap1 and N-Cadherin affects the orientation of migrating neurons undergoing the transition from multipolar to bipolar morphology in the intermediate zone, before initiating radial migration into the cortical plate (Jossin and Cooper, 2011). This migration step may be mediated preferentially by ApoER2, since this is the only receptor that is expressed in the intermediate zone (Hirota et al., 2015).

In addition to Crks and Rap1, biochemical studies identified several molecules that may be involved in Reelin-dependent neuronal migration. These include proteins that regulate cytoskeletal dynamics and cell motility, such as Lis1, Nckβ and N-WASP (Assadi et al., 2003; Pramatarova et al., 2003; Suetsugu et al., 2004), and proteins that downregulate Rap1 due to their GTPase activating protein (GAP) activity. Among Dab1-interacting proteins Lis1, the product of the *PAFAH1b1* gene that is responsible for human lissencephaly type I, may be particularly relevant to cortical development. Lis1 binding to phospho-Dab1 is Reelin-dependent, and genetic interaction between *Dab1* and *PAFAH1b1* demonstrates a functional relationship between these proteins (Assadi et al., 2003). Furthermore, Lis1-interacting *PAFAH1b* alpha subunits bind specifically to VLDLR, potentially promoting the interaction between Lis1 and Dab1 downstream of this receptor (Zhang et al., 2007). Lis1 then affects cytoskeletal dynamics necessary for radial migration through the dynein motor complex (Wynshaw-Boris and Gambello, 2001). Additionally, Dab2IP, a Dab1-binding protein that functions as a Rap GAP, as well as Rap1GAP, were shown to affect neuronal migration in the neocortex (Franco et al., 2011; Jossin and Cooper, 2011; Lee et al., 2012; Qiao et al., 2013). Even though a direct involvement of Rap GAPs in Reelin signaling has not been established, it is likely that this class of proteins regulates Rap1 activity, balancing the GEF activity of C3G and thus enabling proper neuronal orientation and migration through the cortical plate.

REELIN SIGNAL TRANSDUCTION IN THE CONTROL OF DENDRITE AND SPINE DEVELOPMENT

Dendrite outgrowth is disrupted in homozygous *reeler* mice. Dendritic defects are also apparent in immature hippocampal or cortical cultures isolated from mutant mice, but not in mature cultures (Niu et al., 2004; Jossin and Goffinet, 2007; MacLaurin et al., 2007). Since Reelin treatment rescued these defects, these *in vitro* studies first demonstrated that Reelin directly promotes dendrite development. Following studies further demonstrated

that Reelin enables initial dendritic outgrowth by promoting the extension of the Golgi apparatus into apical dendrites (Matsuki et al., 2010), and then orienting and stabilizing the leading processes in the marginal zone (Chai et al., 2015; Kohno et al., 2015; O'Dell et al., 2015). The signal transduction machinery that mediates the activity of Reelin on dendrite development involves the canonical pathway that also controls neuronal migration, including ApoER2/VLDLR, Dab1, SFKs and Crks (Niu et al., 2004; Park and Curran, 2008). Downstream of Dab1, the signaling mechanism that affects dendrite development likely involves the Phosphoinositide 3-kinase (PI3K) and Akt (**Figure 2B**). Earlier studies demonstrated that Reelin activates PI3K and Akt *in vitro* in a manner that is dependent on SFK activity and Dab1 phosphorylation (Beffert et al., 2002; Bock et al., 2003). PI3K may be activated through direct interaction between the regulatory subunit p85α and Dab1 (Bock et al., 2003). Akt is likely activated, at least in part, by the classic PI3K/PDK cascade, however, *in vivo* studies demonstrated that the Crk adaptor proteins are required for Reelin-induced Akt phosphorylation, placing the kinase functionally downstream of these adaptors (Park and Curran, 2008). Downstream of Akt, mTOR and further downstream proteins such as p70S6K and ribosomal protein S6 are robustly induced by Reelin treatment in neuronal cultures and likely contribute to dendrite growth (Jossin and Goffinet, 2007; Ventruti et al., 2011; **Figure 2B**).

Other molecules that have been implicated in Reelin-dependent dendrite outgrowth include the amyloid precursor protein (APP; Hoe et al., 2009), which binds Dab1 via its cytoplasmic tail (Homayouni et al., 1999; Howell et al., 1999b), and the Cdc42/Rac1 guanine nucleotide exchange factor αPIX, which affects dendritic Golgi translocation (Meseke et al., 2013). In addition to outgrowth, dendrite compartmentalization is an important aspect of maturation that is affected by Reelin. In the hippocampus, distal apical dendrites of pyramidal neurons express specific ion channels. Recent studies demonstrated that Dab1/SFK signaling is required for the molecular identity of this dendritic compartment, which regulates the processing of information in hippocampal circuits (Kupferman et al., 2014). Reelin signaling also promotes dendritic spine formation and growth in the cortex and hippocampus of juvenile mice (Niu et al., 2008; Pujadas et al., 2010; Iafrati et al., 2014). The signaling mechanism that underlies this function involves the canonical pathway and possibly additional signaling molecules such as RasGRF1/CamMKII (DiBattista et al., 2015; Kim et al., 2015). Finally, the molecular composition of the dendritic spines is affected by Reelin. Specifically, Reelin promotes the maturation of spines by regulating the NMDA receptor (NMDAR) subunit composition via an unidentified mechanism (Groc et al., 2007; Ventruti et al., 2011).

REELIN SIGNALING AND THE MODULATION OF SYNAPTIC FUNCTION

Heterozygous *reeler* mice exhibit altered hippocampal synaptic plasticity and multiple behavioral abnormalities, such as defects in executive function and contextual fear conditioning learning (Brigman et al., 2006; Krueger et al., 2006; Qiu et al., 2006). Early culture studies demonstrated that Reelin potently enhances hippocampal long-term potentiation (LTP), a cellular mechanism underlying learning and memory, and this effect is dependent on the presence of both, VLDLR and ApoER2 (Weeber et al., 2002). A specific splicing variant of ApoER2 was required for Reelin-induced LTP enhancement and memory formation *in vivo* (Beffert et al., 2005). Mechanistically, it was shown that this ApoER2 variant interacts with the NMDAR through PSD-95, and this complex mediates Reelin–induced Ca^{++} influx through the NMDAR (Beffert et al., 2005; Chen et al., 2005; **Figure 2C**). Genetic studies later demonstrated that Dab1 is also required for Reelin-induced enhancement of hippocampal LTP and for hippocampal-dependent behavioral tasks (Trotter et al., 2013). This study also demonstrated that postnatal Dab1 loss affects basal and plasticity-induced Erk1/2 signaling, suggesting a cross-talk with canonical Reelin signaling. Indeed, Reelin was shown to induce Erk1/2 signaling in a SFK-dependent manner in cultured neurons (Lee et al., 2014). Surprisingly, however, Reelin-induced Erk1/2 phosphorylation did not require the activity of ApoER2 and VLDLR, and it was only partially dependent on Dab1, suggesting the involvement of an unidentified receptor triggering a non-canonical pathway (**Figure 2C**). Erk1/2 activation leads to the expression of synaptic immediate-early genes (IEGs), and thus potentially affects synaptic function (Lee et al., 2014). Others further showed that Reelin induces IEGs expression via a novel enhancer element named LRN (LRP8-Reelin-Neuronal), and that these events affect associative learning. In this model, interaction between the ApoER2 (LRP8) and the NMDAR triggers Ca^{++} influx, Erk1/2 signaling and CREB-dependent IEGs transcription (Telese et al., 2015). In addition, they reported that proteolytical cleavage of ApoER2 by γ-secretase is a crucial component of the synapse-to-nuclear signaling triggered by Reelin. Interestingly, Notch1, another γ-secretase substrate, was also recently shown to contribute to Reelin-mediated synaptic potentiation by interacting with ApoER2 and NMDAR, and stimulating Erk1/2 activity and CREB-dependent transcription (Brai et al., 2015).

In addition to its well-documented postsynaptic effects, Reelin also acts presynaptically, causing a rapid enhancement of spontaneous neurotransmitter release. This effect is due to the mobilization of VAMP7-containing synaptic vesicles, and requires canonical ApoER2/VLDLR receptors, PI3K and Ca^{++} signaling (Hellwig et al., 2011; Bal et al., 2013). Despite robust pre- and postsynaptic effects, acute deletion of the *Reelin* gene in adult mice does not result in impaired synaptic plasticity. However, it renders the adult brain strikingly sensitive to amyloid-induced synaptic suppression, leading to profound learning disabilities (Lane-Donovan et al., 2015). Although specific molecular and physiological mechanisms remain to be further elucidated, these findings indicate that Reelin has the potential to modulate synaptic activity and thus affect memory formation in the adult and aging brain.

AUTHOR CONTRIBUTIONS

GHL wrote the first draft of the manuscript and made the figures. GD wrote and revised the manuscript.

ACKNOWLEDGMENTS

This study was supported by a research fund from Chosun University (2015).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 30 June 2016
doi: 10.3389/fncel.2016.00166

Canonical and Non-canonical Reelin Signaling

Hans H. Bock and Petra May*

Clinic of Gastroenterology and Hepatology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

Reelin is a large secreted glycoprotein that is essential for correct neuronal positioning during neurodevelopment and is important for synaptic plasticity in the mature brain. Moreover, Reelin is expressed in many extraneuronal tissues; yet the roles of peripheral Reelin are largely unknown. In the brain, many of Reelin's functions are mediated by a molecular signaling cascade that involves two lipoprotein receptors, apolipoprotein E receptor-2 (Apoer2) and very low density-lipoprotein receptor (Vldlr), the neuronal phosphoprotein Disabled-1 (Dab1), and members of the Src family of protein tyrosine kinases as crucial elements. This core signaling pathway in turn modulates the activity of adaptor proteins and downstream protein kinase cascades, many of which target the neuronal cytoskeleton. However, additional Reelin-binding receptors have been postulated or described, either as coreceptors that are essential for the activation of the "canonical" Reelin signaling cascade involving Apoer2/Vldlr and Dab1, or as receptors that activate alternative or additional signaling pathways. Here we will give an overview of canonical and alternative Reelin signaling pathways, molecular mechanisms involved, and their potential physiological roles in the context of different biological settings.

Keywords: neuronal development, synaptic plasticity, Alzheimer disease, amyloid precursor protein, integrin, Eph receptor, coreceptor, protein kinase

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers, The State University of New Jersey, USA

Reviewed by:
Shin-ichi Hisanaga, Tokyo Metropolitan University, Japan
Brian W. Howell, SUNY Upstate Medical University, USA

****Correspondence:***
Hans H. Bock
hans.bock@med.uni-duesseldorf.de

Received: *02 March 2016*
Accepted: *08 June 2016*
Published: *30 June 2016*

Citation:
Bock HH and May P (2016) Canonical and Non-canonical Reelin Signaling. Front. Cell. Neurosci. 10:166. doi: 10.3389/fncel.2016.00166

PHYSIOLOGICAL ROLES OF REELIN

Reelin is best known for its role in the developing mammalian cerebral cortex, where it is secreted by Cajal-Retzius cells in the marginal zone and orchestrates the arrangement of postmitotic cortical neurons in an inside-out manner, meaning that younger neurons are located more superficially than the earlier-born neurons. This process is severely disturbed in the spontaneous mouse mutant *reeler*, where disruption of the gene encoding Reelin leads to an approximate inversion of the cortical layering. Other laminated brain structures are affected as well, resulting in a typical "*reeler* phenotype", which includes the eponymous reeling gait as a consequence of cerebellar hypoplasia. Characteristic positioning defects of pyramidal and granule neurons in the hippocampus are found as well (reviewed e.g., by Rice and Curran, 2001; Tissir and Goffinet, 2003; D'Arcangelo, 2005). In addition to regulating layer formation in the neocortex and other laminated brain structures, Reelin functions in the developing and adult brain, where Reelin is highly expressed by GABAergic interneurons in the forebrain and by cerebellar granule neurons (Drakew et al., 1998; Pesold et al., 1998; Ramos-Moreno et al., 2006; Wierenga et al., 2010; Pohlkamp et al., 2014). Its functions include the regulation of filopodia formation, dendrite outgrowth, spine formation and synaptogenesis as well as modulation of synaptic plasticity and neurotransmitter release (reviewed by D'Arcangelo, 2005; Herz and Chen, 2006; Levenson et al., 2008; Forster et al., 2010; Levy et al., 2014).

The study of mutant mice with defects in cortical layering has significantly contributed to our current understanding of corticogenesis (Lambert de Rouvroit and Goffinet, 1998; Hatten and Heintz, 2005; Ogden et al., 2016). However, although the gene affected in *reeler* mice has been identified more than 20 years ago (D'Arcangelo et al., 1995), our knowledge of how precisely Reelin exerts its diverse functions on neuronal positioning and differentiation on a cellular and molecular level is still imperfect (Caffrey et al., 2014). In accordance with its multiple roles during different developmental stages Reelin targets different cell types, including newborn and differentiated neurons, radial glial cells, astrocytes, and possibly neural stem cells (Forster et al., 2002; Kim et al., 2002; Gong et al., 2007; Lakomá et al., 2011; Brunne et al., 2013; Brunkhorst et al., 2015). Of note, many neuropsychiatric diseases have been associated with dysregulated Reelin expression, including schizophrenia, depression, autism, temporal lobe epilepsy, and neurodegenerative disease (Impagnatiello et al., 1998; Guidotti et al., 2000; Fatemi, 2001; Persico et al., 2001; Haas et al., 2002; Sáez-Valero et al., 2003; Botella-López et al., 2006; Knuesel, 2010; Folsom and Fatemi, 2013). Reelin-responsive cells outside the central nervous system remain mostly elusive, although significant amounts of Reelin are detected in plasma and various non-neuronal tissues (Ikeda and Terashima, 1997; Smalheiser et al., 2000; Kobold et al., 2002; Lugli et al., 2003; Botella-Lopez et al., 2008), and functional effects of Reelin on blood cells such as platelets (Tseng et al., 2014), endothelial cells (Ding et al., 2016), or pancreatic cancer cell lines (Sato et al., 2006) have been described.

TABLE 1 | Mouse mutants with a *reeler*-like phenotype.

Mouse	Gene		Reference
reeler-Jackson	*Reln*	spont.	Falconer (1951)
reeler-Orleans	*Reln*	spont.	de Bergeyck et al. (1997) Hirotsune et al. (1995)
reeler-sf transgene	*Reln*	fort.	Miao et al. (1994) D'Arcangelo et al. (1995)
Meox2-Cre-Reln$^{lox/lox}$	*Reln*	targ.	Lane-Donovan et al. (2015)
Apoer2$^{-/-}$; Vldlr$^{-/-}$	*Lrp8, Vldlr*	targ.	Trommsdorff et al. (1999)
yotari	*Dab1*	fort.	Sheldon et al. (1997) Yoneshima et al. (1997)
scrambler	*Dab1*	spont.	Sweet et al. (1996), Sheldon et al. (1997)
Dab1$^{-/-}$	*Dab1*	targ.	Howell et al. (1997)
Dab1$^{5F/5F}$	*Dab1*	targ.	Howell et al. (2000)
Src$^{-/-}$; Fyn$^{-/-}$	*Src, Fyn*	targ.	Kuo et al. (2005)
Nestin-Cre-Crk$^{lox/lox}$; CrkL$^{lox/lox}$	*Crk, CrkL*	targ.	Park and Curran (2008)

The analysis of brain phenotypes of mutant mice proved essential for the elucidation of a core Reelin signaling pathway with LDL receptor gene family members as canonical receptors. Mice lacking either Reelin (ligand, green), Apoer2 and Vldlr (receptor, orange), Dab1 (intracellular phosphoprotein, kinase switch and adapter protein, red), Fyn and Src (Src family nonreceptor tyrosine kinase, blue) or Crk and CrkL (adapter proteins, purple) display a reeler-like phenotype, which is defined by characteristic neuroanatomical defects of laminated brain structures. Inactivation of additional Reelin effector genes causes more circumscribed defects, depending on the degree or spatiotemporal pattern of inactivation, or indicating more specialized functions. Spontaneous (spont.) or targeted (targ.) gene deletions are indicated; reeler-transgene and yotari mice were the result of the fortuitous (fort.) insertion of a mutated Fos transgene (sf) into the Reln locus or a random mutation of the Dab1 gene during the generation of IP3R deficient mice, respectively.

TOWARDS A MOLECULAR UNDERSTANDING OF THE *REELER* PHENOTYPE: THE CORE REELIN SIGNALING CASCADE

The discovery of spontaneous or genetically engineered mutant mouse lines that copy the *reeler* phenotype (**Table 1**) in combination with biochemical approaches for identifying protein interactions proved instrumental in the discovery of a Reelin-dependent core signaling pathway (**Figure 1**) that underlies many of the established biological functions of Reelin in the developing and mature brain.

These include mice lacking the intracellular phosphoprotein Disabled-1 (Dab1) and compound mutant mice that lack both very low density lipoprotein receptor (Vldlr) and apolipoprotein E receptor 2 (Apoer2), which are two close relatives of the low density lipoprotein (LDL) receptor, the prototype of an endocytic receptor and founding member of the LDL receptor gene family (reviewed by Herz, 2001; Herz and Bock, 2002). This discovery is a prime example for the power of mouse genetics and came as a surprise, since so far lipoprotein receptors had not been connected with the transduction of extracellular signals via classical signaling cascades (Howell and Herz, 2001; May et al., 2003a).

By demonstrating that Reelin directly binds to the extracellular domains of Apoer2 and Vldlr (D'Arcangelo et al., 1999; Hiesberger et al., 1999), which interact with the protein interaction/phosphotyrosine-binding (PTB) domain of Dab1 via the tetra-amino-acid NPXY endocytosis motif within their intracellular tails (Howell et al., 1999b; Gotthardt et al., 2000), a linear signaling pathway was established that leads to the tyrosine phosphorylation of Dab1 (Howell et al., 1999a; Rice and Curran, 1999; **Figure 2A**). Alternative models that were compatible with the observed mouse phenotypes (Cooper and Howell, 1999), where the lipoprotein receptors were placed downstream, or in parallel, to a Reelin-Dab1 dependent pathway, could thus be ruled out. Although either Apoer2 or Vldlr alone is sufficient for inducing Reelin-mediated Dab1 tyrosine phosphorylation in primary cultures of cortical neurons (Beffert et al., 2002; Bock and Herz, 2003), the neurodevelopmental phenotypes of the single knockout mice hint at divergent functions of both lipoprotein receptors in the transmission of the Reelin signal. These differences might be attributable to a different regional, cellular and subcellular distribution of both receptors, temporal differences in receptor expression and biochemical properties, including ligand affinities, intra- and extracelullar interaction partners, receptor turnover or processing by proteases (see below).

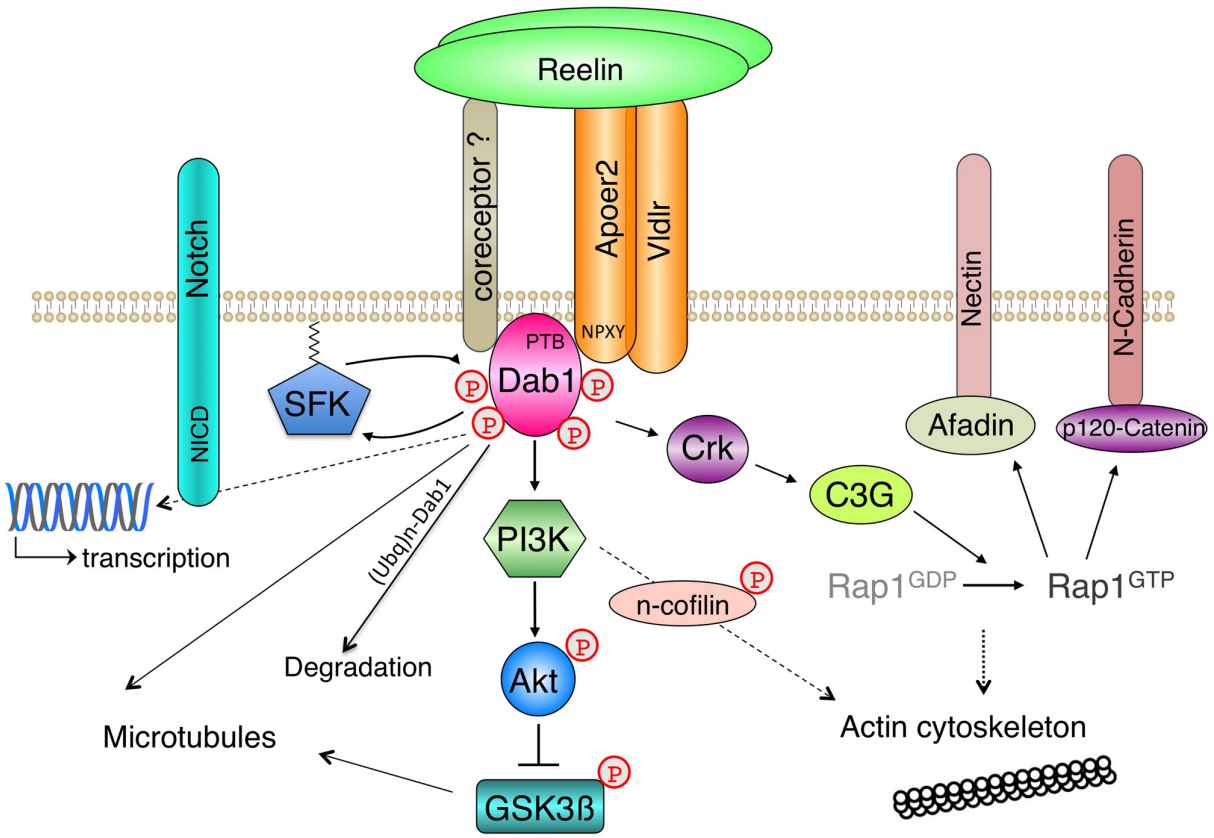

FIGURE 1 | Core Reelin signaling pathway. A simplified scheme of Reelin-induced signaling events in neurons is shown. Based on a combination of phenotypic analysis of mouse mutants and biochemical studies a core Reelin signaling pathway was identified, where lipoprotein receptors interact with the phosphotyrosine-binding (PTB) domain of Disabled-1 (Dab1) via the NPXY motif in their intracellular domains. Oligomerized Reelin induces clustering of the receptor-Dab1 complexes, which induces tyrosine phosphorylation of Dab1 by Src family kinases (SFK) at different sites. This leads to site-specific modification of downstream signaling effectors, which is discussed in more detail in the text. The reciprocal activation of Src kinases (SFK) and Dab1, which underlies the prolonged increase of Dab1 phosphorylation after Reelin stimulation, is negatively regulated by p-Dab1 ubiquitinylation and degradation. It is unclear to what degree Apoer2 and Vldlr are coclustered by Reelin. Inhibition of the serine/threonine kinase Gsk3β via Akt was the first described lipoprotein receptor- and Dab1-dependent linear Reelin signaling cascade in neurons, which targets the cytoskeleton. Reelin binds to additional transmembrane proteins (coreceptors) that also interact with Dab1, which are not essential for the tyrosine phosphorylation of Dab1 but might be involved in receptor crosstalk and/or activation of additional downstream effectors. Long-lasting changes in responsive cells are induced by transcriptional regulation, which involves the gamma-secretase mediated release of receptor intracellular domains. The regulation of transmembrane proteins (N-Cadherin, Nectin) downstream of a Reelin-Dab1-Crk/CrkL-Rap1-dependent signaling cascade has been shown to be essential for Reelin's role during neurodevelopment.

Tyrosine Phosphorylation of Dab1: The Coreceptor Controversy

Subsequent studies focused on the identification of the tyrosine kinase(s) that are required for the phosphorylation of Dab1 at four tyrosyl residues close to its PTB domain, which is essential for Dab1's function during brain development. This was demonstrated by the generation of mice that express a Dab1 protein with phenylalanine substitutions at Tyr185, 198/200, 220 and 232 ($Dab1^{5F/5F}$; Howell et al., 2000). Since, unlike receptor tyrosine kinases, the lipoprotein receptors do not contain a cytoplasmic kinase domain, it was speculated from the beginning that a Reelin coreceptor with associated tyrosine kinase activity (either intrinsic or by forming a complex with a nonreceptor tyrosine kinase) might be required (e.g., Cooper and Howell, 1999, **Figure 2C**). Biochemical studies identified members of the Src family of nonreceptor tyrosine kinases as physiological Dab1 kinases (Arnaud et al., 2003b; Bock and Herz, 2003), which was confirmed genetically by the demonstration of a *reeler*-like phenotype in double-knockout mice lacking both Src and Fyn (Kuo et al., 2005). Members of the gene family of cadherin-related neuronal receptors (CNR), which are expressed in the cortical plate and bind to Fyn with their cytoplasmic domains, were reported to interact with the aminoterminal region of Reelin (Senzaki et al., 1999) and therefore considered as likely coreceptor candidates that would bring Fyn into a Reelin-lipoprotein receptor-Dab1 complex (**Figure 2C**). However, this interaction was not confirmed using an *in vitro* pulldown assay with recombinantly expressed CNR extracellular domain and secreted Reelin (Jossin et al., 2004), and an *in vivo* evaluation of CNR family members as obligatory coreceptors by loss-of-function studies has not been provided. In another study it was suggested that ephrin B receptors,

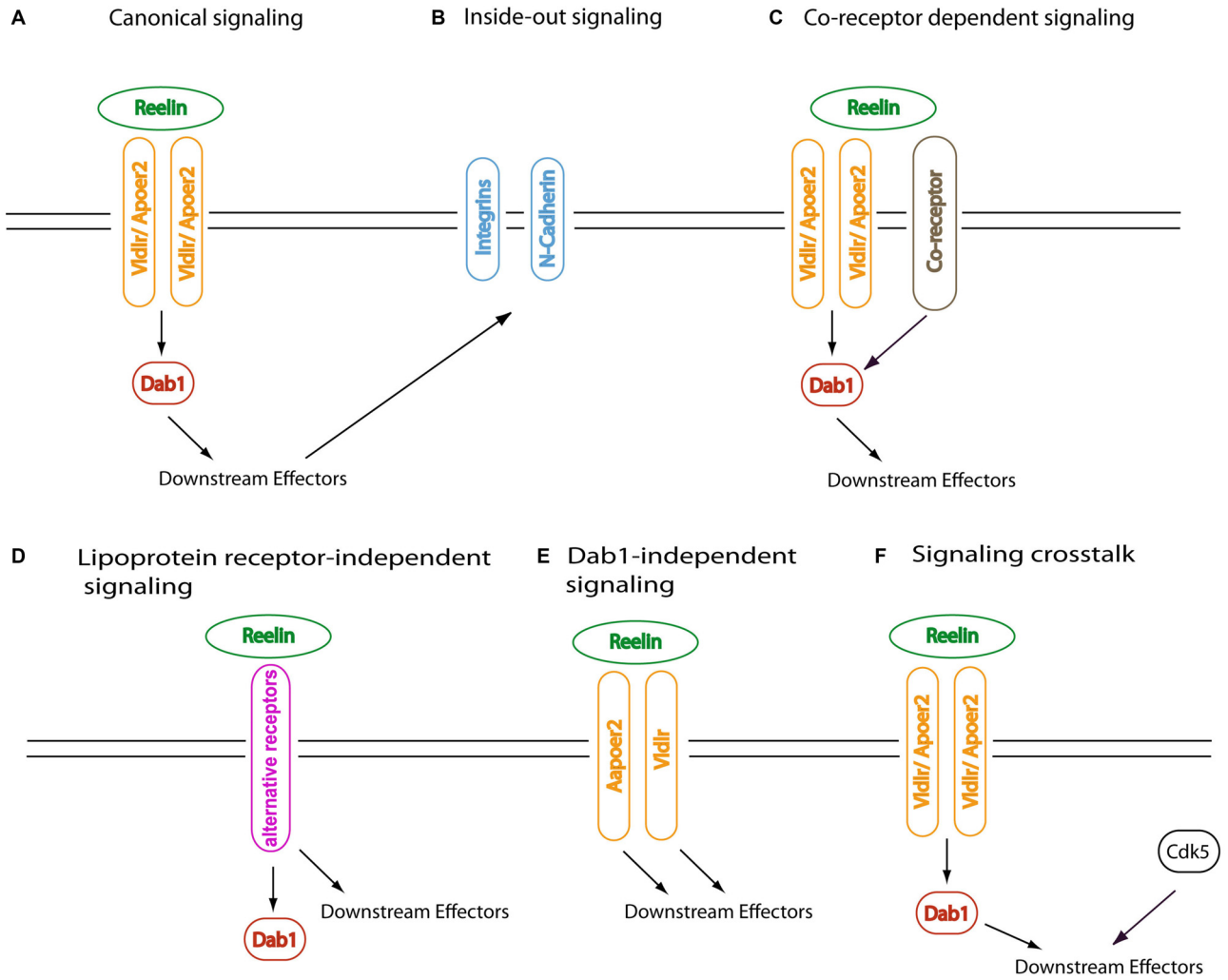

FIGURE 2 | Canoncial and non-canonical Reelin signaling. Alternative schemes of Reelin signaling are shown. Examples for the different concepts are discussed in the text. **(A)** The canonical signaling pathway involves direct binding of Reelin via its central fragment to apolipoprotein E receptor 2 (Apoer2) and very low density lipoprotein receptor (Vldlr), which induces tyrosine phosphorylation of Dab1. This leads to the modulation of downstream signaling effectors. In this model, recruitment of SFK by Reelin-binding coreceptors is not required. **(B)** Other transmembrane receptors can be indirectly activated by Reelin-Dab1 dependent signaling in an inside-out manner; here, the receptors function as downstream effectors of the canonical Reelin signaling cascade. **(C)** In this model, the recruitment of SFK requires Reelin-binding coreceptors in addition to the canonical Reelin receptors Apoer2 and Vldlr. Cadherin-related neuronal receptors (CNR) or ephrin B proteins have been suggested to represent such putative obligatory coreceptors (see text for details). **(D)** Non-canonical activation of Dab1 signaling has been reported; this can either involve other ligands than Reelin that signal via Apoer2 or Vldlr; alternative Reelin receptors, as depicted here; or transphosphorylation of Dab1 via receptor tyrosine kinases. Reelin can also bind to alternative receptors, e.g., via its aminoterminal fragment, and induce Dab1-independent signaling cascades. **(E)** Dab1-independent Reelin signaling via Apoer2 or Vldlr has been postulated, based on phenotypic differences between the respective mouse knockout models. Apoer2-or Vldlr-specific Reelin signaling events have been reported as well, which could involve as yet unidentified receptor-specific adapter proteins. **(F)** Downstream effectors of canonical Reelin signaling function as convergence points for various upstream regulators, allowing responsive cells to fine-tune their response to external and internal stimuli in a context-dependent manner.

transmembrane proteins that interact with Eph receptors to initiate bidirectional signaling (Pasquale, 2008), act as essential Reelin coreceptors, which would be required for the recruitment and activation of Src family kinases (SFK) into the lipoprotein receptor-Dab1 complex at the cell membrane (Sentürk et al., 2011; and Erratum in Nature 2011, 478:274). This model was based on both biochemical and genetic experiments, including precipitation of the *reeler* phenotype in mice carrying only one *reeler* allele on an ephrin B3 knockout background, and the analysis of ephrin B triple knockout mice, which were described to display a *reeler*-like phenotype in the neocortex, hippocampus and cerebellum (Sentürk et al., 2011). In addition, a rescue of the cortical lamination defects in organotypic cortical slice cultures from *reeler* embryos after 2 days of treatment with ephrin B ligand was reported (Sentürk et al., 2011). However, whereas binding of recombinant Reelin to the purified extracellular domain of ephrin B3 was confirmed, the clustered recombinant Ephb3 extracellular domain did

not induce Dab1 tyrosine phosphorylation, as tested by Dab1 immunoprecipitation followed by immunoblotting with the phosphotyrosine antibody 4G10 (Bouché et al., 2013). To verify ephrin B transmembrane proteins as essential components of the canonical Reelin signaling cascade, the independent reproduction of a *reeler*-like phenotype in compound mutant mice would be necessary. In summary, the existence of an additional coreceptor that is required for the tyrosine phosphorylation of Dab1, which is the essential step for the intracellular propagation of the Reelin signal in the canonical pathway, has not been convincingly demonstrated to date.

On the other hand, a considerable body of evidence supports the concept that di- or oligomerization of the lipoprotein receptors Apoer2 and Vldlr, which bind to the central fragment of Reelin (Jossin et al., 2004; Yasui et al., 2010), is sufficient to explain the tyrosine phosphorylation of Dab1 induced by Reelin and its physiological consequences. Reelin is secreted as a disulfide-linked oligomer and forms higher-order multimers (Utsunomiya-Tate et al., 2000; Kubo et al., 2002; Jossin et al., 2004; Yasui et al., 2010, 2011; Manoharan et al., 2015). A conformation-dependent epitope close to the aminoterminal region of Reelin, which is recognized by the function-blocking CR50 monoclonal antibody, is required for homodimerization, and clustered (but not monomeric) receptor-associated protein (RAP), an extracellular lipoprotein receptor ligand, or antibodies directed against the extracellular domains of Apoer2 or Vldlr were able to induce Dab1 tyrosine phosphorylation (Strasser et al., 2004). This was complemented by demonstrating that chemically inducible homodimerization of a Dab 1-FKBP12 fusion protein in heterologous cells was sufficient to induce receptor-independent tyrosine phosphorylation in a SFK-dependent manner (Strasser et al., 2004). Altogether, these studies support the hypothesis that Reelin can induce Apoer2/Vldlr-dependent signaling by clustering Dab1 on the cytoplasmic leaflet of the plasma membrane, bringing it into close proximity with SFK, without the need for an additional coreceptor.

Signaling Events Downstream of Dab1

Here, we will focus on molecular events that regulate Dab1 activation and turnover (and thereby the duration of the Reelin signal) by modulating Src kinase activity, and the Reelin-dependent formation of phospho-Dab1-mediated scaffolding complexes that activate downstream signaling cascades. The Dab1-dependent inside-out activation of other transmembrane receptors involving Rap1 will be discussed in the next chapter.

Feedback Regulation of Reelin Signaling by Dab1 Degradation

Previous studies demonstrated that tyrosine-phosphorylated Dab1 potentiates SFK activation by a positive feedback mechanism (Bock and Herz, 2003), which is limited by the Cullin 5 and SOCS/Rbx2-dependent ubiquitinylation and proteasomal degradation of tyrosine-phosphorylated, activated Dab1 (Arnaud et al., 2003a; Bock et al., 2004; Feng et al., 2007; Simó et al., 2010; Simó and Cooper, 2013; Lawrenson et al., 2015). This places the SFK both upstream and downstream of Dab1. Of note, expression of a mutant Dab1 protein that was phosphorylatable but partly resistant to Cullin-induced degradation in electroporated embryonic brains caused overmigration of cortical neurons, indicating that Dab1 degradation is an essential part of the Reelin-dependent regulation of neuronal positioning in the developing cortical plate (Simó et al., 2010). Accordingly, positioning defects in mice with a conditional deletion of Rbx2 in neuronal progenitors were partially rescued by reducing Dab1 levels through Dab1 heterozygosity, which suggests that accumulation of (phosphorylated) Dab1 by the inability to turn the Reelin signal off causes migration defects (Simó and Cooper, 2013). Conditional deletion of the GTPase-activating protein (GAP) tuberin (Tsc2), which is mutated in the genetic disorder tuberous sclerosis, leads to neuronal migration defects in the cortex, hippocampus and cerebellum that are reminiscent of but not identical with the *reeler* phenotype (Moon et al., 2015). The defects were traced to a defect in Reelin-Dab1-dependent signaling, which is caused by increased activation of mTor kinase and subsequent aberrant expression of the ubiquitin ligase Cullin 5, decreased levels of activated Fyn and phosphorylated Dab1, and increased total Dab1 levels, a biochemical hallmark of defective Reelin-Dab1 signaling (Rice et al., 1998). Interestingly, Reelin also activates mTor in an Akt-dependent manner to regulate growth and branching of hippocampal neurons (Jossin and Goffinet, 2007). An additional link between Reelin and mTor signaling is suggested by the reciprocal regulation of Golgi morphology by Reelin-Dab1 and Lkb1 signaling (Matsuki et al., 2010), which also inhibits mTor by activating Tsc2.

The Relevance of Different Tyrosine Phosphorylation Sites of Dab1

Molecular targets downstream of Dab1 depend on the phosphorylated tyrosine residues. By generating mice with tyrosine to phenylalanine exchanges either at amino acids 185 and 198/200 (ab) or at positions 220 and 232 (cd) of Dab1 (Feng and Cooper, 2009) provided *in vivo* evidence for a dual role of Dab1 both as a SFK activator and a phosphorylation-dependent scaffold for the assembly of downstream signaling complexes. The *Dab1*^{ab} and *Dab1*^{cd} homozygous mice displayed abnormal development of the neocortex and hippocampus, with intermediate cerebellar phenotypes when compared with mice lacking all five tyrosine phosphorylation sites (Howell et al., 2000). Mice hemizygous for each of the mutant alleles (*Dab1*$^{ab/cd}$) showed no morphological brain abnormalities, which indicates that the two sites have individual functions that are required together to support normal brain development. Specifically, the ab site is required for SFK activation (Arnaud et al., 2003b; Bock and Herz, 2003), Dab1 phosphorylation and degradation, and PI3 kinase activation (Beffert et al., 2002; Bock et al., 2003), whereas phosphorylation of the cd site is important for the assembly of Crk/CrkL signaling complexes as well as for the interaction with other adapter proteins such as Nck2 or Crk family members (Pramatarova et al., 2003; Chen et al., 2004; Huang et al., 2004). Recently, Lawrenson et al. (2015) described a role for the tyrosyl residue at position 300 of Dab1, in concert

with Y200, for the interaction with SOCS6 and 7, two SH2-containg proteins that are components of the ubiqutin-E3 ligase complex which is responsible for the proteasomal degradation of phosphorylated Dab1. Mice lacking SOCS6 and SOCS7 display a cortical layer inversion phenotype reminiscent of *reeler* mice and express increased levels of phosphorylated Dab1; however, Reelin-dependent phosphorylation at the Y300 site has not been directly demonstrated yet.

A requirement for PI3 kinase and Akt for normal cortical plate development was demonstrated in an organotypic brain slice culture assay by using chemical inhibitors (Bock et al., 2003; Jossin and Goffinet, 2007), although dominant-negative (DN) kinase-inactive Akt1 expressed under control of a neuron-specific *Dcx* promoter and electroporated into embryonic mouse brain at E12.5 did not affect glia-independent somal translocation (Franco et al., 2011). As would be expected, further branching of the signaling cascade leads to the activation of effectors like cofilin (Chai et al., 2009, 2016), which will not be discussed in detail here. Interestingly, the somatic activation of another Akt isoform, Akt3, in focal malformations of cortical development is responsible for non-cell autonomous cortical migration defects, which lead to drug-resistant epilepsy (Baek et al., 2015). Here, the migration defect is caused by Foxg1-mediated ectopic misexpression of Reelin.

The scaffolding function of Dab1 phosphorylated at the cd sites is essential for normal development. This is underscored by the *reeler*-like phenotype of mice where the adapter proteins Crk and CrkL were simultaneously deleted in the developing nervous system (Park and Curran, 2008). Since they are located downstream of phosphorylated Dab1 in the Reelin signaling cascade, Dab1 protein levels were not increased in the brains of Crk/CrkL double-mutant mice. Reelin-induced phosphorylation not only of the Crk/CrkL-binding guanine nucleotide exchange factor (GEF) C3G, which activates the small GTPase Rap1 (Gotoh et al., 1995), but also of the serine/threonine kinase Akt/protein kinase B (PKB) at position 473 was inhibited in Crk/CrkL-deficient neurons (Park and Curran, 2008). The latter might be a consequence of a loss of the direct interaction of the PI3K regulatory subunit p85 with Crk, which was shown in T lymphocytes (Gelkop et al., 2001).

Regulation by Constrained Localization and Differential Splicing of Dab1

In cultured neurons, Dab1 and Apoer2 are enriched in the distal dendrite of neurons (Howell et al., 1999b; Leemhuis et al., 2010), where Reelin stimulation leads to a localized increase of overall tyrosine phosphorylation (Beffert et al., 2002). The localized action of Reelin signaling is important for the activity-dependent enrichment of HCN1 and GIRK1 ion channels in the distal dendritic tuft of hippocampal CA1 and neocortical layer V pyramidal neurons, thereby modulating the molecular specification of the distal dendritic compartment (Kupferman et al., 2014).

Another level of regulation may be provided by differential splicing of the *Dab1* gene (Bar et al., 2003; Gao and Godbout, 2013). In mice lacking the RNA-binding protein Nova2 a migration defect of late-born cortical neurons was observed. This phenotype was dependent on the overrepresentation of an alternatively spliced form of Dab1 during a critical time window between E14.5 and E16.5, which is normally suppressed by the splicing factor Nova2 (Yano et al., 2010).

Dab1-Dependent Inside-Out Activation of Cell Surface Receptors by Reelin

Several studies addressed the role of Rap1, which is activated through the Reelin-Dab1-Crk-C3G pathway (Ballif et al., 2004), during neocortical development. C3G hypomorphic mice display cortical migration defects resulting in a failure of preplate splitting similar to *reeler* mice, and defects in radial glial processes (Voss et al., 2008). Inactivation of neuronal Rap1 by *in utero* electroporation of the gene encoding *Rap1Gap*, a GAP that specifically inactivates Rap1 by stimulating its GTP hydrolysis, or *Rap1a* shRNA, disrupted glia-independent somal translocation of migrating neurons. This effect was dependent on neuronal Cadherin-2 (NCAD, Cdh2), a transmembrane protein that mediates cell-cell adhesion. Cdh2 overexpression rescued the migration defect caused by Rap1GAP overexpression, placing it downstream of Rap1 as an example of inside-out activation of a cell surface receptor. However, the *Dab1* null phenotype was not rescued by Cdh2 overexpression, which underscores the pleiotropic nature of Reelin-Dab1 signaling affecting different developmental stages, cellular and molecular levels of action (Franco et al., 2011). This is exemplified by two other important studies using *in utero* electroporation of embryonic brains, which confirmed the importance of Reelin-dependent Rap1 activation for neuronal migration in the developing neocortex (Jossin and Cooper, 2011; Sekine et al., 2012). Sekine et al. (2012) found that Cdh2 overexpression did not fully rescue the Rap1 suppression phenotype and provided evidence for an additional role of Dab1-Crk-C3G-Rap1 dependent inside-out activation of integrin β1-α5 receptors for the terminal translocation step of migrating neurons. Moreover, they demonstrated a role for Akt in the regulation of the terminal translocation step, which was rescued by simultaneous coexpression of constitutively active integrin α5 and Akt, but not by either vector alone. It should be mentioned, though, that neuron-specific conditional inactivation of β1 integrin using NEX-Cre mice did not affect the formation of cortical cell layers (Belvindrah et al., 2007). A cell-autonomous role of Dab1 phosphorylation at tyrosine residues 220 and 232 for the detachment of neurons from radial glia was shown, which depends on the downregulation of neuronal integrin α3 levels (Sanada et al., 2004). This provides an example for Reelin-dependent inside-out modulation of a cell surface receptor (**Figure 2B**) by downregulation of its activity.

Jossin and Cooper (2011) showed that Rap1 regulated the membrane localization of N-cadherin, which was required for the transition from multipolar to bipolar migration in the lower intermediate zone. Of note, migration defects of neurons overexpressing a signaling-incompetent, dominant-

negative (DN) Vldlr construct were only partially rescued by transfection with a constitutively active form of Rap1, whereas coexpression of Akt and activated Rap1 overcame the effect of DN-Vldlr (Jossin and Cooper, 2011). In the marginal zone, Reelin-dependent Rap1 signaling influences the interaction of translocating neurons with Reelin-secreting Cajal-Retzius cells by facilitating Cdh2 recruitment to nectin-based adhesion sites, with the Rap1 effector Afadin and its binding partner p120-Catenin serving as a molecular link between the activation of Rap1 and Cdh2 (Gil-Sanz et al., 2013). However, Cdh2 is not only required for the multipolar-bipolar transition and terminal translocation step of cortical neuronal migration but also involved in mediating heterophilic interactions of migrating neurons with radial glia during the locomotion mode of migration, which depends on the regulation of vesicle trafficking by Rab GTPase proteins (Kawauchi et al., 2010). The importance of Rap1 is underlined by the phenotype of conditional knockout mice, which show a complete loss of cortical lamination as a consequence of the loss of radial glial and neuronal polarity (Shah et al., 2016). Knockdown of the RasGAP Dab2IP, a Dab1-interacting protein (Homayouni et al., 2003), has been reported to influence the positioning of later-born cortical neurons by activating Rap1 and integrin signaling (Qiao and Homayouni, 2015), and to regulate the multipolar-bipolar transition in the intermediate zone (Lee et al., 2012). However, no complete *reeler*-like phenotype was reported for conventional Dab2IP (AIP1) knockout mice (Zhang et al., 2008). Other small GTPases like Cdc42 and possibly Rac1 are involved in more specific functions of Reelin during the differentiation of postmitotic neurons (Leemhuis et al., 2010; Jossin, 2011; Leemhuis and Bock, 2011; Meseke et al., 2013; Pasten et al., 2015), where fine-tuning is achieved by the involvement of various GEFs (Rossman et al., 2005).

Role of Dab1 in Receptor Trafficking

Dab1 is a protein with several features of an endocytic accessory factor (Merrifield and Kaksonen, 2014) and might therefore be involved in the regulation of receptor trafficking. This cellular function of Dab1 is relevant to understanding important aspects of Reelin signaling via both canonical and non-canonical Reelin receptors. The aminoterminal phosphotyrosine binding/PTB domain preferentially interacts with the non-phosphorylated NPXY tetra-amino-acid motif (Herz et al., 1988; Chen et al., 1990) in the intracellular tails of transmembrane receptors, including Apoer2 and Vldlr but also the amyloid precursor protein family members and integrins (Rice et al., 1998; Trommsdorff et al., 1998; Howell et al., 1999b; reviewed in Stolt and Bock, 2006; Yap and Winckler, 2015), and also mediates membrane localization by interacting with phosphoinositides (Stolt et al., 2003, 2005; Huang et al., 2005; Xu et al., 2005). In addition, it contains a clathrin binding site within the carboxyterminal domain, which is involved in the regulation of cortical development, since mice expressing only one copy of a carboxyterminally truncated, hypomorphic p45 isoform of Dab1 display migration defects of late-born cortical neurons (Herrick and Cooper, 2002). Morimura et al. (2005) reported data from experiments in cortical neurons and heterologous cells suggesting that tyrosine-phosphorylated Dab1 is recruited to the plasma membrane after Reelin stimulation and that phosphorylation of Dab1 initiates intracellular trafficking of Reelin.

Interaction with Dab1 increases cell surface levels and proteolytic processing of Apoer2 and APP independent of its tyrosine phosphorylation and increases cleavage of extracellular receptor domains (Hoe et al., 2006b). Interaction of tyrosine-phosphorylated Dab1 with the endocytic adapter protein CIN85 in Reelin-treated neurons might contribute to the sorting of Dab1-receptor complexes to early endosomes (Fuchigami et al., 2013). Another regulator of endosomal receptor cycling, sorting nexin 17 (Snx17), binds to the intracellular domain of lipoprotein receptors including Apoer2 (Stockinger et al., 2002), and was reported to contribute to Reelin-induced Apoer2 trafficking, processing and Dab1 dependent signaling (Sotelo et al., 2014). It should be mentioned that based on studies in heterologous cells it was also suggested that Dab1 has two nuclear localization signals (NLS) and two nuclear export signals (Honda and Nakajima, 2006, 2016) and could therefore act as a nucleocytoplasmic shuttling protein. Tyrosine phosphorylation did not affect the intracellular distribution of Dab1 (Honda and Nakajima, 2006), arguing against a direct role of Dab1 in mediating Reelin-dependent regulation of transcription (Telese et al., 2015). *In utero* electroporation of Dab1 harboring a mutant NLS confirmed that excess cytoplasmic Dab1 inhibits neuronal migration (Honda and Nakajima, 2016).

Crosstalk with p35/Cdk5 Signaling

Together, the above-mentioned studies provide important clues about the molecular mechanisms of Reelin's contributions to different steps of neuronal layer formation in the developing neocortex. However, issues such as the the specific contribution of Reelin effectors in different responsive cell types, and the interaction with other signaling pathways (**Figure 2F**) are still insufficiently understood. Activation of Rap1 constitutes an example of signaling crosstalk with Cyclin-dependent kinase 5 (Cdk5) that is fairly well examined. Cdk5 is a serine/threonine kinase that is highly expressed in postmitotic neurons. Its essential role during brain development is obvious from the phenotype of *Cdk5* knockout mice, which display severe defects in laminated brain structures. Like Reelin, Cdk5 also modulates many aspects of neuronal maturation and synaptic transmission in the adult brain (reviewed by Dhavan and Tsai, 2001; Kawauchi, 2014; Shah and Lahiri, 2014). However, in contrast to *reeler* mice, the preplate splits normally during neocortical development (Gilmore et al., 1998), and the failure of Reelin to induce Cdk5 activation (Gilmore et al., 1998) as well as a series of genetic studies support the concept that Reelin and Cdk5 act in parallel rather than in a linear fashion to regulate layer formation in the developing brain (Ohshima

et al., 2002; Beffert et al., 2004; Ohshima, 2015). Rap1 has been shown to be an important convergence point of both pathways. Whereas RapGEF1 (C3G) is activated by Reelin, RapGEF2 is phosphorylated and thereby activated by Cdk5 at Ser1124, which seems to be important for the transition from multipolar to bipolar morphology in the intermediate zone (Ye et al., 2014). Other possible intersection points of Reelin and Cdk5 signaling include collapsin response mediator proteins (CRMP), whose phosphorylation by the Reelin target Gsk3beta (Beffert et al., 2002) needs to be primed by prior Cdk5 phosphorylation (Uchida et al., 2005; Cole et al., 2006); the microtubule-associated phosphoprotein Tau (Sengupta et al., 1997; Li et al., 2006; Plattner et al., 2006), which is involved in the pathogenesis of neurodegenerative disease, and Dab1 itself (Keshvara et al., 2002; Ohshima et al., 2007), although evidence for the *in vivo* relevance of these interaction nodes is sparse. Expression of a carboxyterminally truncated form of Dab1 that lacks the Cdk5 phosphorylation sites rescues the Dab1 knockout phenotype, but mice hemizygous for the truncated gene ($Dab1^{p45/-}$) display a unique migration defect in the neocortex, with normal preplate splitting as in the *Cdk5* knockout mice, and hippocampus (Herrick and Cooper, 2002).

Non-Canonical Reelin Signaling Involving Apoer2/Vldlr, or Dab1

Noncanonical signaling can refer to the involvement of components other than Apoer2/Vldlr as cell surface receptors or Dab1 as intracellular signal transducer of Reelin, which are the essential components of the "classical" Reelin cascade, as outlined above. Indeed, several studies have been published that suggest a role for Reelin binding to its canonical receptors Apoer2 and/or Vldlr without involving Dab1 as the central intracellular mediator of this interaction. Other studies point to a requirement of Dab1 for the transmission of the Reelin signal without the involvement of Apoer2 or Vldlr (**Figures 2D,E**). Rossel et al. (2005) reported that Reelin is required for the second, radial glia-dependent migration step of hindbrain efferent neurons. This phenotype was also observed in Dab1-deficient scrambler mice but not in *Apoer2/Vldlr*-deficient mice, pointing to the involvement of another receptor for Reelin. Possible candidates would be transmembrane proteins that interact with Dab1 via their intracellular tails, e.g., integrins or amyloid precursor protein.

Conversely, a Reelin-dependent effect on the migration of early-generated interneurons in the olfactory bulb was described, which was defective in lipoprotein receptor-deficient mice but was not phenocopied in mice lacking Dab1 (Hellwig et al., 2012), suggesting that context-dependent alternative signal transduction mechanisms for Reelin exist. Still another scenario was described for hypothalamic gonadotropin releasing hormone (GnRH)-positive neurons, with a reduction in number and aberrant position of GnRH neurons in the hypothalamus of *reeler*, but not Dab1 or Apoer2/Vldlr-deficient neurons (Cariboni et al., 2005). A similar situation is found during lymphatic vascular development, which is defective in Reelin-deficient *reeler* mice but not in mice lacking Dab1 or both Apoer2 and Vldlr (Lutter et al., 2012). In none of these cases, the molecular mechanisms underlying the described Reelin-dependent phenotypes have been elucidated.

Reelin-Independent Activation of Lipoprotein Receptor-Dab1 Dependent Signaling

Another "non-canonical" variation of classical Reelin signaling is the activation of the core signaling pathway by ligands other than Reelin itself. Reelin has been described to be important for the so-called chain migration of neuroblasts from the subventricular zone into the olfactory bulb along the rostral migratory stream (RMS; Hack et al., 2002). Here, Reelin induces the detachment of chain-migrating neurons, leading to a switch from chain migration to radial migration in the olfactory bulb. Apoer2 and Dab1 are expressed in the RMS, and Apoer2 and Vldlr seem to be involved in mediating the effect of Reelin on the detachment process (Hellwig et al., 2012). Because Reelin is not present in the RMS (Hack et al., 2002; Andrade et al., 2007), it was suggested that the involvement of Apoer2/Vldlr and Dab1 for proper neuroblast chain formation indicates the requirement for another lipoprotein receptor ligand (Andrade et al., 2007). Since the extracellular matrix (ECM) protein thrombospondin-1 (Tsp1) is expressed in the RMS and Tsp1-deficient mice have a wider and less compact RMS architecture it was examined whether Tsp1 might act as a ligand for Apoer2 and Vldlr, which was shown to be the case (Blake et al., 2008). Tsp1 binding to the receptors was competitive to both Reelin and receptor-associated protein (RAP), a chaperone and universal ligand for LDL receptor family members (Herz et al., 1991), and treatment with Tsp1 induced the tyrosine phosphorylation of Dab1 in primary neurons, probably by promoting receptor multimerization (Blake et al., 2008). Surprisingly, however, other key features of canonical Reelin signaling, such as ligand-induced Dab1 degradation were not observed, and in an *in vitro*-based matrigel assay Tsp1 stabilized neuronal precursor chains (Blake et al., 2008), instead of dissolving them like Reelin does (Hack et al., 2002). The molecular basis of these differences in downstream signaling via Apoer2 and Dab1 remains to be identified and might involve differences in the phosphorylation of the various Dab1 tyrosyl residues, or the different activation of modulating signaling pathways by coreceptors. Another ECM protein that has been reported to bind to Apoer2 was F-spondin (Hoe et al., 2005). The interaction site was mapped to the aminoterminal thrombospondin domains of F-spondin, which are located in the carboxyterminal half of the protein, and was inhibited by the LDL receptor family chaperone RAP, whereas the aminoterminal Reelin and spondin domains bound to the amyloid precursor protein (APP). These interactions were reported to increase the cell surface expression of both receptors and modulated their proteolytic processing (Ho and Sudhof, 2004; Hoe et al., 2005). In chicken ciliary ganglion (CG) neurons, F-spondin induced tyrosine phosphorylation

of Dab1 (Peterziel et al., 2011). Surprisingly, this was likely mediated through binding of F-spondin to APP (**Figure 2D**), since Apoer2 and Vldlr were barely expressed in the GC cells, and the lipoprotein receptor antagonist RAP did not block the neurotrophic effect of F-spondin on GC cells, which was shown to depend on Dab1 phosphorylation (Peterziel et al., 2011).

In another study that was based on the observation of an olfactory bulb layering defect in insulin-like growth factor 1 (IGF1)-deficient mice an effect of IGF1 on Dab1 phosphorylation in OB cell cultures was reported (Hurtado-Chong et al., 2009). However, the phosphosite-specific Dab1 antibodies used in this study were confirmed for overexpresssing cells only and are not specific in primary cortical cultures, and the results should therefore be validated by Dab1 immunoprecipitation followed by phosphotyrosine immunoblotting, or by using OB cells from Dab1-deficient animals. Other ligands that have been described to induce tyrosine phosphorylation of Dab1 via lipoprotein receptor-dependent signaling include fibrillar prion protein fragment, which also reduces total Dab1 levels after prolonged treatment (Gavín et al., 2008), and clusterin/apolipoprotein J, which is present in the adult subventricular zone and might be involved in neurogenesis and neuroblast chain formation (Leeb et al., 2014). Another effect mediated by Apoer2 which does not depend on Reelin was described in the monocytic cell line U937: Activated protein C (APC) induced Dab1 tyrosine phosphorylation and Akt-dependent Gsk3beta phosphorylation in a RAP-sensitive manner (Yang et al., 2009; Sinha et al., 2016), which contributed to APC's anticoagulant activity after endotoxin stimulation *in vitro*. Vldlr did not bind to APC as determined by surface plasmon resonance or solid-phase binding assays (Yang et al., 2009). It remains to be determined if Reelin, which is present in large amounts in the plasma (Smalheiser et al., 2000), has similar effects on circulating blood cells. Vascular endothelial growth factor (VEGF)-induced tyrosine phosphorylation of Dab1 mediated by the receptor tyrosine kinase Flk1 (VEGF receptor-2) in cortical neurons has been reported using a phospho-specific DAB1 antibody (Howell et al., 2013). Again, this finding should be confirmed by Dab1 immunoprecipitation followed by immunoblotting with a phosphotyrosine antibody.

It should be mentioned that selenoprotein P (Sepp1) has been described as an Apoer2 ligand that does not interfere with canonical lipoprotein receptor-Dab1 signaling. Instead, its endocytosis via Apoer2 is essential for selenium supply in the brain and testis (Olson et al., 2007; Masiulis et al., 2009), and is mediated by the Apoer2 beta-propeller domain instead of the ligand binding domain (Kurokawa et al., 2014). Another Apoer2 and Vldlr ligand, the proprotein convertase Pcsk9, binds to the epidermal growth factor-like repeat A of LDL receptor family members next to the ligand binding domain and mediates the intracellular degradation of both receptors by rerouting them to a lysosomal pathway (Cohen and Hobbs, 2013). Whereas this effect was enhanced by the presence of Dab1 in heterologous cells (Poirier et al., 2008), its physiological relevance for Reelin signaling in the brain is unclear (Liu et al., 2010; Kysenius et al., 2012).

Selective Functions of the Canonical Reelin Receptors Apoer2 and Vldlr

Apoer2 and Vldlr are close relatives of the LDL receptor and share a high degree of homology, although important structural differences exist, which are reviewed elsewhere (Bock and Herz, 2008; Reddy et al., 2011). Both receptors bind Reelin as high-affinity receptors, with slightly different binding affinities (Andersen et al., 2003; Benhayon et al., 2003), and can mediate tyrosine phosphorylation of Dab1 as well as activation of Dab1-dependent downstream targets like Akt without the requirement of the presence of the other receptor, as was first shown by using cultured cortical neurons from either *Apoer2* or *Vldlr* single-knockout mice (Beffert et al., 2002). However, the single-knockout mice display strikingly different phenotypes (e.g., Trommsdorff et al., 1999; Weeber et al., 2002; Hack et al., 2007), which is largely explained by the non-overlapping spatiotemporal and subcellular expression pattern of both receptors (Perez-Garcia et al., 2004; Hirota et al., 2015). In addition, differences exist with regard to the subcellular distribution, especially the recruitment to cholesterol-rich microdomains of the plasma membrane (Sun and Soutar, 2003; Mayer et al., 2006), which have important functions in regulating signal transduction and receptor trafficking (reviewed in Lingwood and Simons, 2010). Also, differences in their capacity to mediate endocytosis and degradation of bound ligands likely contribute to their different biological functions (Li et al., 2001; Beffert et al., 2006a; Chen et al., 2010; Duit et al., 2010). Another possible mechanism would be the receptor-specific interaction with different adapter proteins. Important aspects of Apoer2's function as Reelin receptor depend on its differential splicing. The O-linked sugar domain of Apoer2 in close proximity of the transmembrane domain is encoded by a separate exon and required for the extracellular cleavage of Apoer2, which precedes its gamma-secretase mediated proteolytic processing (May et al., 2003b). Mice lacking this exon showed increased Apoer2 abundance in the brain, which was associated with altered synaptic receptor function (Wasser et al., 2014). Activity-dependent alternative splicing of the intracellular exon 19 of Apoer2, which encodes a proline-rich insert that mediates biochemical and functional interaction with NMDA receptors at the synapse (Beffert et al., 2005; Hoe et al., 2006a), represents an additional important means to fine-tune biological responses to Reelin signaling in a context-dependent manner. Scaffold proteins of the JIP and MINT families also bind to Apoer2, but not to Vldlr (**Figure 2E**), in an exon 19-dependent manner (Gotthardt et al., 2000; Stockinger et al., 2000; Verhey et al., 2001; He et al., 2007; Minami et al., 2010). The JIP-JNK recruiting function of the Apoer2 isoform including the proline-rich insert is required for protection against loss of corticospinal neurons (CSN) during normal aging, whereas the same splice form promotes lesion-induced cell death of CSN, as was elegantly shown *in vivo* by using knockin mice with selective alterations of the Apoer2 intracellular domain (Beffert et al., 2006b).

The Dab1 binding site was not involved, suggesting Reelin signaling-independent functions of the Apoer2 intracellular domain. Direct phosphorylation of JNK was demonstrated in hippocampal neurons treated for 1 h with recombinant Reelin. This was blocked by either PI3K inhibition or pertussis toxin, suggesting involvement of heterotrimeric G protein and crosstalk with G-protein coupled receptor signaling (Cho et al., 2015).

On the other hand, Vldlr-specific Reelin signaling responses (**Figure 2E**) have been described. Following demonstration that Reelin and LIS1, the gene product of the *Pafah1b1* gene, genetically and biochemically interact in a phospho-Dab1-dependent manner (Assadi et al., 2003) it was shown that the α1 and α2 catalytic subunits of the Pafahb complex selectively bind the NPXYL motif in the intracellular Vldlr domain (Zhang et al., 2007) and differentially interact with either tyrosine phosphorylated or non-phosphorylated Dab1, which modulates the effect of Reelin-Dab1-Lis1 signaling on microtubule dynamics (Assadi et al., 2008; Zhang et al., 2009). In a recent study, it was reported that Vldlr-dependent Ras signaling affects dendritic spine formation in hippocampal neurons (DiBattista et al., 2015). The authors used a recombinant Reelin fragment, which decreased the biochemical interaction of Vldlr with the GEF RasGRF1; however, it remains to be determined if full-length Reelin exerts the same effect.

The same murine Reelin fragment encompassing amino acids 1221–2661 (i.e., corresponding to the central fragment consisting of the Reelin repeats 3–6 that is able to bind to Apoer2 and Vldlr and to induce Dab1 phosphorylation in cultured neurons (Jossin et al., 2004)) was used to demonstrate coclustering of Apoer2 with other receptors (Divekar et al., 2014). This fragment includes a critical cysteine residue at position 2101 that is required for covalent multimerization and efficient Dab1 phosphorylation (Yasui et al., 2011). Coprecipitation of Apoer2 and Vldlr after stimulation with the Reelin fragment was not observed in heterologous cells and primary neurons, leaving open the question if Reelin induces its two main receptors to form heteroclusters to a significant extent.

Activation of Pathways that Modulate Transcription

Another pivotal protein kinase cascade that relays extracellular signals from the cell surface into cells is the Erk (extracellular signal-regulated kinases) pathway, which was reported to be activated by Reelin in a Src- and Dab1-dependent manner (Simó et al., 2007). Whereas other studies could not detect Erk phosphorylation after stimulation with Reelin-conditioned medium (Ballif et al., 2003; Cho et al., 2015), the activation of Erk in primary neurons was confirmed using highly purified Reelin (Lee et al., 2014; Telese et al., 2015). In contrast to the aforementioned study (Simó et al., 2007), this activation did not depend on Dab1 and was not inhibited by recombinant RAP, a chaperone that blocks binding of extracellular ligands to LDL receptor family members, which suggests involvement of a different Reelin receptor. This is supported by the observation that the lipoprotein receptor-binding central fragment of Reelin was not sufficient to induce Erk phosphorylation (Lee et al., 2014). Reelin-induced Erk activation was accompanied by increased transcription of immediate early genes including *Egr1* and *Arc* (Simó et al., 2007; Lee et al., 2014). In addition, it was demonstrated that Reelin directly potentiates glutamate-induced NMDA receptor-dependent calcium influx (Chen et al., 2005). This was mediated via activation of Src kinases, Dab1 and NMDAR phosphorylation and induced the phosphorylation of cAMP-responsive element binding protein (Creb) at serine 133 (Chen et al., 2005), a transcription factor that modulates many aspects of neuronal development, plasticity and behavior in response to PI3K/Akt and Erk activation (Lonze and Ginty, 2002). Together, these findings suggested that regulation of gene transcription contributes to Reelin's multiple effects on brain development and function. Indeed, transcriptomic profiling of mature cortical neurons confirmed that Reelin induces the expression of synaptic activity-regulated genes in a Src- and NMDAR-dependent manner (Telese et al., 2015). This involved Reelin-induced epigenomic changes that were sensitive to gamma-secretase inhibition, possibly involving nuclear translocation of the intracellular domain of Apoer2 (Telese et al., 2015), which is released by gamma-secretase activity in neurons (May et al., 2003b; Hoe and Rebeck, 2005; Wasser et al., 2014).

Another means of gamma-secretase dependent transcriptional regulation involving Reelin is its crosstalk with Notch signaling, a pathway that regulates many aspects of neural development, cell fate specification, neuronal survival and synaptic plasticity (reviewed by Ables et al., 2011; Pierfelice et al., 2011). A link between Notch and Disabled was first shown in *Drosophila* (Giniger, 1998). In mice, activation of Notch receptors leads to the gamma-secretase mediated release and nuclear localization of their intracellular domains (NICD), which regulates the transcription of target genes. In *reeler* mice, levels of the NICD and its target genes *Hes1* and *Hes5* were reduced, and overexpression of the NICD by electroporation rescued the neuronal migration defect in *reeler* mice or caused by overexpression of the Dab1-5F mutant, which cannot be tyrosine-phosphorylated at Y185, Y198/200, Y220 and Y232 (Hashimoto-Torii et al., 2008). This places NICD, which biochemically interacts with Dab1 (Hashimoto-Torii et al., 2008; Keilani and Sugaya, 2008), downstream of Dab1. Apart from its effect on neocortical migration, the interaction of Reelin and Notch affects the radial glial characteristics of progenitor cells (Keilani and Sugaya, 2008; Sibbe et al., 2009; Lakomá et al., 2011), including expression of brain lipid binding protein (Blbp), an effector of both pathways (Gaiano et al., 2000; Hartfuss et al., 2003). In a recent study it was shown that both Erk and Creb phosphorylation are reduced in the hippocampi of conditional Notch1-deficient mice, and suggested that Notch1 is also required for NMDAR-mediated Reelin signaling at the synapse (Brai et al., 2015).

Non-Canonical Reelin Receptors

The inside-out activation of integrin receptors by Reelin-Dab1-Crk-Rap1 dependent signaling has been discussed above; here, integrin transmembrane proteins act as downstream effectors

of Reelin signaling via the canonical lipoprotein receptor-Dab1-mediated pathway (Sekine et al., 2012). Direct binding of Reelin via its aminoterminal region to the extracellular domain of α3 β1 integrins has also been shown (Dulabon et al., 2000; Schmid et al., 2005). In addition, Dab1 interacts with the NPXY motif in the cytoplasmic tails of beta integrins (Calderwood et al., 2003; Schmid et al., 2005). In α3 β1 integrin-deficient cortical neurons Reelin-induced phosphorylation was not affected, but interestingly, Dab1 levels were downregulated in α3 integrin-deficient brains (Dulabon et al., 2000), as opposed to the characteristic upregulation of Dab1 in mice with genetic defects in the canonical lipoprotein receptor-dependent Reelin signaling cascade (see above). The preplate forms normally in the cortex of α3-deficient mice (Schmid et al., 2004), whereas inactivation of β1 integrin in radial glial cells, which are both neural and glial progenitor cells (reviewed by Dimou and Götz, 2014), develop a disorganized cortex with Cajal-Retzius cell heterotopia (Graus-Porta et al., 2001). Neuron-specific inactivation of β1 integrin produced no phenotype (Belvindrah et al., 2007), which suggests that Reelin-integrin binding alone is not responsible for the neuronal migration defect seen in *reeler* mice (Magdaleno and Curran, 2001) and that β1 integrin expression is pivotal for radial glial function (Forster et al., 2002; Radakovits et al., 2009).

Chronic treatment of hippocampal neurons with Reelin altered the subunit composition of synaptic NMDA receptors, which involved β1 integrin activity as shown by using function-blocking antibodies. The lipoprotein receptor antagonist RAP had no effect (Groc et al., 2007), ruling out inside-out activation of integrins via lipoprotein receptors as underlying signaling mechanism. Moreover, a presynaptic effect of Reelin on neurotransmitter release, which was blocked by a cyclic integrin-inhibiting arginylglycylaspartic acid (RGD) peptide (Ruoslahti, 1996), has been reported (Hellwig et al., 2011). In mouse brain synaptosome preparations Reelin enhanced the local translation of *Arc* mRNA (Dong et al., 2003), an immediate early gene that regulates synaptic plasticity (Shepherd and Bear, 2011), and this was inhibited by echistatin, an RGD-containing peptide that functions as a competitive integrin receptor antagonist (Gan et al., 1988). Hence, integrins might modulate the functions of Reelin at the synapse. However, a selective increase of spontaneous neurotransmission by Reelin was reported to depend both on Vldlr and Apoer2, which was also expressed presynaptically, and PI3 kinase activity, and required the vesicular SNARE protein Vamp7 (Bal et al., 2013). Apart from brain-specific roles, integrins are prime candidate receptors for Reelin functions in "peripheral" organs (Lin et al., 2016), many of which do not express detectable amounts of essential components of the canonical signaling cascade.

Reelin as a Ligand for Amyloid Precursor Protein (APP)

The demonstration that the interaction of Reelin with lipoprotein receptors at the adult synapse enhances long-term potentiation through Dab1- and Fyn-dependent phosphorylation of NMDA receptors (Weeber et al., 2002; Beffert et al., 2005; Chen et al., 2005) suggested that Reelin signaling in the adult brain modulates learning and memory performance, which has subsequently been demonstrated *in vivo* using different experimental approaches including conditional knockout of Reelin, Dab1, or Reelin overexpression in adult mice (Brosda et al., 2011; Rogers et al., 2011, 2013; Trotter et al., 2013; Pujadas et al., 2014; Lane-Donovan et al., 2015; Imai et al., 2016). As LDL receptor family members are main receptors for Apo E (reviewed by Herz and Willnow, 1994; Hussain et al., 1999), whose E4 variant is a strong genetic risk factor for the sporadic form of Alzheimer disease (AD; (Strittmatter et al., 1993), a causal connection between disturbed Reelin signaling and neurodegeneration has been proposed ever since Apoer2 and Vldlr were first described as Reelin receptors (Cooper and Howell, 1999; Bothwell and Giniger, 2000; Herz, 2001). This view, summarized in several reviews (e.g., Rogers and Weeber, 2008; Krstic et al., 2013; Lane-Donovan et al., 2014) is supported by a multitude of *in vitro* and *in vivo* studies, which collectively suggest that Reelin has an overall neuroprotective role in the adult brain. The amyloid-beta peptide, which is considered to be central for the development and progression of AD (Selkoe, 2000), is generated by sequential proteolytic cleavage from a type I transmembrane receptor named APP (O'Brien and Wong, 2011). Importantly, the inhibition of hippocampal long-term potentiation (LTP) by soluble amyloid-beta oligomers (reviewed by Selkoe, 2008) is overcome by coapplication of Reelin in acute slices (Durakoglugil et al., 2009), and selective retardation of cell surface recycling of endocytosed Reelin-ApoE receptor complexes by ApoE4-containing lipoproteins at least partially explains the negative effect of this isoform on synaptic function (Chen et al., 2010). Inactivation of Reelin in the adult brain by tamoxifen-inducible conditional gene knockout precipitated amyloid-beta neurotoxicity in transgenic mice overexpressing an AD-associated mutant form of APP (Lane-Donovan et al., 2015). In a reverse approach, inducible overexpression of Reelin was shown to overcome toxic effects of amyloid-beta (Pujadas et al., 2014). In this study, Reelin delayed the formation of amyloid fibrils, the main constituents of senile plaques, which were previously shown to accumulate Reelin both in animal models of AD and during normal aging (Wirths et al., 2001; Knuesel et al., 2009). Importantly, however, loss of Reelin in the conditional knockout model did not accelerate amyloid plaque deposition at the studied age of 7 months (Lane-Donovan et al., 2015). This rules out the possibility that Reelin's protective role at the synapse is predominantly mediated by regulating plaque abundance.

Beside its function as a preproprotein whose processing generates biologically active soluble fragments (Haass et al., 2012) and a transcriptionally active intracellular domain (Müller et al., 2008) the role of the full-length amyloid precursor protein as a cell surface receptor is being increasingly recognized (Deyts et al., 2016), although its normal physiological functions remain largely unknown. Reelin has been reported to be one of several candidate ligands of full-length APP (Hoe et al., 2009) and modulates the proteolytic processing of APP *in vivo* and *in vitro* (Kocherhans

et al., 2010; Rice et al., 2013), partly through the interaction of APP with Dab1 (Rice et al., 1998; Trommsdorff et al., 1998; Howell et al., 1999b), Fe65, and lipoprotein receptors (Hoe et al., 2006b, 2008; Parisiadou and Efthimiopoulos, 2007; Kwon et al., 2010; Minami et al., 2011). The direct binding of Reelin to the extracellular domain of APP involves its central fragment, which also interacts with the ligand binding domains of Apoer2 and Vldlr (Hoe et al., 2009). A possible function of this interaction, which was reported to involve α3 β1 integrins, might be the regulation of neurite outhgrowth (Hoe et al., 2009). An excess of the APP intracellular domain blocked the inhibitory effect of cell surface-immobilized Reelin on neurite outgrowth, possibly by sequestering Dab1 in the nucleus (Hoareau et al., 2008). The genetic interaction of *Dab1* and *App*, and the effect of *App* knockdown or overexpression on neuronal migration (Young-Pearse et al., 2007; Pramatarova et al., 2008) support additional functions for Reelin-APP and APP-Dab1 interactions during neurodevelopment.

Interaction with Eph Receptors

Mice lacking members of the EphB transmembrane tyrosine kinase receptor family, which mediate a variety of interactions regulating brain development and function (reviewed by Klein, 2004; North et al., 2013), display deficits in hippocampal morphogenesis (Catchpole and Henkemeyer, 2011; Bouché et al., 2013). This observation led to the identification of EphB proteins as Reelin receptors. Binding to the extracellular domain of EphB2 is mediated by the aminoterminal part of Reelin and induces EphB forward signaling (**Figure 2D**) in heterologous cells expressing EphB2 and in primary neurons (Bouché et al., 2013). Principally, the interaction with the aminoterminal domain of Reelin allows for the simultaneous binding of lipoprotein receptors or APP, which interact with Reelin via its central fragment. The composition of the putative supramolecular Reelin-receptor complex might be further modulated through regulated proteolytic cleavage of Reelin (Lambert de Rouvroit et al., 1999; Jossin et al., 2007; Kohno et al., 2009; Krstic et al., 2012; Tinnes et al., 2013; Koie et al., 2014; Trotter et al., 2014; Sato et al., 2016) and gamma-secretase dependent intramembrane proteolysis of its receptors (Haass and De Strooper, 1999; May et al., 2003b; Hoe and Rebeck, 2005, 2008; Litterst et al., 2007; Xu et al., 2009; Bouché et al., 2013; Wasser et al., 2014), which opens up new avenues of crosstalk with other important neuronal signaling receptor systems (Larios et al., 2014).

Whereas the hippocampal defect in EphB1; EphB2 compound deficient mice that could be attributable to defective Reelin-mediated EphB forward signaling is limited to the CA3 region, other functions of this non-canonical Reelin-receptor interaction in the central nervous system might relate to the positioning of Cajal-Retzius cells by EphB-dependent contact repulsion during brain development (Villar-Cerviño et al., 2013), or the fine-tuning of NMDA receptor signaling at the synapse (Cissé et al., 2011; Nolt et al., 2011). The interaction might also be relevant to organs and tissues outside of the nervous system (Jung et al., 2011).

SUMMARY AND PERSPECTIVE

The discovery of the canonical linear lipoprotein receptor/Dab1-dependent Reelin signaling cascade has enabled us to decipher many of the cellular and molecular mechanisms underlying Reelin's multiple functions in the developing and adult brain, and is now one of the best-characterized signaling pathways involved in shaping the developing brain (Ayala et al., 2007). However, even for well-established Reelin targets, many open questions regarding their exact functional relevance remain, which can be attributed to the pleiotropic actions of Reelin at different stages of development, different Reelin-responsive cells, different requirements for specific Reelin domains depending on the developmental stage (Kohno et al., 2015), functional redundancies of signaling components, posttranslational modifications of Reelin (Botella-López et al., 2006), and various technical obstacles (Baek et al., 2014). The biological functions of non-canonical Reelin signaling cascades, as outlined in detail above, are even less well defined and require further investigation.

Many of the non-canonical Reelin-receptor interactions are possibly related to the expression of Reelin outside the brain, which has first been acknowledged shortly after the Reelin gene was discovered (Ikeda and Terashima, 1997). Various tissues and organs contain Reelin at relatively high concentrations, including plasma, blood cells, liver and intestine (Smalheiser et al., 2000; Lugli et al., 2003; Underhill et al., 2003; García-Miranda et al., 2010; Böttner et al., 2014; Ding et al., 2016), and altered expression, glycosylation and processing of peripheral Reelin under pathophysiological conditions has been described (Botella-Lopez et al., 2008). Moreover, an association of (mostly reduced) Reelin expression and malignancy of various tumors has been reported, even in tissues that normally do not express Reelin, which suggests a possible role in the control of tumorigenesis and/or metastasis via unknown mechanisms (Wang et al., 2002; Sato et al., 2006; Perrone et al., 2007; Dohi et al., 2010; Stein et al., 2010; Okamura et al., 2011; Castellano et al., 2016; Lin et al., 2016). In many of these tissues the canonical mediators of Reelin signal transduction are not expressed, whereas interactions of hitherto unknown significance, such as low-affinity binding to the LDL receptor (D'Arcangelo et al., 1999), might turn out to be of physiological significance and hint to as yet unknown or poorly defined functions of Reelin that might be unrelated to signaling, e.g., sequestering of coagulation factors (Tseng et al., 2014).

AUTHOR CONTRIBUTIONS

HHB and PM: designed, wrote and approved the review.

FUNDING

Work in the laboratory of HHB and PM is supported by the Bundesministerium für Bildung und Forschung (BMBF) (ReelinSys, 0316174C) and the Deutsche Forschungsgemeinschaft (DFG) (SFB974/B10).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 10 March 2016
doi: 10.3389/fncel.2016.00065

Neurochemical Phenotype of Reelin Immunoreactive Cells in the Piriform Cortex Layer II

Hector Carceller[1], Laura Rovira-Esteban[1†], Juan Nacher[1], Eero Castrén[2] and Ramon Guirado[2*]*

[1] Departamento de Biologia Celular, Spanish National Network for Research in Mental Health, CIBERSAM, Fundación Investigación Hospital Clínico de Valencia, INCLIVA, Universitat de Valencia Valencia, Valencia, Spain, [2] Neuroscience Center, University of Helsinki, Helsinki, Finland

Reelin, a glycoprotein expressed by Cajal-Retzius neurons throughout the marginal layer of developing neocortex, has been extensively shown to play an important role during brain development, guiding neuronal migration and detachment from radial glia. During the adult life, however, many studies have associated Reelin expression to enhanced neuronal plasticity. Although its mechanism of action in the adult brain remains mostly unknown, Reelin is expressed mainly by a subset of mature interneurons. Here, we confirm the described phenotype of this subpopulation in the adult neocortex. We show that these mature interneurons, although being in close proximity, lack polysialylated neural cell adhesion molecule (PSA-NCAM) expression, a molecule expressed by a subpopulation of mature interneurons, related to brain development and involved in neuronal plasticity of the adult brain as well. However, in the layer II of Piriform cortex there is a high density of cells expressing Reelin whose neurochemical phenotype and connectivity has not been described before. Interestingly, in close proximity to these Reelin expressing cells there is a numerous subpopulation of immature neurons expressing PSA-NCAM and doublecortin (DCX) in this layer of the Piriform cortex. Here, we show that Reelin cells express the neuronal marker Neuronal Nuclei (NeuN), but however the majority of neurons lack markers of mature excitatory or inhibitory neurons. A detail analysis of its morphology indicates these that some of these cells might correspond to semilunar neurons. Interestingly, we found that the majority of these cells express T-box brain 1 (TBR-1) a transcription factor found not only in post-mitotic neurons that differentiate to glutamatergic excitatory neurons but also in Cajal-Retzius cells. We suggest that the function of these Reelin expressing cells might be similar to that of the Cajal-Retzius cells during development, having a role in the maintenance of the immature phenotype of the PSA-NCAM/DCX neurons through its receptors apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR) in the Piriform cortex layer II during adulthood.

Keywords: Reelin, Cajal-Retzius cells, PSA-NCAM, piriform cortex, DCX

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Edited by:
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Reviewed by:
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***Correspondence:**
Eero Castrén
eero.castren@helsinki.fi;
Ramon Guirado
ramon.guirado@helsinki.fi

†Present address:
Laura Rovira-Esteban, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

Received: *13 November 2015*
Accepted: *29 February 2016*
Published: *10 March 2016*

Citation:
Carceller H, Rovira-Esteban L, Nacher J, Castrén E and Guirado R (2016) Neurochemical Phenotype of Reelin Immunoreactive Cells in the Piriform Cortex Layer II. Front. Cell. Neurosci. 10:65. doi: 10.3389/fncel.2016.00065

INTRODUCTION

Reelin is a secreted extracellular matrix glycoprotein, identified 20 years ago by the study of the "reeler" mouse (D'Arcangelo et al., 1995). Previous studies of this particular rodent model found that Reelin is expressed and secreted massively during the early stages of the development by the Cajal-Retzius cells on different brain regions like the neocortex, the hippocampus and the cerebellum (Ogawa et al., 1995; Del Río et al., 1997; Soriano and Del Río, 2005). During this phase, Reelin acts as a chemoattractant of the recently generated neurons, allowing for the correct pattern of neuronal migration and lamination of the main structures of the brain. After early development, the expression of Reelin is downregulated, as Cajal-Retzius cells mainly disappear after their developmental function by cell death or are transformed to functional neurons adopting a new phenotype (Schiffmann et al., 1997; Alcántara et al., 1998; Yabut et al., 2007; Frotscher, 2010). Although the study of Reelin has been classically focused on early development, recent studies have showed an emerging role of Reelin in the adult brain, especially in processes related with neural plasticity like modulation of synaptic plasticity, memory and learning processes (Borrell et al., 1999; Weeber et al., 2002; Niu et al., 2008) and disease (Impagnatiello et al., 1998; Hethorn et al., 2015; Lane-Donovan et al., 2015). In the adult rodent brain, Reelin expression is found sparsely across the telencephalon (Alcántara et al., 1998; Pesold et al., 1998; Ramos-Moreno et al., 2006). However, the Piriform cortex displays a very distinct pattern of expression of Reelin in the rodent adult cortex, although the exact phenotype of the Reelin expressing cells in this area remains unknown.

The Piriform cortex receives synaptic input from the olfactory bulb in the layer I; then through its layer III projects widespread along all the telencephalon (Bekkers and Suzuki, 2013). Of special interest is its layer II, where a subpopulation of immature neurons expressing polysialylated neural cell adhesion molecule (PSA-NCAM), doublecortin (DCX) or cyclic nucleotide-gated (CNGA3) can be found (Nacher et al., 2001; Luzzati et al., 2009; Klempin et al., 2011). However, unlike the neurogenic niches of the adult brain (the subventricular zone and the subgranular zone of the dentate gyrus) these cells are mostly generated during early stages of development (Gómez-Climent et al., 2008; Bonfanti and Peretto, 2011; Rubio et al., 2015). Interestingly, the function and fate of these immature neurons in the Piriform cortex layer II remains unknown (Bonfanti and Nacher, 2012).

In order to improve our understanding about the neural population that express Reelin and its relationship with these immature neurons, we have analyzed the neurochemical phenotype of these cells expressing Reelin in the adult neocortex and Piriform cortex.

MATERIALS AND METHODS

Animals and Histological Procedures

Ten male CD-1 mice and three male Sprague-Dawley rats were caged in a standard lighted environment (12 h light/dark cycle) and free access to food and water. All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de Valencia and by the Animal Ethical Committee of Southern Finland.

At the age of 4 months old all the animals were deeply anesthetized with sodium pentobarbital (200 mg/Kg). Animals were perfused transcardially; first briefly with saline and then with 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4 (PB). The brains were extracted, postfixed for 30 min at 4°C and stored in PB 0.1 M and sodium azide 0.05%. Then, brains were cut in 60 μm thick sections using a vibratome (Leica VT 1000E, Leica). That same procedure was followed with three CD-1 mice pups at 6 days of age after birth (P6).

Stereotaxic Viral Injections

Six mice were injected with adeno-associated viruses in two different regions of the right hemisphere. First, we injected 1 μL of AAV6-CAG-GFP in the olfactory bulb (Antero-Posterior −4.3 mm and Lateral −1 mm relative to Bregma, Deep −0.8 mm with an angle of −16 degrees) targeting the mitral cell layer. We used this virus as an anterograde tracer, since it allow us to follow the projection of these neurons to the Piriform cortex. Those same animals were also injected in the Piriform cortex of the right hemisphere (AP −0.46 mm and L −2 mm relative to Bregma, and 4.6 mm deep with an angle of −16 degrees) with 1 μL of AAV5-CMV-mCherry targeting its layer II, to study the morphology of those cells expressing Reelin.

Immunohistochemistry

Free-floating sections were processed as it follows: after washing with phosphate saline buffer (PBS), non-specific bindings were blocked by 10% normal donkey serum (NDS; Abcys), 0.2% Triton-X100 (Sigma) in PBS for 1 h. Sections were then incubated for 48 h at 4°C with different primary antibody cocktails diluted in PBS—0.2% Triton-X100 (see **Table 1**). After washing, sections were incubated for 2 h at room temperature with different secondary antibody cocktails also diluted in PBS—0.2% Triton-X100 (see **Table 1**). Finally, sections were washed in PB 0.1 M, mounted on slides and coverslipped using fluorescence mounting medium (Dako).

Confocal Analysis and Quantification

We analyzed the co-expression of different cell markers in Reelin expressing cells both in the Piriform cortex and the neocortex. We obtained z-stacks of single confocal planes using a confocal microscope (Leica TCS SPE or Zeiss LSM 700). At least three different coronal sections were analyzed per animal between +0.5 and −2.0 mm Antero-Posterior relative to Bregma. From each section 20 cells were analyzed in the Piriform cortex, while in the neocortex all Reelin expressing cells of each stack where analyzed to obtain the percentages describing the neurochemical phenotype, expressed over the total of Reelin expressing cells.

To analyze the animals injected with different adeno-associated viruses, we first obtained single confocal planes at each

TABLE 1 | List of primary and secondary antibodies used in the study.

Anti	Host	Isotype	Dilution	Company
Primary antibodies				
Reelin	Mouse	Monoclonal IgG1	1:2000	Millipore
TBR1	Rabbit	Polyclonal IgG	1:1000	Abcam
DCX	Rabbit	Polyclonal IgG	1:1000	Abcam
DCX-C18	Goat	Polyclonal IgG	1:250	Santa Cruz
GAD67	Mouse	Monoclonal IgG2a	1:500	Millipore
Glu2R/3	Rabbit	Polyclonal IgG	1:100	Chemicon
PSA-NCAM	Mouse	Monoclonal IgM	1:700	DSHB
CNGA3	Rabbit	Polyclonal IgG	1:2000	Alomone
GFAP	Rabbit	Polyclonal IgG	1:500	Millipore
NeuN	Rabbit	Polyclonal IgG	1:1000	Millipore
VLDL	Rabbit	Polyclonal IgG	1:100	Abcam
APOER2	Rabbit	Polyclonal IgG	1:100	Abcam
Secondary antibodies				
Goat IgG	Donkey	Alexa555	1:400	Invitrogen
Mouse IgG1	Donkey	DyLight 649	1:400	Jackson
Mouse IgG2A	Donkey	Alexa555	1:400	Invitrogen
Mouse IgM	Donkey	Alexa488	1:400	Invitrogen
Rabbit IgG	Donkey	Alexa555	1:400	Invitrogen
Rabbit IgG	Donkey	Alexa488	1:400	Invitrogen

injection site to confirm the correct location of each injection. Then we obtained z-stacks in the Piriform cortex to study the dendritic arbor of dendrites expressing mCherry and axons expressing green fluorescent protein (GFP).

RESULTS

In the Neocortex Reelin is Expressed Mostly by Interneurons

In the mouse neocortex, Reelin-expressing cells were found scattered in all layers, although with a higher density in the Layer I (**Figure 1**). When analyzing the neurochemical phenotype of these neocortical Reelin-expressing cells, we have found that few of them (10 ± 2.5%) expressed the Glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors 2/3, only expressed in excitatory neurons (Leranth et al., 1996; Toth et al., 1997). On the other hand, most of these Reelin-expressing cells in the neocortex, especially in layer I, expressed the 67 KDa isoform of the glutamic acid decarboxylase enzyme (GAD67; 51 ± 9%), which is exclusively expressed by interneurons (**Figure 2A**).

We then analyzed whether these Reelin expressing interneurons would also express the PSA-NCAM. We found that the vast majority of Reelin expressing interneurons do not express PSA-NCAM (4.1 ± 2.5%), although in very few cases we found a very weak PSA-NCAM expression in Reelin expressing interneurons (**Figure 2B**).

In the Piriform Cortex Layer II Reelin Expressing Cells Lack Mature and Immature Neuronal Markers

In the Piriform cortex, Reelin-expressing cells were densely located in the layer II, being scarce in the layers I and III (**Figure 3A**). We found that a small fraction of these Reelin expressing cells, mainly located in the layer II, expressed the excitatory marker GluR2/3 (8 ± 3%). On the contrary, in layer I, most of the Reelin expressing cells expressed the interneuronal marker GAD67 (74 ± 14.5%). However, when considering all three layers only few expressed GAD67 (5.8 ± 2.8%; **Figure 3A**), indicating that most of the cells expressing Reelin in the Piriform cortex layer II expressed neither of these markers.

Then, we analyzed the expression of the glial fibrillary acidic protein (GFAP). We found out that none of these Reelin expressing cells expressed GFAP, therefore we discarded the possibility that these neurons would correspond to glial cells (**Figure 3B**).

We also analyzed the expression of immature neuronal markers. We found that none of the Reelin expressing cells were immunoreactive for PSA-NCAM, the A3 subunit of the CNGA-3 ion channel (**Figures 3B,D**) or DCX (**Figure 3C**).

However, we found that these Reelin expressing cells in the Piriform cortex express the Neuronal Nuclei protein (NeuN) even if in some cells this expression is faint (**Figure 3E**).

Reelin Expressing Cells Extend their Dendritic Arbor Towards Layer I and Receive Olfactory Input

We analyzed the connectivity of these neurons by transfecting mCherry under a generic promoter (CMV) to the Piriform cortex combined with Reelin immunohistochemistry (**Figure 4A**). We observed that very few neurons in the upper layer II were transfected. Those expressing mCherry and Reelin displayed a dendritic arbor (**Figure 4B**) that extended towards the layer I. By transfecting the mitral cell layer of the olfactory bulb with GFP under the CAG promoter (**Figure 4A**), we could find axons from these cells in the layer I of the Piriform cortex where they appear in apposition to the dendrites of Reelin expressing cells (**Figure 4B**). All these

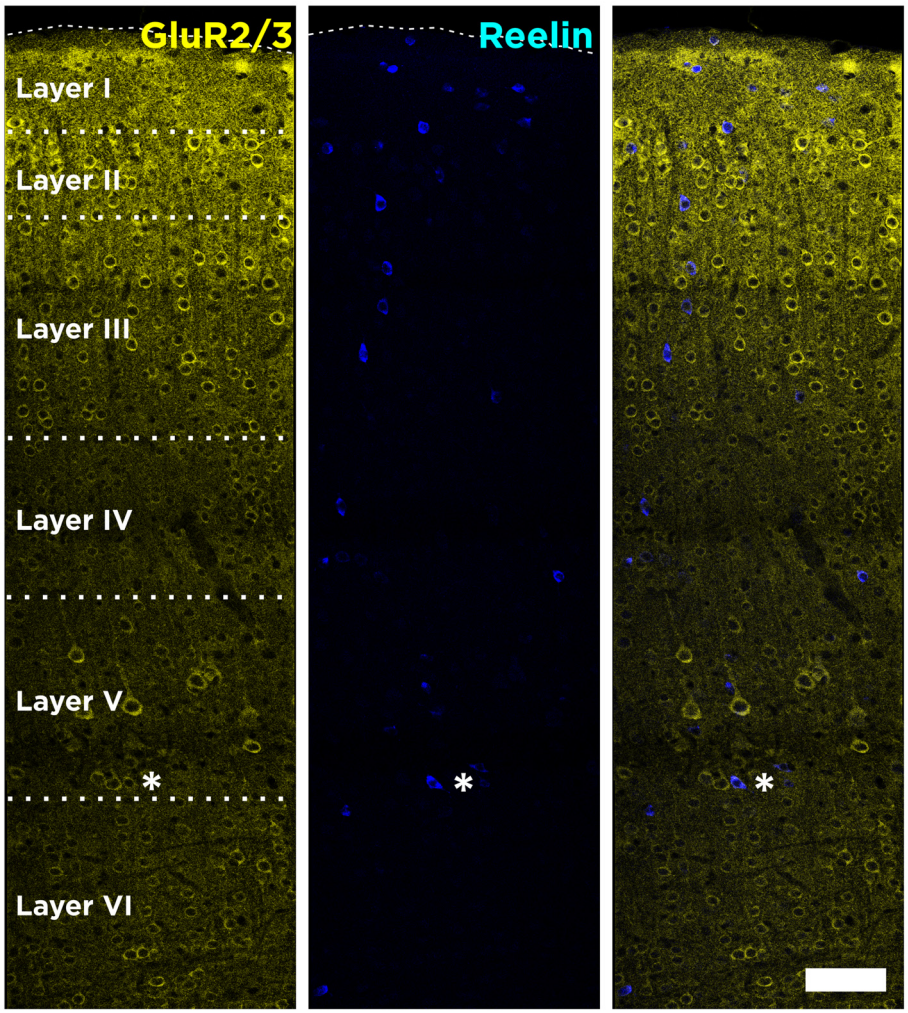

FIGURE 1 | Distribution of Reelin-positive cells in the neocortex. Tiled image composition of single confocal planes showing the expression of Reelin and GluR2/3 in all layers of the neocortex of adult mice. GluR2/3 expression defines clearly the boundaries between the different layers. Asterisk indicates a neuron co-expressing Reelin and GluR2/3. Scale bar = 80 μm.

results suggesting that these neurons expressing mCherry correspond to semilunar neurons. Interestingly however, we found some of these axons from the olfactory bulb innervating deeper layers of the Piriform cortex, contacting perisomatically the Reelin expressing cells located in the layer IIb or III (**Figure 4C**).

Reelin Expressing Cells in the Piriform Cortex Layer II Resemble the Cajal-Retzius Cells from the Developing Brain

We analyzed the expression of the neuron-specific transcription factor T-box brain 1 (TBR-1) as it is expressed by neurons of pallial origin and by Cajal-Retzius neurons during brain development (Hevner et al., 2001). Interestingly, we found that the immense majority of Reelin expressing cells in the Piriform cortex layer II express TBR-1 in the adulthood (89.5 ± 2.5%; **Figure 5A**).

We also analyzed the expression of Reelin and TBR-1 at postnatal brain development (P6). We confirm that Cajal-Retzius cells in the marginal zone of the neocortex express both Reelin and TBR-1 (**Figure 5C**). Interestingly, we found none or very few Cajal-Retzius cells in the marginal layer of the Piriform cortex at P6. On the other hand, the abundant population of Reelin/TBR-1 neurons in the layer II, which neurochemical phenotype resembles that of Cajal-Retzius cells, is already present at this early stage (**Figure 5B**).

Immature Neurons from the Piriform Cortex and the Role of Reelin

The detailed analysis of PSA-NCAM expression in the Piriform cortex shows not only the close proximity of both subpopulations: an upper layer II densely packed with Reelin expressing cells and a lower layer II with a numerous immature neurons expressing PSA-NCAM and DCX (**Figure 6A**).

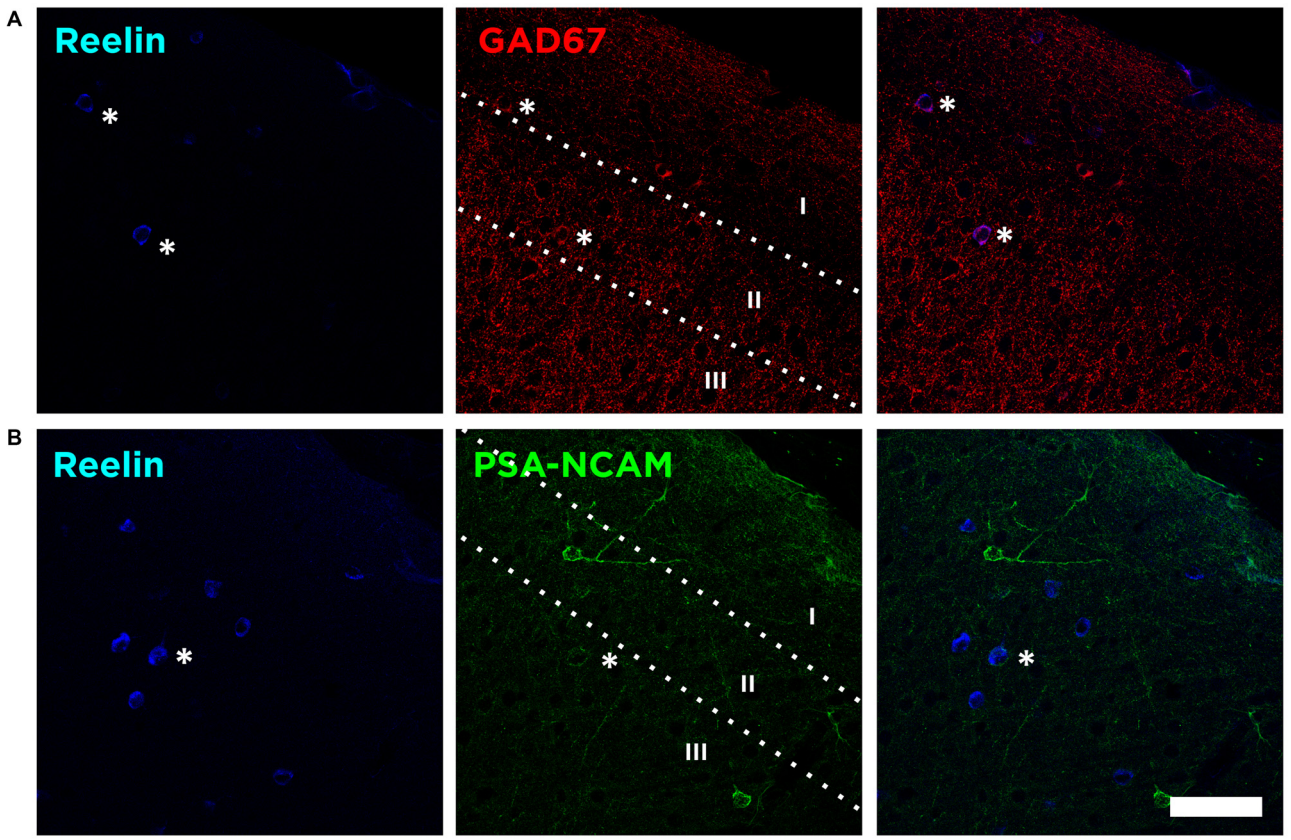

FIGURE 2 | Reelin is expressed by interneurons lacking PSA-NCAM. (A) Single confocal planes showing the expression of Reelin, and glutamic acid decarboxylase (GAD67) in the layers I–III of the neocortex. Asterisks indicate co-localization of these markers with Reelin. **(B)** Single confocal planes showing the lack of co-localization of PSA-NCAM and Reelin positive cells in the neocortex. Scale bar = 60 μm.

It also reveals the existence of long thick processes forming a *pseudo-radial glia* that expresses PSA-NCAM (**Figure 6A**), connecting the lowest part of the external capsule and the layer II of the Piriform cortex, in which end we find the Reelin expressing cells (**Figure 6A**), resembling the canonical function during development of Reelin expressed by Cajal-Retzius cells.

Interestingly, although not limited to the immature neurons of the Piriform cortex, we find expression of both Reelin receptors, apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR), on the surface of PSA-NCAM expressing neurons in the layer II (**Figures 6B,C**). These results suggest that the secretion of Reelin might be involved in the control, through these receptors, of the maturation process of the subpopulation expressing PSA-NCAM and DCX in the Piriform cortex layer II.

DISCUSSION

In the present study, we describe in detail the neurochemical phenotype of Reelin expressing cells in the neocortex and the Piriform cortex in the adult mouse brain. In the neocortex we confirm a widespread distribution of Reelin positive cells among all layers of the neocortex, confirming previous results based in the study of Reelin expression in the adult rodent brain (Alcántara et al., 1998; Pesold et al., 1998; Ramos-Moreno et al., 2006). There are divergent results regarding the nature of these cells according to the species studied. There is solid evidence that Reelin is expressed both by GABAergic and glutamatergic neurons in rats and non-human primates (Pesold et al., 1998; Martínez-Cerdeño and Clascá, 2002; Ramos-Moreno et al., 2006). However, the only previous study that focused in the adult mouse brain found that Reelin is expressed exclusively in GABAergic neurons in the neocortex (Alcántara et al., 1998). We provide here evidence that, at least to a certain extent, there are also few glutamatergic excitatory neurons expressing Reelin in the mouse neocortex.

Reelin is expressed widely during development but is restricted to a subpopulation of neurons in the adult brain (Pesold et al., 1998; Frotscher, 2010). Nevertheless, under different paradigms of adult brain plasticity, there is an increase of Reelin expression suggesting a role in plasticity involving its receptors ApoER2 and VLDL through PSD-95 with N-Methyl-D-aspartate (NMDA) receptors, promoting LTP (Weeber et al., 2002; Hoe et al., 2006; Li et al., 2007; Qiu and Weeber, 2007; Tiraboschi et al., 2013; Hethorn et al., 2015).

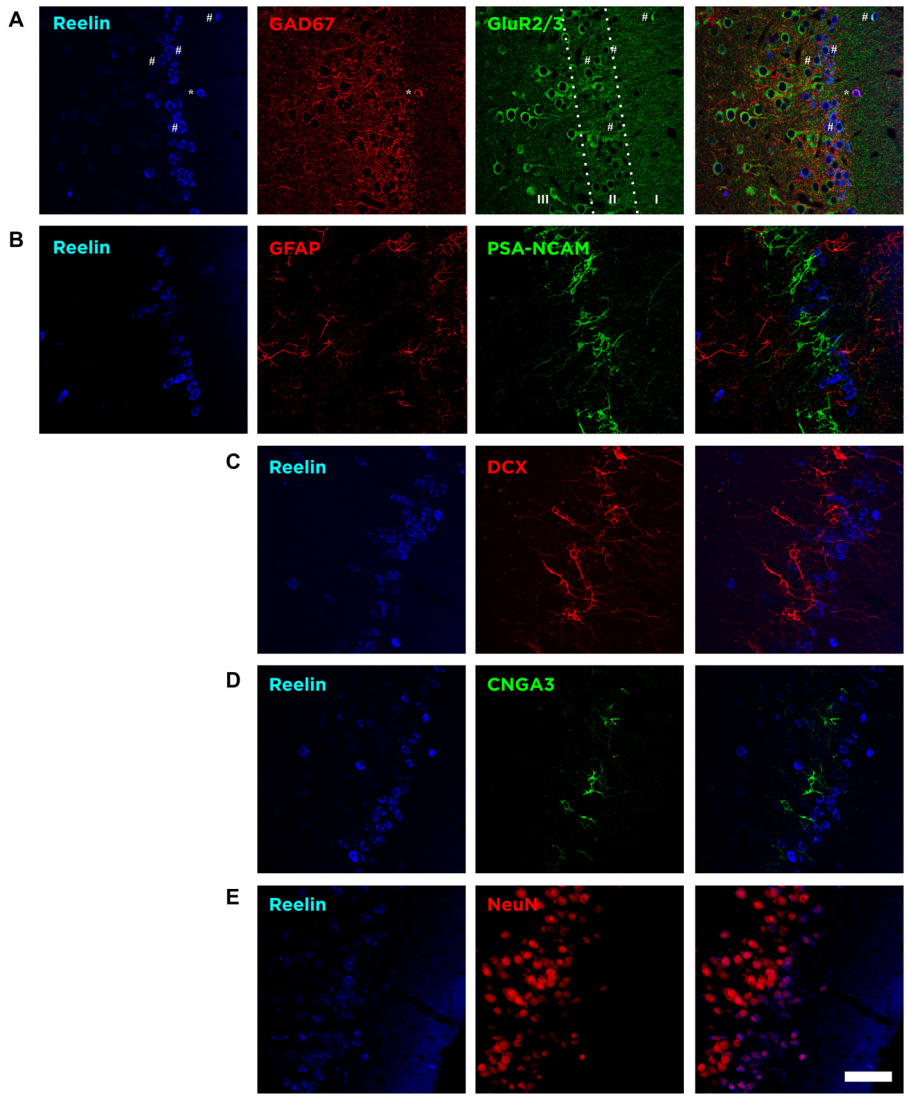

FIGURE 3 | The majority of Reelin expressing cells in the layer II express Neuronal Nuclei (NeuN) but lack expression of proteins related to mature excitatory and inhibitory neurons, glia and inmmature neurons. (A) Single confocal planes showing the expression of Reelin, GAD67 and GluR2/3. Reelin expression was particularly high in the uppermost part of the layer II and few Reelin expressing cells expressed either GluR2/3 or GAD67. Hashes indicate Reelin cells expressing GluR2/3 and asterisks indicate the co-expression of Reelin and GAD67. **(B)** Single confocal planes showing Reelin expressing cells lack do not express either PSA-NCAM or glial fibrillary acidic protein (GFAP). **(C)** Single confocal planes showing lack of DCX expression by Reelin expressing cells. **(D)** Single confocal planes showing Reelin expressing cells lack expression of CNGA3. **(E)** Single confocal planes showing the co-localization of NeuN and Reelin positive cells. Scale bar = 60 μm.

Similar to Reelin, PSA-NCAM is expressed by inhibitory neurons in the adult neocortex and it has been suggested to play an important role in adult brain plasticity as well (Nacher et al., 2013). Moreover, both molecules have robustly been related to changes in the synaptic activity of the cells where they are expressed in the adulthood (Qiu and Weeber, 2007; Guirado et al., 2014). Thus, we investigated whether this subpopulation of mature interneurons expressing these molecules might be the same. However, although the distribution of these two subpopulations is remarkably similar, we found that the vast majority of Reelin expressing cells do not express PSA-NCAM.

In the present study, we also analyze the nature of Reelin expressing cells in the Piriform cortex, which has never been studied in detail before. We found Reelin positive cells scattered in the layers I and III and, as previously described, a large density of these cells packed in the layer II (Alcántara et al., 1998). In the layer I, as in the neocortex, Reelin expressing cells also express the inhibitory marker GAD67 indicating that Reelin-positive cells in the Piriform cortex layer I are mainly interneurons, as indicated in that same study

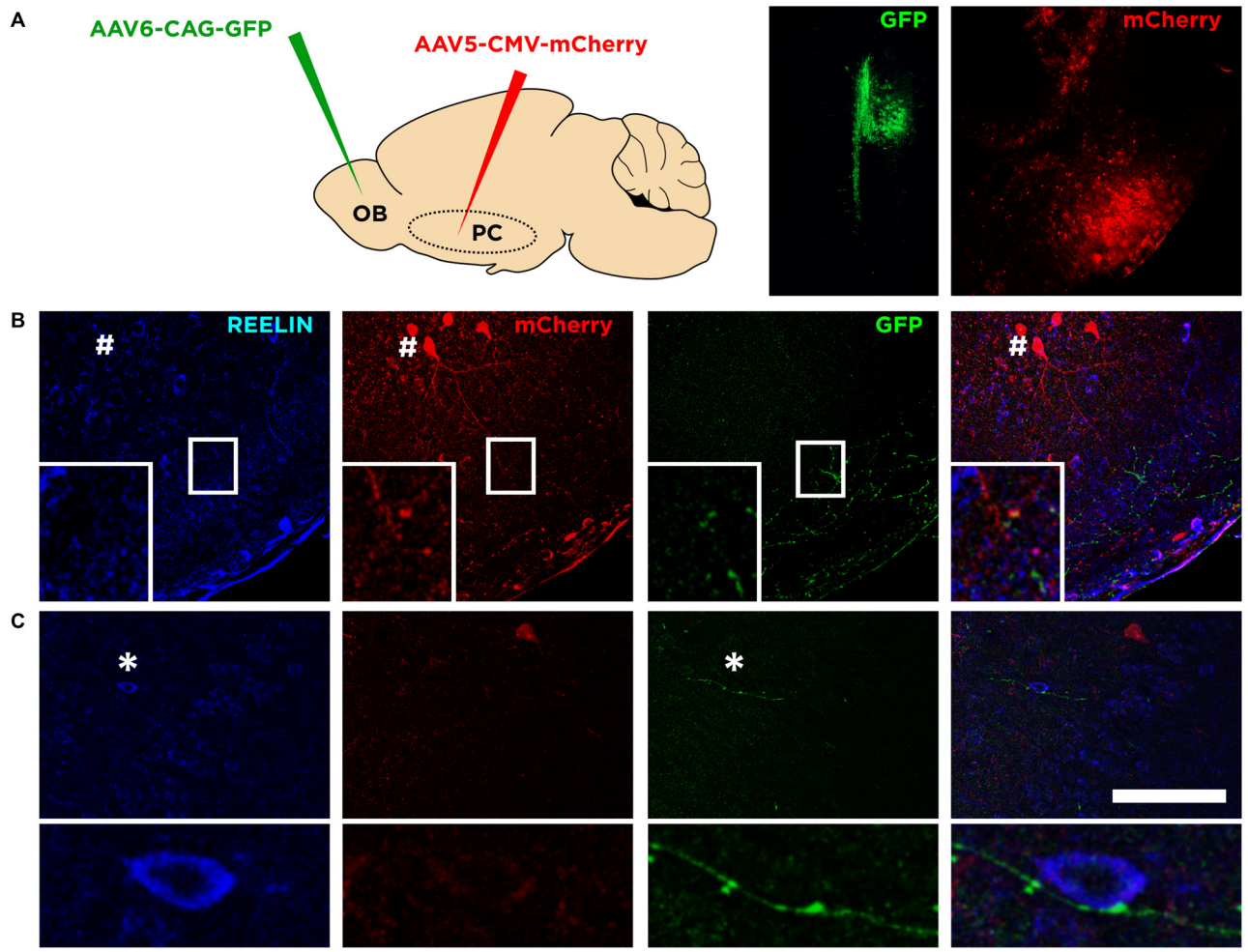

FIGURE 4 | Connectivity of Reelin expressing cells. (A) Diagram showing the viral strategy employed and the injection site of each of the virus in the olfactory bulb and the Piriform cortex (left and right pictures respectively). **(B)** Confocal images showing the morphology of a Reelin expressing cell transfected with mCherry (indicated by a hash symbol) and a possible connection between an axonal bouton from the olfactory bulb expressing GFP and a dendrite of a Reelin expressing cell (inset). **(C)** Confocal images showing an axon from the olfactory bulb expressing GFP and a possible contact with a soma expressing Reelin in deep layer II (shown in the inset; indicated by an asterisk symbol). Scale bar = 100 μm.

(Alcántara et al., 1998), suggesting a common origin with those of the neocortex.

Since the neurons in the layer II lack expression of GAD67 and the large number of cells present there, it was previously assumed these neurons would correspond to excitatory neurons. However, the neurochemical phenotype of these neurons has not ever been investigated. We found that Reelin expressing neurons of the layer II displayed indeed a divergent neurochemical phenotype: most of the Reelin expressing neurons of the layer II expressed neither GAD67 nor GluR2/3. Only few of these neurons express GluR2/3; a marker of excitatory neurons (Leranth et al., 1996). In fact, our viral strategy to study the morphology revealed that the virus did have low preference for the neurons located in the uppermost part of layer II. Only few neurons expressed mCherry and these neurons resembled glutamatergic semilunar neurons, extending dendrites towards the layer I to receive synaptic input from the olfactory bulb, as these semilunar neurons had been previously described (Suzuki and Bekkers, 2011). It is likely then that these semilunar neurons represent the fraction (8%) of Reelin expressing cells expressing GluR2/3 in the layer II of the Piriform cortex.

To explore further the phenotype of these Reelin expressing cells we discarded the possibility that these neurons would correspond to glia, and in fact our results showed that there was no colocalization with GFAP. Then we considered whether Reelin expressing cells might correspond to the immature neurons described in the layer II of paleocortex (Nacher et al., 2002; Luzzati et al., 2009; Klempin et al., 2011). However, our results indicate that these neurons do not express classical

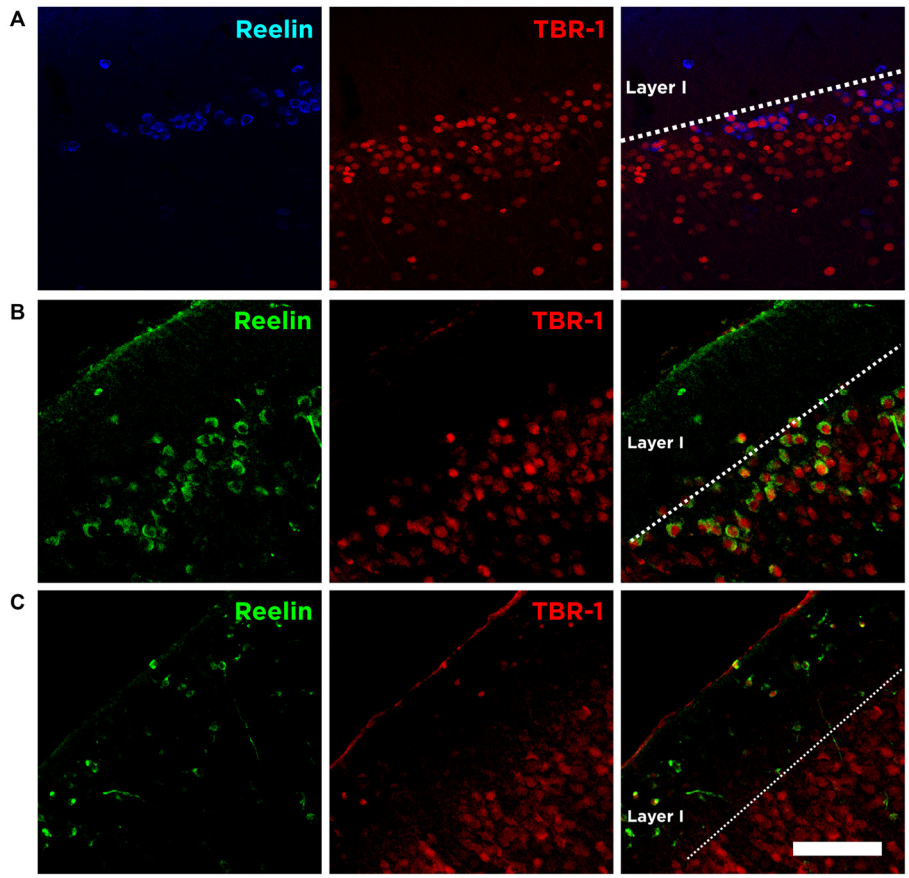

FIGURE 5 | Expression of Reelin and TBR-1 at different developmental stages. (A) Single confocal planes showing the expression of Reelin and TBR-1 in the adult Piriform cortex, **(B)** in the developing Piriform cortex (P6) and **(C)** in the developing neocortex. Scale bar = 60 μm.

immature markers such as PSA-NCAM, CNGA-3 or DCX.

The presence of cortical guide scaffolds expressing PSA-NCAM resembling radial glia intuitively suggests a pathway from subventricular areas to the Piriform cortex as a diversion from the elbow of the rostral migratory stream as suggested previously (Shapiro et al., 2007). However, in a previous study, we demonstrated that these immature neurons expressing PSA-NCAM, CNGA-3 and DCX are generated during embrionary development, and not during adult life (Gómez-Climent et al., 2008). Interestingly, we found that these immature neurons seem to express Reelin receptors such as ApoER2 and VLDL, so we hypothesize that Reelin might play a role in the maturation of these immature neurons. However, there is still controversy whether these neurons mature throughout life: some laboratories have suggested that these neurons mature into interneurons (Xiong et al., 2008; Cai et al., 2009; Zhang et al., 2009) while others have suggested that these neurons mature into excitatory neurons (Gómez-Climent et al., 2008; Luzzati et al., 2009).

Considering these results, we decided to study the expression of the neuron-specific transcription factor TBR-1, a molecule found on pallium-derived neurons committed to differentiate into excitatory neurons and in Cajal-Retzius cells (Hevner et al., 2001; Bedogni et al., 2010). To our surprise we found that the majority of Reelin expressing neurons in the layer II of the Piriform cortex also express TBR-1. Therefore, although the morphology in terms of soma size is different, these Reelin/TBR-1 cells resemble the neurochemical phenotype of Cajal-Retzius cells during development (Hevner et al., 2003). In fact, at postnatal day 6 we observe Cajal-Retzius cells Reelin/TBR-1+ in the marginal layer of the neocortex, but practically absent in the Piriform cortex, where we can find already the Reelin expressing cells in the uppermost part of layer II, suggesting that at that developmental stage the function of the Cajal-Retzius cells is carried out by our subpopulation of Reelin/TBR-1 cells.

Altogether our results indicate that Reelin is expressed in the Piriform cortex layer II by different subpopulations of neurons including, but not limited to, semilunar glutamatergic neurons and interneurons. These results also suggest that Reelin might be involved through ApoER2 and VLDL in the maturation stage of the subpopulation of immature neurons located in the Piriform cortex layer II. Future experiments controlling the secretion of

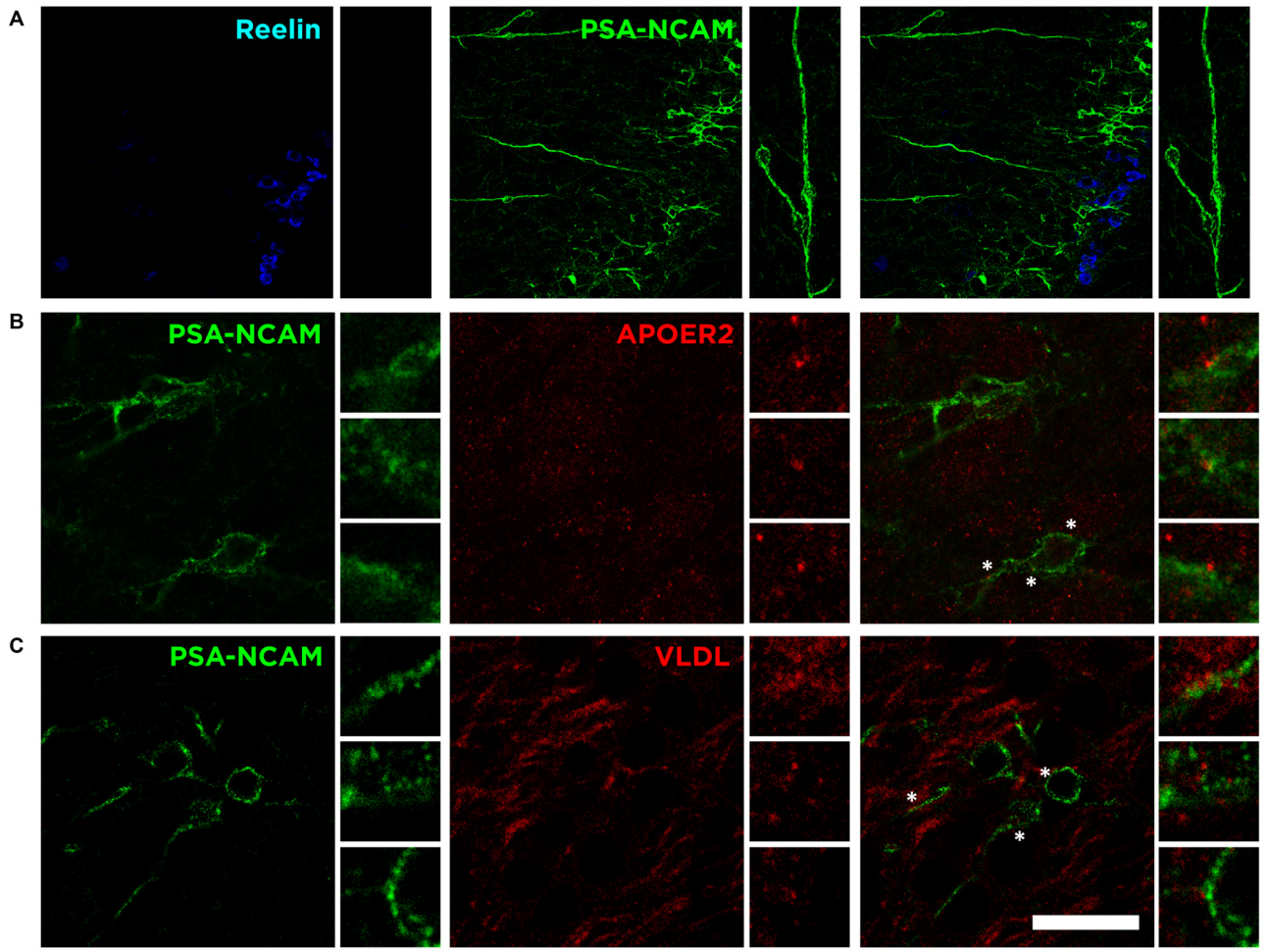

FIGURE 6 | Possible influence of Reelin on immature elements expressing PSA-NCAM. (A) Single confocal image showing the expression of Reelin, PSA-NCAM. *Pseudo-radial glia* (inset) expressing PSA-NCAM might act as an scaffolding cortical guide with Reelin secretion at the end, resembling the canonical function of Reelin during development. **(B)** Single confocal plane showing the expression of PSA-NCAM and ApoER2 in the layer II of the Piriform cortex. **(C)** Singles confocal plane showing the expression of PSA-NCAM and VLDL. Asterisks indicate inset where expression of Reelin receptors is in close proximity to PSA-NCAM expressing cells. Scale bar = 60 μm in **(A)** and 20 μm in **(B,C)**.

Reelin will be needed to understand yet the physiological role of Reelin in the adult brain.

AUTHOR CONTRIBUTIONS

RG designed the study; RG, HC and LR-E performed the experiments; RG and HC analyzed the data; RG, EC and JN wrote the manuscript.

FUNDING

This study was supported by ERC Grant No 322742—iPLASTICITY, Sigrid Juselius foundation and Academy of Finland Grant #257486. The Spanish ministry of economy and competitiveness: BFU2012-32512 and MICINN-PIM2010ERN-00577/NEUCONNECT and Generalitat Valenciana: PROMETEO2013/069.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 30 May 2016
doi: 10.3389/fncel.2016.00138

Reelin Exerts Structural, Biochemical and Transcriptional Regulation Over Presynaptic and Postsynaptic Elements in the Adult Hippocampus

Carles Bosch[1,2,3], Ashraf Muhaisen[1,2,3,4], Lluís Pujadas[1,2,3], Eduardo Soriano[1,2,3,4,5] and Albert Martínez[1]**

[1] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain, [2] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain, [3] Vall d'Hebron Institut de Recerca, Barcelona, Spain, [4] Institute of Neurosciences, University of Barcelona, Barcelona, Spain, [5] Institució Catalana de Recerca i Estudis Avançats Academia, Barcelona, Spain

Reelin regulates neuronal positioning and synaptogenesis in the developing brain, and adult brain plasticity. Here we used transgenic mice overexpressing Reelin (Reelin-OE mice) to perform a comprehensive dissection of the effects of this protein on the structural and biochemical features of dendritic spines and axon terminals in the adult hippocampus. Electron microscopy (EM) revealed both higher density of synapses and structural complexity of both pre- and postsynaptic elements in transgenic mice than in WT mice. Dendritic spines had larger spine apparatuses, which correlated with a redistribution of Synaptopodin. Most of the changes observed in Reelin-OE mice were reversible after blockade of transgene expression, thus supporting the specificity of the observed phenotypes. Western blot and transcriptional analyses did not show major changes in the expression of pre- or postsynaptic proteins, including SNARE proteins, glutamate receptors, and scaffolding and signaling proteins. However, EM immunogold assays revealed that the NMDA receptor subunits NR2a and NR2b, and p-Cofilin showed a redistribution from synaptic to extrasynaptic pools. Taken together with previous studies, the present results suggest that Reelin regulates the structural and biochemical properties of adult hippocampal synapses by increasing their density and morphological complexity and by modifying the distribution and trafficking of major glutamatergic components.

Keywords: dendritic spines, spine apparatus, NMDA receptors, axon terminal, electron microscopy

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers University, USA

Reviewed by:
Menahem Segal, Weizmann Institute of Science, Israel
Alev Erisir, University of Virginia, USA

****Correspondence:***
Albert Martínez
albmartinez@ub.edu;
Eduardo Soriano
esoriano@ub.edu

Received: *15 March 2016*
Accepted: *10 May 2016*
Published: *30 May 2016*

Citation:
Bosch C, Muhaisen A, Pujadas L, Soriano E and Martínez A (2016) Reelin Exerts Structural, Biochemical and Transcriptional Regulation Over Presynaptic and Postsynaptic Elements in the Adult Hippocampus. Front. Cell. Neurosci. 10:138. doi: 10.3389/fncel.2016.00138

INTRODUCTION

Reelin, a large secreted glycoprotein of the extracellular matrix, controls neuronal migration and brain development (D'Arcangelo et al., 1995; Alcantara et al., 1998; Rice and Curran, 2001; Soriano and Del Rio, 2005; Cooper, 2008; D'Arcangelo, 2014). Reelin binds to lipoprotein family receptors apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDL; D'Arcangelo et al., 1999; Hiesberger et al., 1999), and induces the phosphorylation of the adaptor protein mDab1 (Howell et al., 1999; Ballif et al., 2004). The Reelin cascade therefore includes several signaling pathways, including distinct members of the Src Kinase family (Arnaud et al., 2003),

Erk1/2 (Simo et al., 2007; Lee et al., 2014) and AKT/Gsk3 (Beffert et al., 2002; Gonzalez-Billault et al., 2005). The duration of the Reelin signaling is regulated by ubiquitination and degradation of p-mDab1, triggered by the Cul5 cascade (Feng et al., 2007; Simo et al., 2010; Simo and Cooper, 2013).

In the adult cerebral cortex, Reelin is expressed mainly by γ-amino-butyric acid (GABA) interneurons (Alcantara et al., 1998; Pesold et al., 1998). Reelin promotes the maintenance of synaptic connectivity by modulating synaptic plasticity (Beffert et al., 2005; Bender et al., 2010; Pujadas et al., 2010) and by regulating the composition and traffic of NMDA and AMPA receptor subunits (Chen et al., 2005; Qiu et al., 2006b; Groc et al., 2007; Qiu and Weeber, 2007). Moreover, *in vitro* and *in vivo* studies have revealed that both mDab1 mutant mice and Reeler mice show a reduction in spine density, thereby supporting the notion that Reelin is involved in synaptic development and/or maturation (Niu et al., 2004, 2008; Ventruti et al., 2011). Similarly, local *in vivo* injections of Reelin in wild-type mice promote an increase in spine density and synapse formation (Rogers et al., 2011, 2013). In parallel, transgenic mice overexpressing Reelin (Reelin-OE) show hypertrophy of dendritic spines in the adult hippocampus (Pujadas et al., 2010). It is known that dendritic spine morphology is linked to synaptic efficacy (Bourne and Harris, 2008; Colgan and Yasuda, 2014). For example, spines are enlarged after long-term potentiation (LTP) – induced stimulation (Matsuzaki et al., 2004; Yang et al., 2008), and both the spine head volume and the number of AMPA-type glutamate receptors increase synaptic strength (Takumi et al., 1999; Matsuzaki et al., 2001). Taken together, these findings suggest that Reelin modulates synaptic efficacy not only by regulating the density of dendritic spines but also by controlling dendritic spine and synaptic architecture. Finally, recent studies point to the participation of Reelin in Alzheimer's disease and in the synaptopathies associated with this condition (Knuesel, 2010; Pujadas et al., 2014; Lane-Donovan et al., 2015).

To understand the role of this protein in adult neural function, there is a need for a better knowledge of the fine regulation of the structural and molecular characteristics of dendritic spines and synapses, specifically in the adult brain *in vivo*. Here we take advantage of transgenic mice that overexpress Reelin exclusively in late postnatal and adult forebrain, with no impact on neural migration (Pujadas et al., 2010) to determine the effects of Reelin overexpression on the structural and molecular phenotype of dendritic spines and synaptic terminals in the hippocampus. Our data provide the first evidence that Reelin modulates the structural and molecular properties of adult synapses by altering the synaptic distribution of glutamate receptors and associated proteins rather than by controlling the protein and gene expression levels of the same.

MATERIALS AND METHODS

Animals

Transgenic Reelin-OE mice of either sex (TgRln) used in this study have been previously described (Pujadas et al., 2010). This line overexpresses Reelin ectopically under the calcium–calmodulin-dependent kinase IIa promoter (pCaMKIIα) through a tTA transactivator. Feeding for 1 week with doxycycline-containing feed (Bio-Serv, 200 mg per kg) successfully blocks the transgene expression (Pujadas et al., 2010). Mice were housed in groups (2–6 mice per cage) and maintained in a 12-h light-dark cycle with access to food and water *ad libitum*. All procedures were performed in accordance to the protocols approved by local ethics committee and in compliance with the European guidelines for humane treatment of laboratory animals.

Synaptosome Extracts and Western Blots

Hippocampal synaptosome-enriched protein extracts were obtained following a protocol described elsewhere (Niu et al., 2008). Briefly, adult mice were sacrificed by decapitation, brains were removed from the skull, and hippocampi were dissected in ice-cold dissection buffer and pooled (four hippocampi per sample). Hippocampi were homogenized in 1.5 ml of SP buffer with an Eppendorf tissue grinder. A 150 μl aliquot of homogenate was kept for validation purposes. The homogenate was centrifuged twice at 800*g* for 5 min at 4°C in a F45-24-11 rotor on an Eppendorf® 5415R centrifuge, and pellets were discarded. The cleaned supernatant was then centrifuged at 5769*g* for 11 min at 4°C in a TLA-55 rotor on a Beckmann OPTIMA TLX ultracentrifuge. The supernatant was then carefully removed, and the pellet was resuspended in 250 μl of lysis buffer.

Western blots against various proteins of interest were performed using the following antibodies: Actin (mouse monoclonal "mAb" clone C4; 1:100000; Millipore), Synapsin 2 (rabbit polyclonal "poly"; 1:500; Stressgen Bioreagents), SNAP25 (mouse mAb clone SMI-81; 1:750; Becton-Dickinson), Synaptopodin (rabbit poly; 1:750; Synaptic Systems), NR2a (rabbit poly; 1:500; Millipore), NR2b (rabbit poly; 1:500; Millipore), p-Cofilin on serine 3 (rabbit poly; 1/100; Santa Cruz), NR1 (mouse mAb clone 54.1; 1/1000; BD Biosciences), GluR1 (rabbit mAb clone C3T; 1/500; Millipore), GluR2/3 (rabbit poly; 1/1000; Chemicon), postsynaptic density (PSD)-95 (mouse mAb clone 7E3-1B8; 1:1000; Millipore), CaMKII (mouse mAb clone 6G9; 1/2000; Affinity Bioreagents), LIMK-1 (rabbit poly; 1/100; Santa Cruz), phospho-LIMK1 (Thr508)/LIMK2 (Thr505; rabbit poly; 1/500; Cell Signaling Technology) and Cofilin (rabbit poly; 1/500; Millipore).

Immunohistochemistry

Five-month-old Reelin transgenic mice ($n = 3$), control littermates ($n = 3$), and Reelin transgenic mice treated with doxycycline (DOX) for 7 days ($n = 3$) were anesthetized and perfused with 4% paraformaldehyde in 0.1 M Phosphate buffer. Brains were removed, post-fixed overnight in the same solution, cryoprotected, and frozen. They were then coronally sectioned at 30 μm, distributed into 10 series, and maintained at −20°C in cryoprotectant solution (PB 0.1 M, sucrose 30% and glycerol-30% ethylene glycol). For

the immunodetection of Synaptopodin, sections were blocked for 2 h at RT with PBS containing 10% of normal goat serum (NGS) and 0.2% of gelatin and then incubated with rabbit anti-Synaptopodin (1:500, Synaptic Systems) overnight at 4°C with PBS–5% NGS. Next, they were incubated with goat anti-rabbit fluorochrome-labeled secondary antibodies (1:700, Molecular Probes), mounted in Mowiol, and stored at −20°C. Sections were viewed in a Leica SP2 confocal scanning laser microscope. The acquisition of confocal stacks of 3–5 dorsal hippocampi was achieved in a Leica SP2 microscope. Images were taken at a 20× magnification with a z-spacing of 2 μm. To allow comparison between all the animal groups, sections were immunolabeled in bulk and imaged in identical conditions. Acquisition *x*- and *y*-resolution was set at 1.46 μm/px. From each stack, the intensity of two to three confocal slices was z-projected. Regions of interest (ROIs) were defined across various layers (5 to 10 ROIs per layer), avoiding histological artifacts such as vessels or cell nuclei from which gray values were recorded. Intensities were normalized to average slice intensity to retrieve a local contrast index comparable across acquisitions.

Gene Expression Microarray Analysis of Reelin-OE Mice

The differential expression of genes directly associated with the term "Synapse" (GO:0045202) was examined from data collected in the analysis of the genome-wide expression pattern of whole hippocampus. Briefly, mouse hippocampi (n = 5–7 mice per group) were dissected out and immediately frozen in liquid nitrogen. mRNAs were extracted using a Trizol (Invitrogen)-based protocol, quantified in Qubit Fluorometer (Life Technologies), and subjected to quality control using a Bioanalyzer (Agilent). cDNAs were synthesized and amplified by Ovation System (NuGEN) and hybridized to GeneChip Mouse Genome 430 2.0 array (Affymetrix) at the *Functional Genomics Core Facility* (IRB Barcelona). GeneChips were scanned in a GeneChip Scanner 3000 (Affymetrix). Full analysis of data was performed at the *Biostatistics/Bioinformatics Unit* (IRB Barcelona), as previously described (Rossell et al., 2008). Of the 396 genes directly associated with GO:0045202, 360 gene probe sets with 871 independent probes are represented in the GeneChip (details in **Supplementary Table S1**). In addition, probes corresponding to genes Limk1/Limk2/Cfl1/Cfl2 were also analyzed (details in **Supplementary Table S2**). Thresholds for considering differential expression were set at *probability* of differential expression >0.95, and absolute fold change >1.25.

Electron Microscopy (EM) and Post-embedding Immunogold Immunohistochemistry

Adult Reelin transgenic mice (n = 3), control littermates (n = 3), and Reelin transgenic mice treated with DOX for 7 days (n = 3) were perfused with 2% glutaraldehyde-2% paraformaldehyde in 0.12 M phosphate buffer. After post-fixation in the same solution overnight, tissue slices were transferred to 2% osmium tetroxide, stained with 2% uranyl acetate, dehydrated, and embedded with araldite. Ultrathin sections were then obtained from at least two araldite blocks per hippocampus, and stained with lead citrate. Electron micrographs (at 25,000×) of each hippocampal layer were randomly taken, and the area, circularity index and phenotype of spines receiving at least one synaptic contact were determined (n = 112–161 spines for each layer and group). Moreover, the density of dendritic spines showing spine apparatus (SA; n = 30–70 electron micrographs analyzed per layer and group) and the area of this organelle (n = 44–83 spine apparatuses) were calculated. In this case, a SA was considered to be measured if at least two tubules of smooth endoplasmic reticulum were detected in close apposition (Deller et al., 2006). The analyzed images corresponded to randomly selected fields within the neuropil in the layer of interest. We excluded the sampling of serial, consecutive sections, to avoid the possibility that any given item could be counted twice. Areas of spine apparatuses were measured using ImageJ software (Schindelin et al., 2012). Finally, we analyzed the area and circularity index of axon terminals and the length of their synaptic contacts (n = 92–171 synaptic terminals for each layer and group).

For post-embedding immunostaining analysis, adult Reelin transgenic mice (n = 2), control littermates (n = 2) and Reelin transgenic mice treated with DOX for 7 days (n = 2) were perfused with 0.1% glutaraldehyde-4% paraformaldehyde in 0.12 M phosphate buffer and processed. After removal, brains were cryoprotected gradually in sucrose and cryofixed by immersion in liquid propane. Freeze substitution was performed at −90°C for 3 days in an "Automatic Freeze Substitution System" (AFS, Leica), using methanol containing 0.5% uranyl acetate as substitution medium. Brains were infiltrated in Lowicryl HM20 at −50°C and then polymerized with UV lamps. Ultrathin sections were obtained from one block per hippocampus, collected and processed for post-embedding immunostaining. Samples were incubated with either rabbit anti-NR2a (1:5, Millipore Bioscience Research Reagents), rabbit anti-NR2b (1:5, Millipore Bioscience Research Reagents), or rabbit anti-p-Cofilin (1:5, Cell Sign Tech) antiserum. Sections were then incubated with goat anti-rabbit 18-nm gold-tagged antibody (1:20; British Biocell International). These sections were then counterstained with uranyl acetate and lead citrate. Electron micrographs (30,000×) were obtained in the stratum radiatum (SR) of the hippocampus CA1 region, and the number of dots per dendritic spine was counted in those spines that contained at least two gold particles (n = 20–35 spines per group). We classified each in-spine dot as belonging to one out of three compartments based on the features that lied within a 20 nm radius from the dot: synaptic (PSD), extrasynaptic (plasma membrane) or cytoplasmic (intracellular; dots not meeting the criteria for either synaptic or extrasynaptic).

Statistical Analyses

In all analyses, animal genotype was blind to the experimenter. The number of animals used in each experiment is detailed above.

Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal–Wallis test) followed by Dunns's post-test.

RESULTS

Reelin Overexpression Modulates the Structural Complexity of Presynaptic Terminals

We recently reported that Reelin overexpression induces a higher density of synaptic contacts in the adult hippocampus (Pujadas et al., 2010). Here we first further analyzed the ultrastructural and molecular phenotypes of synaptic elements in the different layers of the hippocampus of control and Reelin-OE mice. We also examined Reelin-OE mice in which the transgene had been switched off 1 week earlier by DOX treatment. Regarding presynaptic elements, axon terminals in Reelin-OE mice were larger (16–62%) than those in controls in all hippocampal layers (**Figures 1A–D**). The greatest increase was found in the stratum lacunosum-moleculare (SLM). The circularity index—used as a measure of the morphological complexity of axon terminals—tended to be lower in Reelin-OE mice than in controls, although this reduction was significant only in the SR and stratum oriens (SO; 7–8%; **Figure 1E**). Both the axonal bouton area and the circularity index returned to normal values 1 week after transgene inactivation, thus reinforcing the specificity of the observed phenotypes (**Figures 1D,E**). In two hippocampal layers, however, the circularity index in the Reelin-OE-DOX group surpassed the control values, a feature often observed for other parameters in both the present study and previously (Pujadas et al., 2010). Finally, we measured the length of the synaptic contacts, observing a significant increase in the SLM of Reelin-OE mice (**Figure 1F**).

To elucidate whether the molecular presynaptic machinery was also altered in Reelin-OE mice, we performed Western blot analyses against distinct synaptic proteins in lysates from hippocampal synaptosomal fractions. The protein expression levels for a number of presynaptic markers, including Synapsin 2a and 2b, and SNAP-25 (**Figures 1G,H**), Synaptophysin, VAMP-2, and Syntaxin 1 (data not shown), remained unchanged across the experimental groups. These results suggest that Reelin overexpression modulates the structural features of hippocampal axon terminals but does not substantially alter the protein levels of the presynaptic machinery linked to exocytosis and neurotransmitter release.

Reelin Overexpression Regulates Postsynaptic Dendritic Spine Morphology

By reconstructing hippocampal dendritic segments, we recently found that dendritic spines were hypertrophied in the SR of Reelin-OE mice (Pujadas et al., 2010). To substantiate these findings, we conducted a detailed fine structural analysis on postsynaptic dendritic spines in the different hippocampal layers (**Figure 2**). The surface of dendritic spines was larger in most hippocampal layers in Reelin-OE mice than in control mice (**Figures 2A,B**). Differences were greater in the layers receiving entorhinal input, i.e., the medial molecular layer (MML), the outer molecular layer (OML) and the SLM (27–35% of increase; **Figure 2D**). Furthermore, in Reelin-OE mice treated with DOX dendritic spine surfaces returned to control values (**Figures 2C,D**). The circularity index of dendritic spine heads, a parameter that reflects their complexity, was lower in all the hippocampal layers of Reelin-OE mice than in their littermate controls (**Figure 2E**). Again, Reelin-OE-DOX animals exhibited spine circularity index values close to those detected in control mice (**Figure 2E**).

Dendritic spine shapes are associated with spine function and plasticity (Bourne and Harris, 2008). We thus analyzed the phenotype of hippocampal dendritic spines in Reelin-OE mice. The most widely used nomenclature to distinguish the morphological diversity of dendritic spines is based on the relative size (and shape) of the spine head and neck (Harris et al., 1992; Bourne and Harris, 2008). Following these criteria, dendritic spines are classified as thin, stubby, or mushroom type. However, this grouping requires fully imaged dendritic spines. Since our analysis was based on single section EM micrographs, we implemented a new spine classification criteria based on the curvature of the postsynaptic density (PSD), a parameter suitable for the analysis of sectioned spines. On the basis of the curvature of the PSD and postsynaptic region, we distinguished the following types of spines: type I, showing a convex PSD (**Figure 2F**); type II, showing a straight or weakly concave PSD (**Figure 2G**); and type III, showing a PSD with a sufficiently concave curvature to fit an area of the pre-synaptic bouton large enough to accommodate an entire synaptic vesicle (**Figure 2H**). These synaptic types are likely to correspond to the classical thin, stubby, and mushroom types, respectively (Marrone and Petit, 2002; Marrone et al., 2004; Medvedev et al., 2010a). Following this classification, we observed that while some layers showed significant differences in the percentage of types I and II (**Supplementary Figure S1**), the number of type III dendritic spines was higher in all the hippocampal layers of Reelin-OE mice (from 1.5 to 8.2-fold, see **Figure 2I**), compared to their control littermates. These spine phenotypes were largely reverted by a 1-week treatment with DOX (**Figure 2I**). All together, these findings indicate that Reelin increases the structural complexity of pre- and postsynaptic elements and shifts a proportion of spines toward a type III phenotype, whose higher complexity recalls that of mushroom spines.

Reelin Modulates the Complexity of Spine Apparatus in a Lamina-Specific Manner

The SA (**Figure 3A**), a specialized form of endoplasmic reticulum comprising several stacks (Spacek and Harris, 1997), has been implicated in dendritic spine function and plasticity as it regulates the synthesis and trafficking of glutamate receptors, among other

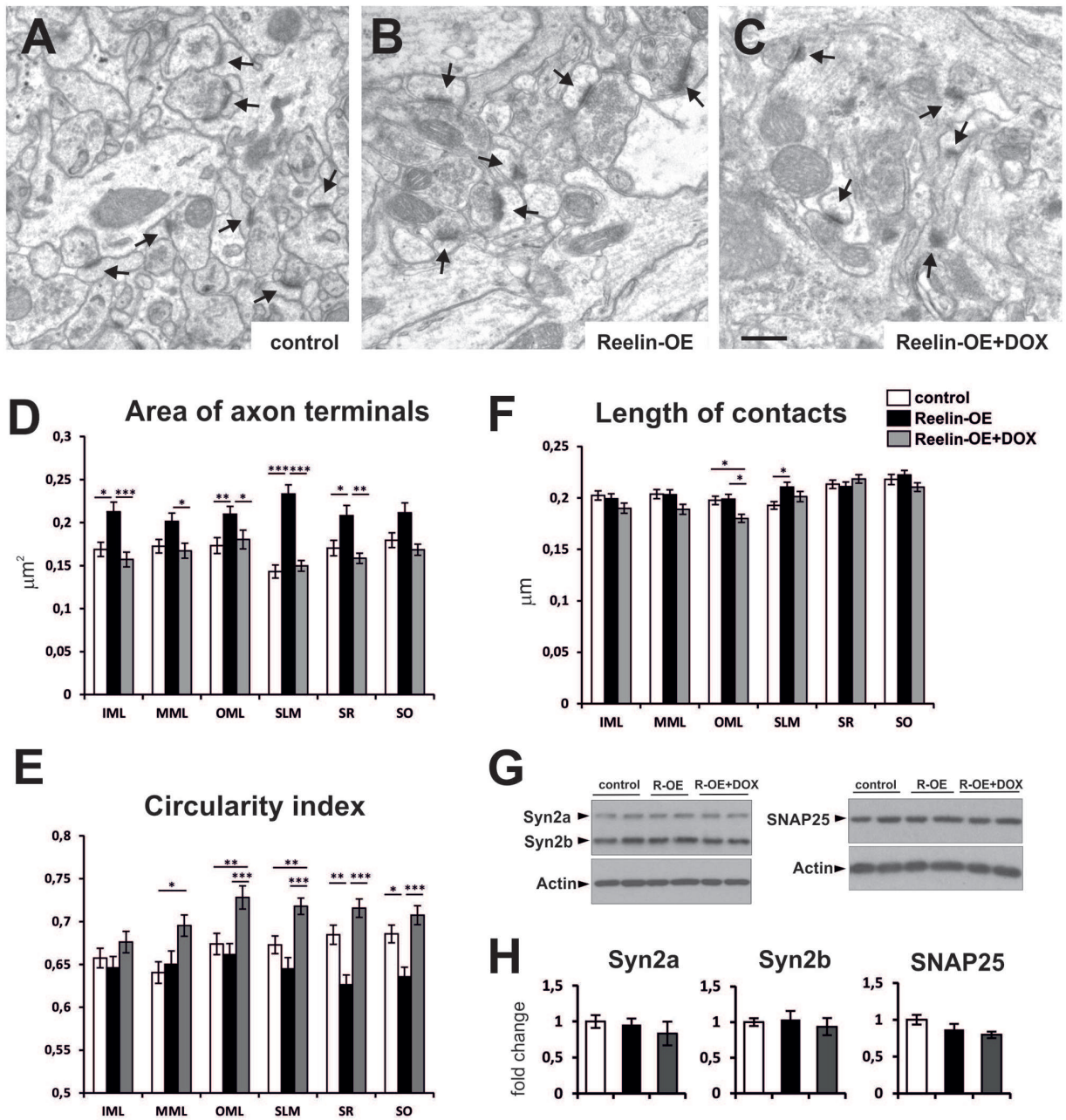

FIGURE 1 | Fine structure and molecular phenotype of axon terminals in Reelin-OE mice. (A–C) Electron micrographs illustrating axon terminals establishing synaptic contacts (arrows) in the inner molecular layer of control, Reelin-OE, and DOX-treated Reelin-OE mice. **(D–F)** Histograms showing area **(D)**, circularity index **(E)**, and length of synaptic contact **(F)** of axon terminals in different hippocampal layers in control, Reelin-OE, and DOX-treated Reelin-OE mice (mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA test). **(G)** Western-blot analysis of a and b isoforms of Synapsin 2, and SNAP25 in synaptosomal fractions of the hippocampus. **(H)** Quantification of Synapsin 2a, Synapsin 2b, and SNAP-25 values from triplicate experiments. Values are normalized to the levels of actin and expressed as percentage of control mice (mean ± SEM, ANOVA test). IML, inner molecular layer; MML, medial molecular layer, OML, outer molecular layer; SLM, stratum lacunosum-moleculare; SO, stratum oriens; SR, stratum radiatum. Scale bar in **(A–C)**, 0.5 µm.

functions (Jedlicka et al., 2008; Segal et al., 2010). Interestingly, we found an increase in the percentage of spines containing SA in all hippocampal layers of Reelin-OE mice, this increase being statistically significant in the OML (**Figure 3B**). Moreover, analysis of the surface occupied by SAs (e.g., **Figure 3A**) revealed that these organelles were significantly larger in Reelin-OE mice in the layers receiving entorhinal input, i.e., the MML, OML, and SLM (**Figure 3C**). Both quantitative measurements reverted to control values when Reelin-OE mice were treated with DOX (**Figures 3B,C**).

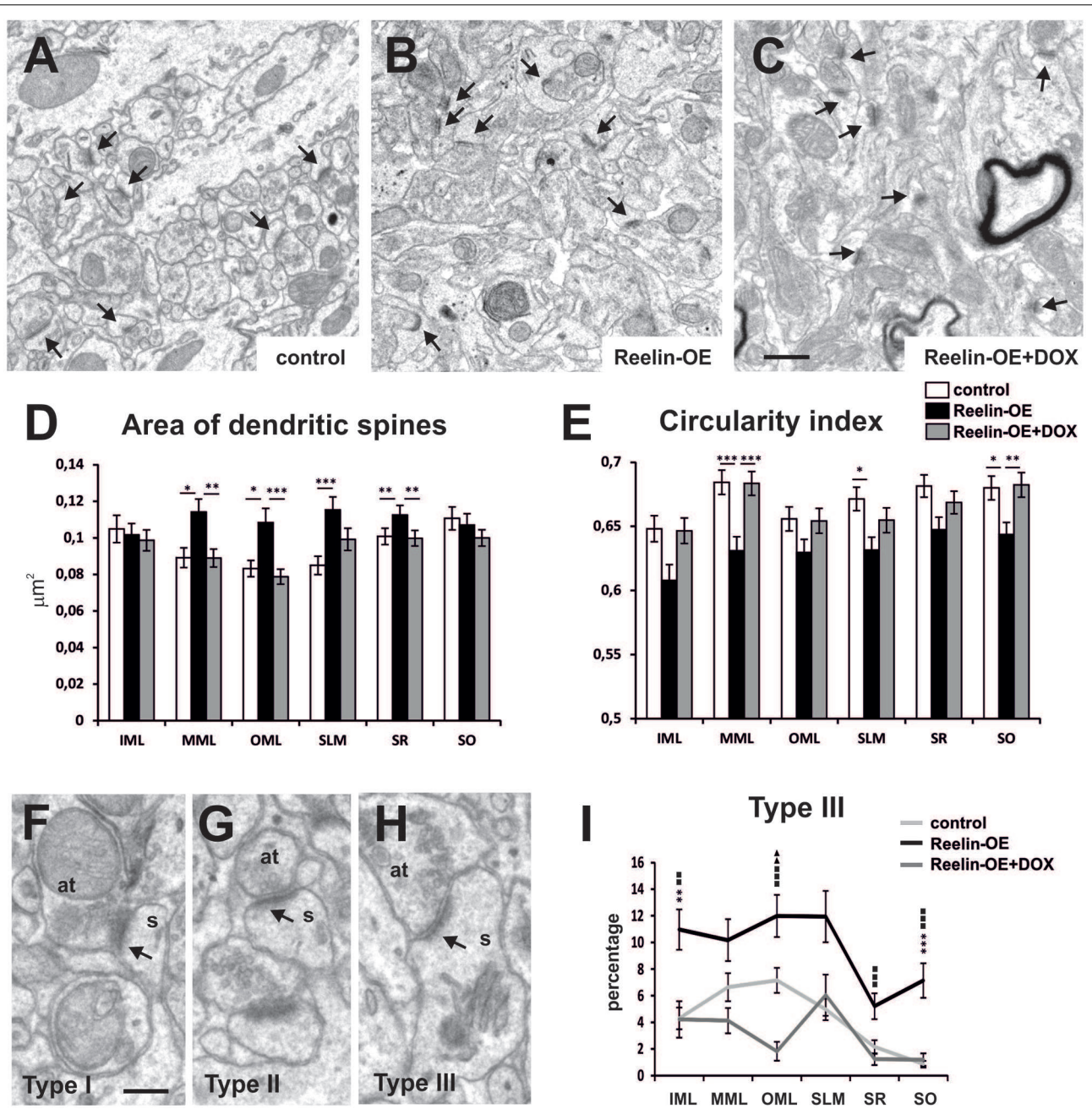

FIGURE 2 | Fine structure of dendritic spines in Reelin-OE mice. (A–C) Electron micrographs illustrating dendritic spines receiving synaptic inputs (arrows) in the inner molecular layer of control, Reelin-OE, and DOX-treated Reelin-OE mice. **(D,E)** Histograms showing area **(D)** and circularity index **(E)** of dendritic spines in different hippocampal layers in control, Reelin-OE, and DOX-treated Reelin-OE mice (mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; ANOVA test). **(F–H)** Electron micrographs showing three types of defined dendritic spines depending on PSD curvature (see text): type I **(F)**, type II **(G)**, and type III **(H)**. **(I)** Histogram illustrating the percentage of type III dendritic spines in all the hippocampal layers in control, Reelin-OE, and DOX-treated Reelin-OE mice (mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for control vs. Reelin-OE mice; squares for Reelin-OE vs. Reelin-OE+DOX; triangles for control vs. Reelin-OE+DOX mice; ANOVA test). IML, inner molecular layer; MML, medial molecular layer, OML, outer molecular layer; SLM, stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale. Scale bars, **(A–C)** = 0.5 µm; **(F–H)** = 200 nm.

An essential component of the SA is the actin-associated protein Synaptopodin, which is also involved in synaptic plasticity (Mundel et al., 1997; Jedlicka et al., 2008; Segal et al., 2010; Zhang et al., 2013). Synaptopodin mRNA is expressed by both hippocampal pyramidal neurons and granule cells (Mundel et al., 1997), where it shows a lamina-specific distribution (Deller et al., 2000; Roth et al., 2001; Bas Orth et al., 2005). To determine whether Reelin overexpression altered

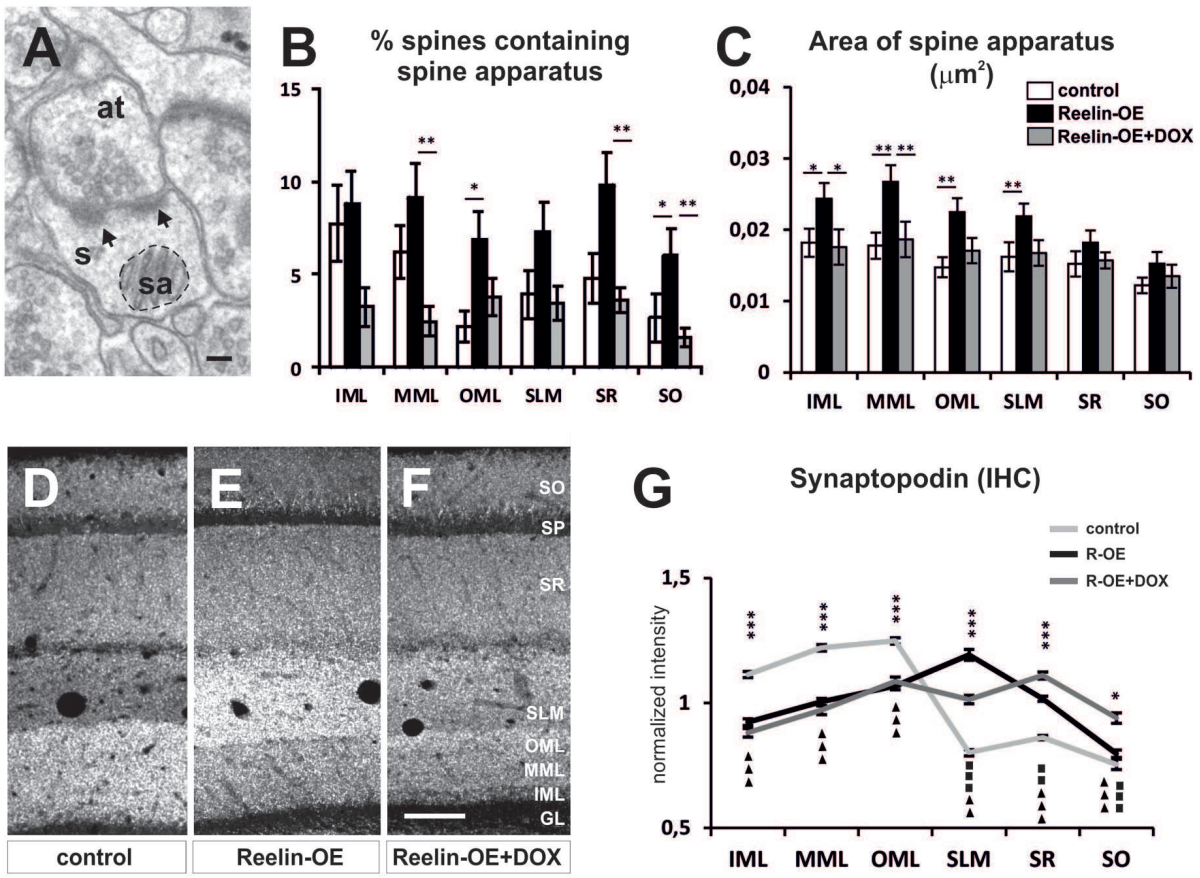

FIGURE 3 | Spine apparatus and Synaptopodin in dendritic spines of Reelin-OE mice. (A) Electron micrograph of dendritic spines (s) containing a spine apparatus (sa) and receiving two synaptic contacts (arrows) from an axon terminal (at). Dashed-line circle represents an example of the area of spine apparatus used to perform analysis in **(C)**. **(B,C)** Histograms illustrating the percentage of dendritic spines containing spine apparatus **(B)** and the area of these organelles **(C)** in distinct hippocampal layers of control, Reelin-OE, and DOX-treated Reelin-OE mice (mean ± SEM; $*p < 0.05$, $**p < 0.01$; ANOVA test). **(D–F)** Immunolabeling for Synaptopodin in hippocampal sections of control, Reelin-OE, and DOX-treated Reelin-OE mice. **(G)** Quantitative determination of immunofluorescence signals in control, Reelin-OE, and Reelin-OE+DOX mice. Diagram shows continuous linear profiles of fluorescence intensities in vertical stripes of hippocampal sections from the stratum oriens to the granule layer for Synaptopodin. The intensity of fluorescence is represented in gray levels (mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for control vs. Reelin-OE mice; squares for Reelin-OE vs. Reelin-OE+DOX; triangles for control vs. Reelin-OE+DOX mice; ANOVA test). GL, granular layer; IHC, immunohistochemistry; IML, inner molecular layer; MML, medial molecular layer; OML, outer molecular layer; SLM, stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale; WB, western blot. Scale bars, **(A)** = 100 nm; **(F,G)** = 100 μm.

Synaptopodin expression in the hippocampus, we performed immunohistochemical analyses and Western blot assays in the synaptosomal fractions. No significant differences were found in the overall level of protein expression among genotypes (**Supplementary Figure S2**). However, immunohistochemical analyses showed layer-specific alterations in the distribution of Synaptopodin in Reelin-OE mice (**Figures 3D–F**). Thus, in agreement with earlier studies (Deller et al., 2000; Bas Orth et al., 2005), control mice showed a highest expression of Synaptopodin in the molecular layer of the dentate gyrus (DG), with diffuse staining in the CA1 region (**Figures 3D,G**). In contrast, Reelin-OE mice showed a marked increase in Synaptopodin expression in the SLM (**Figures 3E,G**) and decreased expression in the DG. These differences were partially reversed in Reelin-OE mice treated with DOX (**Figures 3F,G**). Taken together, these results indicate that Reelin overexpression leads to a higher complexity of the SA and to a redistribution of Synaptopodin expression, being concentrated in the SLM, without altering the overall hippocampal expression levels of this protein.

Reelin Regulates the Synaptic Distribution of NMDA Receptors

To study whether the molecular composition of postsynaptic elements is affected by Reelin-overexpression in the hippocampus, we performed Western blot assays on synaptosomal fractions. Thus, we analyzed the protein levels of several glutamate receptor subunits (GluR1, GluR2/3, GluN1, NR2a, and NR2b), scaffolding proteins (PSD-95), and downstream effector components (CaMKII, Cofilin, p-Cofilin, LIMK1, and p-LIMK1/LIMK2) (**Supplementary Figure S3**; **Figures 4A,B** and **5A,B**). Noteworthy, none of these proteins

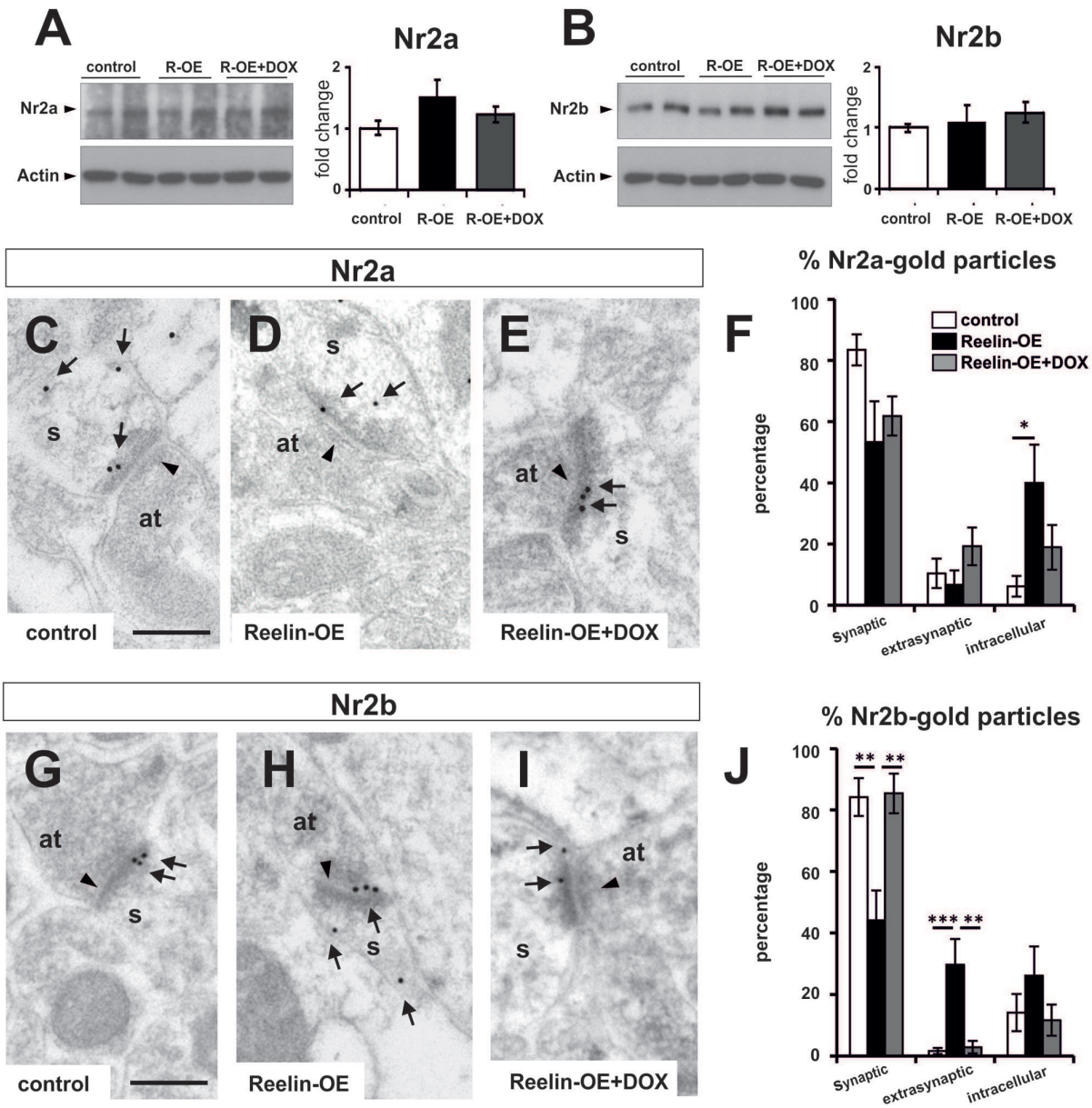

FIGURE 4 | Expression of NMDA receptor subunits NR2a and NR2b in Reelin-OE mice. (A) Left, immunoblot analysis of NR2a in control, Reelin-OE, and DOX-treated Reelin-OE mice. Right, histogram showing densitometric analysis ($n = 4$ animals per group) by fold change in the three groups of mice (mean ± SEM; ANOVA test). **(B)** Left, immunoblot analysis of NR2b in control, Reelin-OE, and DOX-treated Reelin-OE mice. Right, histogram showing densitometric analysis ($n = 3$ animals per group) by fold change in the three groups of mice (mean ± SEM; ANOVA test). **(C–E)** Examples of immunogold labeling against NR2a in dendritic spines (s) receiving a synaptic contact from an axon terminal (at) in the SR of control **(C)**, Reelin-OE **(D)**, and DOX-treated Reelin-OE **(E)** mice. **(F)** Histogram illustrating the proportion of NR2a-gold particles in the synaptic contact, the extrasynaptic membrane and the intracellular domain of dendritic spines in the SR of Reelin-OE and DOX-treated Reelin-OE mice and their control littermates (mean ± SEM; $^{*}p < 0.05$; ANOVA test). **(G–I)** Examples of immunogold labeling against NR2b in dendritic spines (s) receiving a synaptic contact from an axon terminal (at) in the SR of control **(G)**, Reelin-OE **(H)**, and DOX-treated Reelin-OE **(I)** mice. **(J)** Histogram illustrating the proportion of NR2b-gold particles in the synaptic contact, the extrasynaptic membrane and the intracellular domain of dendritic spines in the SR of Reelin-OE and DOX-treated Reelin-OE mice and their control littermates (mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA test). In **(C–E,G–I)**, note synaptic contacts pointed by arrowheads and immunolabeling dots labeled by arrows. Scale bars, 250 nm.

showed altered expression levels in purified synaptosomal fractions.

Given that Reelin expression has been linked to the surface mobility of NMDA receptors *in vitro* (Groc et al., 2007; Qiu and Weeber, 2007), we next addressed whether this protein influences the distribution of these receptor subunits *in vivo*. For this purpose, we performed immunogold EM staining against the NR2a and NR2b subunits, and the

distribution of these subunits in the SR was plotted. The distribution of NR2a subunits was altered in Reelin-OE mice, compared to controls (**Figures 4C–E**). Thus, while 83% of NR2a receptors were found in postsynaptic densities in control spines, Reelin-OE mice showed a marked decrease in this location, concomitant with a higher number of intracellular (40%) receptor subunits (**Figure 4F**). Immunogold analyses of the NR2b subunits showed a similar altered distribution of synaptic versus intracellular subunits in Reelin-OE mice; moreover, Reelin-overexpression lead to marked increase in extrasynaptic NR2b subunits (**Figures 4G–J**). The effects of Reelin overexpression on NR2a/b synaptic/extrasynaptic distribution were largely reversed after arresting transgene expression with DOX (**Figures 4E,F,I,J**).

Cofilin, an F-actin severing protein, promotes the stabilization of mature dendritic spines. It is preferentially found in postsynaptic densities (Chai et al., 2009; Shi et al., 2009; Rust et al., 2010), and its phosphorylation has been proposed to be regulated by Reelin (Chai et al., 2009; Leemhuis and Bock, 2011). We thus tested Cofilin protein levels and distribution in dendritic spines in the SR. While overall protein and p-Cofilin levels were not altered in synaptosomal fractions (**Figures 5A,B**), immunogold staining showed a redistribution of p-Cofilin in Reelin-OE mice, passing from synaptic to extrasynaptic/intracellular locations when compared to controls (**Figures 5C–F**). The distribution of p-Cofilin in Reelin-OE-DOX mice was similar to that in controls (**Figure 5F**). These observations indicate that p-Cofilin redistributes in Reelin-OE mice, similar to NR2a/b receptor subunits.

Gene Expression of Synaptic-Associated Genes Is Not Altered in Reelin-OE Mice

The above protein expression experiments indicated that Reelin does not alter the expression of several important pre- and postsynaptic proteins in the adult brain. To confirm and extend these findings, we performed a genome-wide transcriptomic analysis, comparing mRNA abundance in the hippocampus of control, Reelin-OE, and Reelin-OE-DOX mice. In addition to the genes tested in Western blots, we screened for possible differential expression of 360 genes directly associated with the GO term "Synapse" (GO:0045202), which include presynaptic proteins and SNAREs, receptors, scaffolding and signaling proteins, and other synapse-related genes. As shown in **Figure 6** and **Supplementary Tables S1** and **S2**, the patterns of gene expression of these genes remained largely unaltered in Reelin-OE mice, compared to controls or Reelin-OE-DOX mice. Only a few gene probes (corresponding to Syt7, EphB2, Lrrc4, and Syn2 genes) showed minor, though

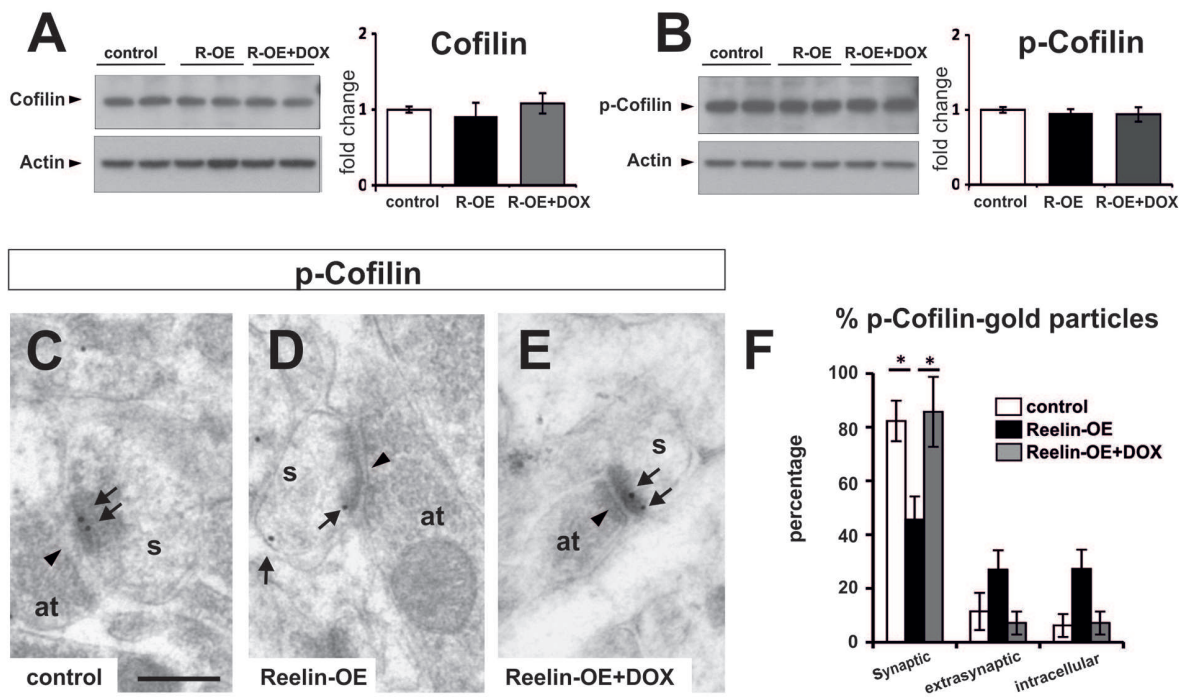

FIGURE 5 | Expression of Cofilin and p-Cofilin in Reelin-OE mice. (A) Left, immunoblot analysis of Cofilin in control, Reelin-OE, and DOX-treated Reelin-OE mice. Right, histogram showing densitometric analysis ($n = 3$ animals per group) by fold change in the three groups of mice (mean ± SEM; ANOVA test). **(B)** Left, immunoblot analysis of p-Cofilin in control, Reelin-OE, and DOX-treated Reelin-OE mice. Right, histogram showing densitometric analysis ($n = 3$ animals per group) by fold change in the three groups of mice (mean ± SEM; ANOVA test). **(C–E)** Examples of immunogold labeling against p-Cofilin in dendritic spines (s) receiving a synaptic contact from an axon terminal (at) in the SR of control **(C)**, Reelin-OE **(D)**, and DOX-treated Reelin-OE **(E)** mice. Note synaptic contacts indicated by arrowheads and immunolabeling dots by arrows. Scale bar, 250nm. **(F)** Histogram illustrating the proportion of dots in the synaptic contact, the extrasynaptic membrane and the intracellular domain of dendritic spines in the SR of Reelin-OE and DOX-treated Reelin-OE mice and their control littermates (mean ± SEM; $^*p < 0.05$; ANOVA test).

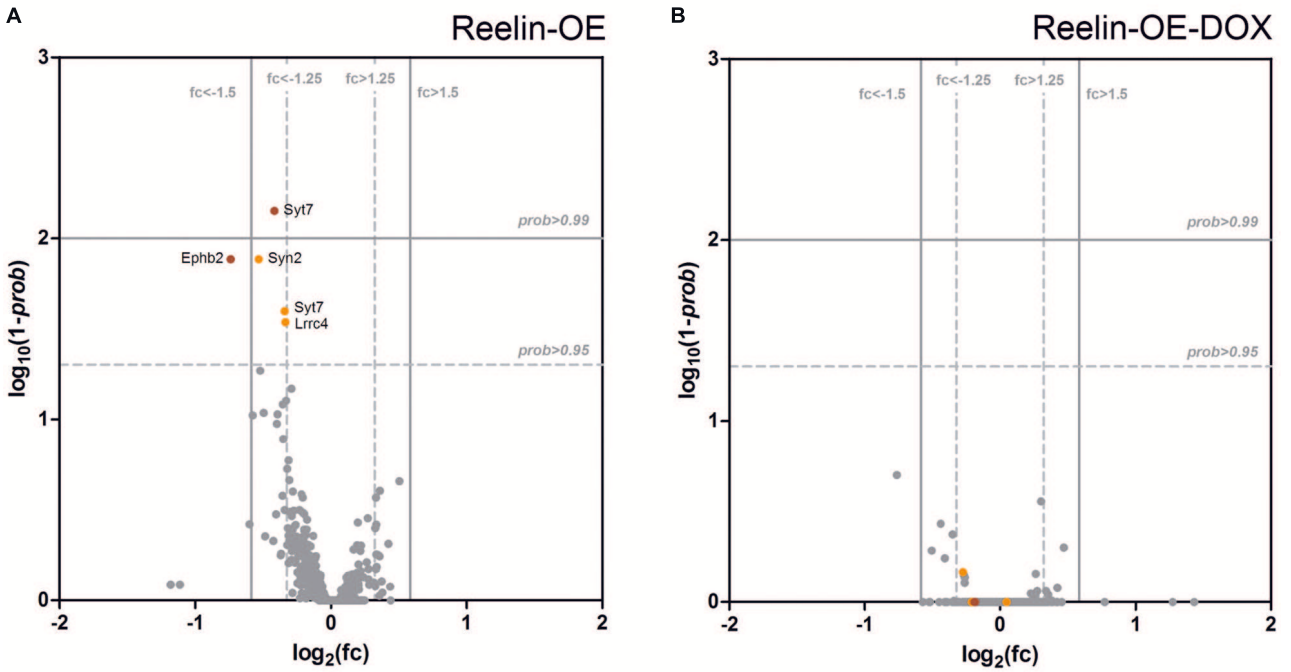

FIGURE 6 | Analysis of differential expression of genes directly associated with "Synapse" (GO:0045202) in the hippocampus of Reelin-OE mice. **(A,B)** Volcano plots showing the magnitude of differential gene expression compared with control mice. Each dot represents an independent probe for a GO:0045202 gene. The vertical and horizontal lines mark the thresholds for defining up-/down-regulation for probability (*prob*) and fold change (fc), respectively. **(A)** Reelin-OE mice show most of the probes with non-differential expression (gray dots). Five probes show down-regulation (orange/red dots). **(B)** Reelin-OE mice treated for 1 week with doxycycline (Reelin-OE-DOX) do not show differences in gene expression with control mice.

statistically significant, regulation by Reelin levels. We conclude that the overall transcript expression for synapse-related genes is not altered by Reelin expression levels in the adult hippocampus.

DISCUSSION

Reelin Confers Unique Structural Properties to Adult Hippocampal Dendritic Spines

Despite the role of Reelin in the developing brain in spine formation and maturation has been tackled consistently (Niu et al., 2004; Jossin and Goffinet, 2007; Niu et al., 2008), only recently it has started to be elucidated its impact on adult dendritic spine structure and physiology. In a previous study, we reported that spines of individual CA1 pyramidal cell dendrites in the SR of adult Reelin-OE mice were hypertrophied (Pujadas et al., 2010). These results were supported by studies in mDab1 KO mice showing that the size of dendritic spines was reduced (Trotter et al., 2013). Using a singular mouse model with increased Reelin expression specifically in the adult forebrain, here we show that sustained *in vivo* Reelin overexpression triggers larger dendritic spines and a lower circularity index (as an index of complexity) in all hippocampal layers, including the molecular layer of the DG. These observations thus support that these effects are a general consequence of the Reelin cascade in adult neurons. Enlargement of dendritic spines is a characteristic feature of synaptic plasticity mechanisms, including LTP (Toni et al., 2001; Popov et al., 2004; Yang et al., 2008; Hill and Zito, 2013), and is believed to confer a greater postsynaptic strength (Matsuzaki et al., 2004; Bourne and Harris, 2008; Lee et al., 2009; O'Donnell et al., 2011). In fact, Reelin-OE mice exhibit increased LTP responses *in vivo* (Pujadas et al., 2010). Moreover, synaptic plasticity is associated with changes in the proportion of dendritic spine types (Medvedev et al., 2010a,b). We found that Reelin overexpression in mice increases the percentage of dendritic spine types to type III (more than a 100% increase)—which most likely represent mushroom-type of dendritic spine—again in all layers (see Results). In contrast to thin dendritic spines, mushroom dendritic spines are considered to be the most mature spines, acting as stable "memory" spines (Matsuzaki et al., 2004; Bourne and Harris, 2007; Yasumatsu et al., 2008; Kasai et al., 2010). Moreover, recent studies report that LTP increases the proportion of mushroom spines (Medvedev et al., 2010a,b). Finally, we found a substantial increase in the percentage of spines bearing SAs and in the size of the SAs themselves in Reelin-OE mice. The SA is a Ca^{2+}-sequestering organelle that regulates spine Ca^{2+} signaling, synaptic efficacy, and post-translational modification of receptors, among other processes, and that is enriched in mushroom spines (Spacek and Harris, 1997; Fiala et al., 2002; Nimchinsky et al., 2002; Korkotian et al., 2014). This finding again reinforces the notion that Reelin promotes

the formation and stabilization of physiologically more efficient dendritic spines.

Reelin increases postsynaptic glutamatergic responses and LTP (Weeber et al., 2002; Beffert et al., 2005; Qiu et al., 2006a,b; Groc et al., 2007; Pujadas et al., 2010; Rogers et al., 2011, 2013). Moreover, infusion of Reelin enhances cognitive performance in wild-type mice (Rogers et al., 2011), while overexpression fully rescues cognitive deficits in mouse models of Alzheimer's disease and during normal aging (Pujadas et al., 2014). Together with these findings, the present data suggest that one of the mechanisms by which Reelin enhances cognitive performance in a range of conditions, as well as potentiating glutamatergic neurotransmission, is by forming more stable and complex, mushroom-type dendritic spines, thus enabling stronger postsynaptic responses. Finally, the finding that dendritic spine phenotypes were dramatically reversed when the Reelin transgene was switched off supports the notion that the structural changes observed were effectively caused by the activation of the Reelin cascade.

Reelin Exerts Presynaptic Effects in the Adult Brain

Reelin-deficient reeler mice exhibit decreased SNAP25 SNARE protein levels and reduced neurotransmitter release, which are rescued upon addition of recombinant Reelin (Hellwig et al., 2011). Here we found that Reelin overexpression did not alter the expression levels of SNAP25 protein or other SNARE components in Reelin-overexpressing adult mice. This observation suggests that while the absence of Reelin modifies SNAP25 levels, overactivation of the Reelin pathway is not sufficient to trigger increases in SNARE proteins. In contrast, here we report that axon terminals in the hippocampus of Reelin-OE mice were larger and exhibited increased complexity. Taken together with previous findings showing a higher density of axon terminals in reelin-OE mice (Pujadas et al., 2010), our results suggest that presynaptic numbers and complexity could be regulated independently from gene expression and protein synthesis. Possible mechanisms may include re-distribution of synaptic proteins and activation of synaptic proteins by post-translational modifications (such as phosphorylation) that may led to presynaptic functional and structural changes. In fact, Reelin increases short-term synaptic facilitation, a process believed to be caused essentially by presynaptic mechanisms (Pujadas et al., 2010; Rogers et al., 2011). Finally, a novel Reelin-dependent presynaptic mechanism has recently been reported in which the Reelin pathway stimulates spontaneous, action potential-independent neurotransmission via a Ca^{2+}/VAMP7-dependent signaling cascade (Bal et al., 2013).

Reelin Levels Do Not Regulate Synaptic Protein Expression Levels but do Determine Their Synaptic/Extrasynaptic Distribution

Here we show that Reelin levels do not regulate the expression of genes encoding for synaptic proteins, including presynaptic proteins such as SNAREs, glutamatergic receptor subunits, and postsynaptic scaffolding, cytoskeletal, and signaling proteins. Of a collection of 396 genes analyzed, only 4 genes (Syt7, Ephb2, Lrrc4, and Syn2) showed a moderate increase (∼1.2-fold) in expression. These results were validated at the protein levels for several SNAREs, glutamate receptor subunits, and postsynaptic signaling proteins. Although the Reelin pathway is believed to activate the CREB transcription factor pathway (Rogers et al., 2011) and gene expression alterations have been described in reeler mice (Kuvbachieva et al., 2004) the present study supports that Reelin does not control the expression of target genes coding for synaptic proteins, at least in adult mice.

Recent studies show that Reelin increases synaptic activity through altering NMDA and AMPA receptor activation and surface trafficking activity *in vitro* (Beffert et al., 2005; Chen et al., 2005; Qiu et al., 2006b; Groc et al., 2007; Qiu and Weeber, 2007; Campo et al., 2009). Our results in the adult hippocampus *in vivo* support these observations by showing that Reelin induces a decrease in NR2a/b postsynaptic subunits, concomitant with an increase in extrasynaptic and cytosolic NR2a/b content. The fact that no significant differences are found in the total number of immunogold dots in spines for NR2a or NR2b proteins (data not shown) or in receptor subunit expression by WBs, suggests that Reelin leads to a re-distribution of the existing proteins rather than to *de novo* protein synthesis. Again, this Reelin-dependent localization was reversed after blocking Reelin transgene expression for 1 week, thereby suggesting that the Reelin pathway promotes both the lateral diffusion and internalization of these proteins *in vivo*. Notably, this recovery was found much more robust for NR2B than NR2A subunits. Taking into account that Reelin has been shown to trigger the mobility of NR2B subunits (Groc et al., 2007) and that Reelin overexpression *in vivo* recovers the chronic stress-induced reduction in hippocampal NR2B-mediated currents (Teixeira et al., 2011), our results support the notion that the Reelin pathway might interact differently with both NMDA receptor subunits. The fine mechanisms by which Reelin regulates NMDAR trafficking are still poorly understood, although they may include receptor subunit phosphorylation and the modulation of scaffolding and signaling proteins. It has been proposed that Reelin activates LIMK1, which phosphorylates Cofilin at serine residues (Meng et al., 2004; Chai et al., 2009), which in turn stabilizes the actin cytoskeleton in dendritic spines (Shi et al., 2009) and increases AMPAR surface trafficking (Gu et al., 2010; Wang et al., 2013). Although we did not detect differences in LIMK1 and Cofilin phosphorylation levels in Reelin-OE mice, we did observe Reelin-dependent changes in p-Cofilin distribution that mimicked those of NMDA receptor subunits. Thus, taken together with the above findings, the present findings in adult Reelin-OE mice support that, rather than inducing changes in the expression of synaptic protein components, Reelin signaling modulates glutamatergic neurotransmission by regulating post-translational mechanisms, including the trafficking and assembly of receptor subunits, Ca^{2+}-dependent neurotransmitter release, and structural modifications of pre- and postsynaptic spine components.

Is There a Layer-Specific Synaptic Responsiveness to Reelin?

Most of the pre- and postsynaptic alterations reported here in Reelin transgenic mice [including pre- and postsynaptic areas, length of contacts, area of SA and percentage of spines containing SA, and those observed in our previous study (Pujadas et al., 2010)] were more dramatic in the SLM, OML, and MML layers. These three layers correspond to principal cell dendrites receiving entorhinal input, in contrast to the SR, SO, and inner molecular layer (IML), which receive commissural/associational input. These differences could be explained by a differential, layer-specific distribution of Reelin signaling components or of Reelin itself. However, neither transgenic Reelin in our mice (Pujadas et al., 2010) nor the APOER2/VLDL receptors or mDab1 show clear layer-specific distributions (Borrell et al., 2007; Trotter et al., 2013). However, we cannot exclude that other signaling components are enriched in particular dendritic domains of pyramidal and granule cell neurons.

Perhaps the most dramatic layer-specific synaptic alteration reported here refers to the Reelin-dependent distribution of Synaptopodin. An actin-binding protein tightly associated with SA, Synaptopodin is critical for the formation of this organelle, which has been linked to the structural and physiological properties of dendritic spines (Deller et al., 2000, 2003, 2006; Zhang et al., 2013). Here we found that Reelin induces a dramatic shift in Synaptopodin distribution, with it being enriched in the SLM while decreasing in the dentate molecular layer. These alterations in distribution were not accompanied by changes in gene or protein expression. Although the present experiments do not allow us to offer a mechanistic explanation for these findings, taken together with the above observations, our results support the notion that Reelin modulates synaptic structure and function differentially in distinct dendritic domains of the same neuron and that this modulation is correlated with the type of afferent synaptic input.

CONCLUSION

Our results highlight the participation of Reelin in the structural modulation of synaptic terminals and dendritic spines *in vivo* in adult mice. Importantly, they support the idea that, in addition to modulating presynaptic terminals, Reelin promotes the appearance of large, mushroom-type dendritic spines with large SAs and increased extrasynaptic NMDA receptors. Altogether, these data provide the structural basis to unravel the contribution of Reelin to normal neurotransmission and synaptic facilitation/LTP forms of plasticity and also to pathological conditions.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CB, ES, and AMa. Performed the experiments: CB, LP, AMu, and AMa. Analyzed the data: CB, ES, AMu, and AMa. Wrote the paper: CB, ES, and AMa.

FUNDING

This work was supported by grants from Spanish MINECO (SAF2013-42445R), CIBERNED (ISCIII), IMSERSO and La Marató de TV3 Foundation to ES.

ACKNOWLEDGMENT

We thank Tom Yohannan for editorial.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2016.00138

FIGURE S1 | Histograms illustrating the percentage of dendritic spines types I and II in all the hippocampal layers in control, Reelin-OE, and DOX-treated Reelin-OE mice (mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for control vs. Reelin-OE mice; squares for Reelin-OE vs. Reelin-OE+DOX; triangles for control vs. Reelin-OE+DOX mice; ANOVA test). IML, inner molecular layer; MML, medial molecular layer, OML, outer molecular layer; SLM, stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale.

FIGURE S2 | Western blot analysis and quantification of Synaptopodin. (A) Immunoblot analysis of Synaptopodin in Reelin-OE, DOX-treated Reelin-OE mice and control littermates. **(B)** Histogram showing densitometric analysis ($n = 3$ animals per group) by fold change in the three groups of mice (mean ± SEM; ANOVA test).

FIGURE S3 | Western blot analysis and quantification of distinct post-synaptic proteins. (A–G) Immunoblot analysis and its corresponding quantification of NR1 **(A)**, GluR1 **(B)**, GluR2/3 **(C)**, PSD-95 **(D)**, CaMKII **(E)**, LIMK1 **(F)**, and p-LIMK1/LIMK2 **(G)** from synaptosomal fractions of the hippocampus ($n = 3$) in control, Reelin-OE, and DOX-treated Reelin-OE mice. Values in histograms are normalized to the levels of actin and expressed as percentage of control mice (mean ± SEM; ANOVA test).

TABLE S1 | Gene expression of GO synapse genes. The fold-change (fc) and the probability (prob) of differential expression are presented for all the probes in the Genechip Mouse Genome 430 2.0 array corresponding to GO:0045202 genes. Reelin-OE mice (R-OE) and Reelin-OE mice treated for one week with doxycycline (R-OE+1w(DOX)) were compared with controls.

TABLE S2 | Gene expression of selected genes. The fold-change (fc) and the probability (prob) of differential expression are presented for all the probes in the Genechip Mouse Genome 430 2.0 array corresponding to Limk1/Limk2/Cfl1/Clf2 genes. Reelin-OE mice (R-OE) and Reelin-OE mice treated for one week with doxycycline (R-OE+1w(DOX)) were compared with controls.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 28 July 2016
doi: 10.3389/fncel.2016.00183

Seizure-Induced Motility of Differentiated Dentate Granule Cells Is Prevented by the Central Reelin Fragment

*Catarina Orcinha[1,2,3,4†], Gert Münzner[1,2,3†], Johannes Gerlach[1,2,3], Antje Kilias[3,4,5], Marie Follo[2,6], Ulrich Egert[4,5,7] and Carola A. Haas[1,2,4,7]**

[1] Experimental Epilepsy Research, Department of Neurosurgery, Medical Center, University of Freiburg, Freiburg, Germany, [2] Faculty of Medicine, University of Freiburg, Freiburg, Germany, [3] Faculty of Biology, University of Freiburg, Freiburg, Germany, [4] Bernstein Center Freiburg, University of Freiburg, Freiburg, Germany, [5] Laboratory for Biomicrotechnology, Department of Microsystems Engineering, University of Freiburg, Freiburg, Germany, [6] Lighthouse Core Facility, Department of Medicine I, Medical Center, University of Freiburg, Freiburg, Germany, [7] BrainLinks-BrainTools, Cluster of Excellence, University of Freiburg, Freiburg, Germany

Granule cell dispersion (GCD) represents a pathological widening of the granule cell layer in the dentate gyrus and it is frequently observed in patients with mesial temporal lobe epilepsy (MTLE). Recent studies in human MTLE specimens and in animal epilepsy models have shown that a decreased expression and functional inactivation of the extracellular matrix protein Reelin correlates with GCD formation, but causal evidence is still lacking. Here, we used unilateral kainate (KA) injection into the mouse hippocampus, an established MTLE animal model, to precisely map the loss of reelin mRNA-synthesizing neurons in relation to GCD along the septotemporal axis of the epileptic hippocampus. We show that reelin mRNA-producing neurons are mainly lost in the hilus and that this loss precisely correlates with the occurrence of GCD. To monitor GCD formation in real time, we used organotypic hippocampal slice cultures (OHSCs) prepared from mice which express enhanced green fluorescent protein (eGFP) primarily in differentiated dentate granule cells. Using life cell microscopy we observed that increasing doses of KA resulted in an enhanced motility of eGFP-positive granule cells. Moreover, KA treatment of OHSC resulted in a rapid loss of Reelin-producing interneurons mainly in the hilus, as observed *in vivo*. A detailed analysis of the migration behavior of individual eGFP-positive granule cells revealed that the majority of these neurons actively migrate toward the hilar region, where Reelin-producing neurons are lost. Treatment with KA and subsequent addition of the recombinant R3–6 Reelin fragment significantly prevented the movement of eGFP-positive granule cells. Together, these findings suggest that GCD formation is indeed triggered by a loss of Reelin in hilar interneurons.

Keywords: temporal lobe epilepsy, hippocampus, granule cell dispersion, migration, kainate, motility

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers University, USA

Reviewed by:
Nicola Berretta, Fondazione Santa Lucia, Italy
Jack M. Parent, University of Michigan, USA

****Correspondence:***
Carola A. Haas
carola.haas@uniklinik-freiburg.de

† These authors have contributed equally to this work.

Received: *31 March 2016*
Accepted: *08 July 2016*
Published: *28 July 2016*

Citation:
Orcinha C, Münzner G, Gerlach J, Kilias A, Follo M, Egert U and Haas CA (2016) Seizure-Induced Motility of Differentiated Dentate Granule Cells Is Prevented by the Central Reelin Fragment. Front. Cell. Neurosci. 10:183. doi: 10.3389/fncel.2016.00183

INTRODUCTION

Characteristic features of mesial temporal lobe epilepsy (MTLE) are recurrent focal seizures and Ammon's horn sclerosis (AHS) characterized by neuronal loss and granule cell dispersion (GCD), an abnormal broadening of the dentate granule cell layer (GCL; Houser, 1990; Haas et al., 2002). There is recent evidence that a loss of the extracellular matrix protein Reelin is involved in the development of GCD, since the Reelin-deficient *reeler* mouse (D'Arcangelo et al., 1995; Hirotsune et al., 1995) shows a disorganized GCL, reminiscent of GCD (Frotscher et al., 2003). Moreover, GCD formation has been shown to be accompanied by a loss of Reelin-producing neurons in the hippocampus of MTLE patients (Haas et al., 2002) and in rodent epilepsy models (Heinrich et al., 2006; Gong et al., 2007; Antonucci et al., 2008; Duveau et al., 2011). A local reduction of GCD has been achieved by infusion of recombinant Reelin into the rodent hippocampus during epileptogenesis, pointing to a causal role of Reelin in maintaining lamination in the adult brain (Müller et al., 2009).

Reelin is a key regulator of neuronal positioning during brain development, but Reelin is also important for synaptic function and memory formation in the adult brain (Herz and Chen, 2006; Lane-Donovan et al., 2015). Reelin is synthesized and secreted by Cajal-Retzius (CR) cells and interneurons into the extracellular matrix (Alcántara et al., 1998; Pesold et al., 1998), where full-length Reelin is proteolytically cleaved into smaller isoforms, an important prerequisite for activation of target cells (Jossin et al., 2004, 2007). The full-length Reelin molecule consists of an N-terminal F-spondin-like sequence, followed by eight Reelin repeats. Cleavage of Reelin can occur at two sites: N-terminal between the second and the third repeat, and C-terminal between the sixth and seventh repeat, generating five possible isoforms depending on the protease in action (Lambert de Rouvroit et al., 1999; Jossin et al., 2004). Specifically, the central region of Reelin (R3–6) has been described to be very important for receptor binding (D'Arcangelo et al., 1999; Jossin et al., 2004; Lee and D'Arcangelo, 2016).

Reelin signaling occurs after binding to lipoprotein receptors, very-low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), tyrosine phosphorylation of the intracellular adaptor protein Disabled-1 (Dab1) by Src family kinases, and the subsequent activation of downstream effectors, which target the actin and microtubule cytoskeleton (Tissir and Goffinet, 2003; Stolt and Bock, 2006; Jossin and Goffinet, 2007; Leemhuis and Bock, 2011). Recently, binding of Reelin to Ephrins has been reported, although the physiological functions of these interactions remain poorly understood (Sentürk et al., 2011; Bouché et al., 2013).

Granule cell dispersion can be induced experimentally in adult mice by unilateral injection of kainate (KA), an agonist of the excitatory neurotransmitter glutamate (Bouilleret et al., 1999; Heinrich et al., 2006; Häussler et al., 2012). In this animal model, AHS including neuronal cell loss and GCD develops within 3 weeks after KA injection in spite of the loss of dentate neurogenesis (Kralic et al., 2005; Heinrich et al., 2006; Nitta et al., 2008). In addition, spontaneous, focal epileptic seizures develop similar to human MTLE (Riban et al., 2002; Häussler et al., 2012). GCD can also be induced *in vitro* in organotypic hippocampal slice cultures (OHSCs) by KA application (Tinnes et al., 2011, 2013). In this *in vitro* model, GCD has been shown recently to occur via somal translocation of differentiated granule cells (Murphy and Danzer, 2011; Chai et al., 2014), but so far the molecular mechanism has remained unclear.

In the present study, we show in the intrahippocampal KA mouse model that GCD formation and loss of reelin mRNA-producing neurons are spatially correlated and that this loss mainly affects the hilus. In addition, we present evidence in OHSC that, like *in vivo*, KA treatment causes a complete loss of Reelin-producing hilar neurons. Moreover, we show in real time by life cell microscopy that differentiated enhanced green fluorescent protein (eGFP)-positive granule cells actively migrate toward the Reelin-free hilar region and that this migration process can be prevented by application of the central R3–6 Reelin fragment.

MATERIALS AND METHODS

Animals

Experiments were performed with C57BL/6 and Thy1-eGFP mice (M-line, C57BL/6 background). All animal procedures were carried out in accordance with the guidelines of the European Community's Council Directive of 22 September 2010 (2010/63/EU) and were approved by the regional council (Regierungspräsidium Freiburg).

Intrahippocampal Kainate Injection

Adult male (7–12 weeks of age) C57BL/6 mice were used for unilateral intrahippocampal KA injections as described previously (Heinrich et al., 2006; Häussler et al., 2012). In brief, anesthetized mice (ketamine hydrochloride 100 mg/kg, xylazine 5 mg/kg, atropine 0.1 mg/kg body weight, i.p.) were stereotaxically injected with 50 nl (1 nmol) of a 20 mM KA solution (Tocris) in 0.9% sterile saline into the right dorsal hippocampus [coordinates relative to bregma: anterio-posterior (AP) = −2.0 mm, medio-lateral (ML) = −1.4 mm, dorso-ventral (DV) = −1.8 mm]. Controls were injected with 0.9% saline. After recovery from anesthesia, mice were kept under observation for several hours. Behavioral *status epilepticus* (SE) was verified, characterized by mild convulsive movements, chewing, rotations or immobility, as previously described (Riban et al., 2002; Heinrich et al., 2006). Only mice that had experienced SE after KA injection were kept for further experiments.

In situ Hybridization

Localization of reelin mRNA was performed by *in situ* hybridization (ISH) with digoxigenin (DIG)-labeled cRNA probes as described earlier (Haas et al., 2002; Heinrich et al., 2006). Three weeks after KA injection, mice were deeply anesthetized (see above), transcardially perfused for 10 min with paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, followed by post-fixation of isolated brains for 4 h at 4°C in PFA, cryoprotection (20% sucrose in PB overnight at 4°C) and sectioning (50 μm; coronal plane) on a cryostat.

Slices were collected in culture dishes containing 2x SSC (1x SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), followed by incubation with a 1:1 mixture of 2x SSC and hybridization buffer (50% formamide, 4x SSC, 50 mM NaH_2PO_4, 250 μg/ml heat-denaturated salmon sperm DNA, 100 μg/ml tRNA, 5% dextransulfate, 1% Denhardt's solution) for 15 min. Prehybridization in hybridization buffer for 50 min at 45°C was followed by hybridization in the same buffer supplemented with DIG-labeled antisense reelin cRNA probe (100 ng/ml) at 45°C. Brain sections were washed twice in 2x SSC, followed by stringent washing at 55°C with 2x SSC and 50% formamide, 0.1x SSC and 50% formamide, and 0.1x SSC. Immunological detection of the hybrids was performed with an anti-DIG antibody conjugated with alkaline phosphatase and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Tissue sections were mounted on slides, air dried, and embedded in Kaiser's gelatine (Roche).

Quantification of Reelin mRNA-Expressing Neurons and Correlation with GCL Width

Reelin mRNA-expressing neurons were quantified in the whole hippocampus of control animals (NaCl-injected) and after KA injection. Cells were counted at three positions along the septohippocampal axis (septal, intermediate, and temporal). Cell numbers were determined in two regions of interest (ROI) per section: ROI1 included the *strata moleculare*, *lacunosum moleculare* and *radiatum*, whereas ROI2 comprised the hilus (see Supplementary Figure S1). All reelin mRNA-expressing neurons were counted in each ROI using the *ImageJ* analysis software (NIH, public domain). Cell densities were determined in three sections per position by relating cell numbers to the area of the respective ROI. These data were imported to *GraphPad Prism 5* software for statistical analysis.

Next, the mean GCL thickness was determined in the same tissue sections used for the quantification of reelin mRNA-producing neurons. Both GCL blades were subdivided into portions with constant thickness (see Supplementary Figure S2). Their width was measured with *ImageJ*. The respective values were multiplied with the length of the respective GCL portion. The multiplication-products from all CGL parts were summed up and divided by the total GCL length: $(Length_1 \times Width_1 + Length_2 \times Width_2 \ldots + Length_n \times Width_n)/Total\ Length$. Finally, the mean GCL thickness was correlated with the corresponding density of reelin mRNA-positive neurons in the hilus using *GraphPad Prism 5* software. We fitted the curve of a non-linear regression analysis (exponential growth equation) into the data points, since we found this equation to be the most suitable approximation to the real data.

Organotypic Hippocampal Slice Cultures

Eight-days-old (P8) male and female Thy1-eGFP mouse pups were used for OHSC preparation as described previously (Chai et al., 2014). In brief, brains were removed from the skull following decapitation under isoflurane anesthesia. The hippocampi were dissected and sliced (400 μm) perpendicular to the longitudinal axis of the hippocampus using a *McIlwain* tissue chopper. Only slices from the mid region of each hippocampus were used. The slices were placed onto culture inserts (Millipore) and transferred to 6-well plates with 1 ml/well of nutrition medium containing 50% minimal essential medium (MEM), 25% basal medium Eagle (BME), 25% heat-inactivated horse serum (Invitrogen) supplemented with 0.65% glucose and 2 mM glutamate (pH 7.2). OHSC were incubated as static cultures (Stoppini et al., 1991) in 5% CO_2 at 37°C for 7 days *in vitro* (DIV) before experiments started; the medium was changed every second day.

Production and Purification of the Recombinant Reelin Fragment R3–6

Human embryonic kidney 293 (HEK 293) cells were grown to subconfluency and were transiently transfected with an expression vector containing the myc-tagged R3–6 Reelin fragment (central fragment; Jossin et al., 2004; Bouché et al., 2013). One day after transfection, the fetal calf serum (FCS)-containing cell culture medium was replaced by serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 1.0 g/l glucose. After 2 DIV, the cell culture medium containing the R3–6 Reelin fragment was harvested and concentrated by centrifugation using 100 kDa cutoff centrifugal filters (Merck Millipore), sterile filtered, and stored at −20°C until further use (Leemhuis et al., 2010).

Purity and size of the recombinant R3–6 Reelin fragment was confirmed by Western blot analysis. To this end, recombinant full length Reelin and R3–6 Reelin were size-fractionated by a 3–8% Tris-acetate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Invitrogen) and blotted onto a polyvinylidene fluoride (PVDF) membrane (Roche). For immunodetection, the PVDF membrane was blocked with I-Block buffer (Tropix) and incubated with the monoclonal Reelin antibody R4B (2:1), produced in our laboratory as described by Jossin et al. (2007). It was followed by the incubation with the appropriate alkaline-phosphatase-conjugated secondary antibody (1:10,000; Tropix) and CDP Star (Tropix) was used as substrate for chemiluminescent detection by the *Chemismart* System (Peqlab Biotechnologies; see Supplementary Figure S3).

Concentration Assessment of the Recombinant R3–6 Reelin Fragment

The concentration of the recombinant R3–6 Reelin fragment was assessed by a dot blot assay followed by immunofluorescence detection. In brief, recombinant mouse Reelin protein with a known concentration (100 μg/mL, R&D Systems) and purified recombinant R3–6 Reelin were serially diluted and spotted onto nitrocellulose membrane (Santa Cruz Biotechnology). For immunodetection, the membrane was probed with the same antibody as for the Western blot, followed by incubation with the respective alkaline-phosphatase-conjugated secondary antibody (1:10,000; Tropix). CDP Star (Tropix) was used as substrate for chemiluminescent detection by the *Chemismart* System. Densitometric evaluation of the dot blot signals was performed by optical density (OD) measurement using *ImageJ* software.

The concentration was calculated based on the comparison of OD values obtained for the recombinant mouse Reelin standard curve and the recombinant R3–6 Reelin fragment.

Immunohistochemistry

Tissue sections (50 μm) from perfused KA-injected mouse brains (see above) or whole OHSC from Thy1-eGFP mice were immunolabeled using a free-floating protocol (Heinrich et al., 2006; Tinnes et al., 2011). OHSC (13 DIV) were fixed with 4% PFA in PB (4 h, RT). After pre-incubation (0.25% Triton X-100, 10% normal serum in PB, 2 h), tissue sections or slices were incubated for 24 h (RT) with mouse monoclonal anti-Reelin antibody (G-10, 1:1000; Chemicon) or rabbit polyclonal anti-Prox1 (1:1000, Abcam). Antibody binding was visualized by incubation with an appropriate Cy3-conjugated secondary antibody (1:400, Jackson ImmunoResearch Laboratories) in the dark (6 h, RT). Tissue sections were counterstained with DAPI. Sections and whole slices were coverslipped with anti-fading mounting medium (IMMU-Mount, Thermo Fisher Scientific) and analyzed using an epifluorescence (Axio Imager 2, Carl Zeiss) or confocal microscope (Olympus FluoView FV10i).

Quantification of Reelin-Immunofluorescence in OHSC

Reelin-expressing neurons were quantified in whole slices under control conditions and after KA (10 μM) application for 45 min, followed by incubation in fresh medium for 8 h. To quantify Reelin fluorescence intensity, confocal z-stacks were acquired from whole OHSC with an Olympus FV10i confocal laser scanning microscope (Olympus) using a 10x objective at high resolution (1024 × 1024 pixel; 8× frame-average) with constant exposure time and z-stack settings. Stacks were converted to grayscale and the signal intensity of Reelin immunolabeling along the hippocampal fissure (HF) and in the hilus was quantified as integrated density using the *ImageJ* analysis software. Values were corrected by background subtraction: integrated density – (measured area × mean background signal). The mean background was calculated for each experiment and for both areas of the HF and hilus. Background signals were measured in areas without Reelin signal.

Live Cell Imaging

Organotypic hippocampal slice cultures were prepared from Thy1-eGFP mouse pups (P8) and cultivated for 7–18 DIV. Immediately before live cell imaging, OHSC were exposed to KA (3 or 10 μM) for 45 min, followed by addition of fresh medium or fresh medium including recombinant R3–6 Reelin fragment (1 nM) followed by live cell imaging for 8 h. For imaging, OHSC were placed, on the stage of a Leica SP2 confocal microscope, and enclosed in an aerated chamber at 37°C and with humidified atmosphere containing 5% CO_2. eGFP-positive granule cells were imaged along the Z-axis with a spacing of 6 μm, using a 10x objective at 2x optical zoom, at 45 min intervals over a period of 8 h. To monitor the positions of individual granule cells, confocal image stacks taken at the different time points were imported into the image analysis software *Fiji,* which is based on *ImageJ* (NIH, public domain). Using the *MtrackJ*© plugin (Erik Meijering), six individual granule cells/OHSC were randomly selected. Their positions were marked at all 10 time points over a period of 8 h to allow for the calculation of their migration distances.

To investigate preferred directions of migration, we identified individual granule cells in the superficial and deep GCL (three cells per sublayer/OHSC) after exposure to KA or control conditions. Cell motility was quantified by calculating the total length of the traveled path over 8 h and the effective distance of the migration as the length of the resulting vector between start and end point of the cell. To differentiate the direction of migration, we marked the border between the hilus (H) and GCL and calculated the distance of the start and end point of the cells to this hilar-GCL border. Cells that reduced their distance to the border over time [$H_{distance}(t = 0$ min) – $H_{distance}(t = 450$ min)] were considered to migrate toward the hilus, whereas cells showing an increased distance in relation to the hilar-GCL border moved toward the molecular layer (ML). We used custom Matlab® software to perform this data analysis (Matlab® 2014a, The-Mathworks).

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed with *GraphPad Prism 5* software. Differences between groups were tested for statistical significance (Student's *t*-test or one-way ANOVA with Tukey's multiple comparison test). Significance levels were set to $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Quantification of Reelin mRNA-Expressing Neurons Along the Septotemporal Axis After Intrahippocampal KA Injection

In previous studies, a loss of reelin-producing neurons has been shown in the septal hippocampus after KA injection on the mRNA and protein level (Heinrich et al., 2006; Antonucci et al., 2008; Duveau et al., 2011). Yet, these reports focused only on the area close to the KA injection site. There is, however, a septotemporal gradient of GCD and cell loss in the intrahippocampal KA mouse model (Häussler et al., 2012; Marx et al., 2013). Therefore, we aimed at precisely determining the spatial distribution of reelin-expressing neurons along the septotemporal axis of the hippocampus after KA injection by ISH and immunohistochemistry. To this end, reelin mRNA-positive neurons were counted in tissue sections at three positions (septal, intermediate, and temporal) along the hippocampal septotemporal axis at 21 days after KA injection, a time point when GCD has fully developed (Heinrich et al., 2006) and we correlated the GCL width with the number of reelin mRNA-positive hilar neurons. NaCl-injected mice were used as controls (see Materials and Methods, Supplementary Figure S1).

In tissue sections of control animals, many reelin mRNA-expressing neurons were observed in *stratum oriens*, *stratum lacunosum moleculare* along the HF and in the hilus at all positions along the septotemporal axis of the hippocampus (**Figures 1A,C,E**). After KA injection, a drastic loss of reelin mRNA- and Reelin-expressing neurons was evident all over the ipsilateral septal hippocampus, only at the HF, reelin mRNA- and Reelin-positive neurons were preserved (**Figures 1B,I**). Interestingly, this loss was confined to the septal portion of the hippocampus, where GCD was present (**Figures 1B,D,F,I–K**). Cell counting revealed that the density of reelin mRNA-expressing neurons was decreased ipsilaterally at the HF at all three positions when compared to controls (septal; control: 228.5 ± 31.26, KA: 194.0 ± 12.6; intermediate; control: 165 ± 24.45, KA: 132 ± 7.1; temporal; control: 188 ± 9.9, KA: 147.0 ± 8.6 cells/mm^2), but this decrease did not reach significance (**Figure 1G**). In the hilus, however, an almost complete loss of reelin mRNA-synthetizing neurons was detectable in sections of the septal and intermediate KA-injected hippocampus when compared to controls (septal; control: 469.3 ± 69.8, KA: 22.92 ± 3.2; intermediate; control: 413.2 ± 56.1, KA: 27.56 ± 5.9 cells/mm^2), whereas at the temporal position the loss was not significant in comparison to controls (temporal; control: 335.9 ± 71.9, KA: 209.6 ± 15.56 cells/mm^2; **Figure 1H**). Next, we determined the GCL width ipsilaterally in the same tissue sections and related it to the number of reelin mRNA-positive hilar neurons. We found an inverse correlation of GCL width and number of reelin mRNA-expressing neurons in the hilus (**Figure 1L**).

In summary, our detailed quantification along the septotemporal axis revealed that the loss of reelin mRNA-positive neurons was strongest in the hilus in regions exhibiting GCD and this loss precisely correlates with the occurrence of GCD.

Distribution of Reelin-Expressing Neurons in KA-Treated OHSC

Next, we used OHSC from transgenic Thy1-eGFP mice to investigate the effect of the KA treatment on Reelin-producing neurons *in vitro*. KA (10 μm) was applied to OHSC for 45 min, followed by incubation in fresh medium for 8 h. Slices were fixed with PFA and whole OHSC were immunolabeled for Reelin. In control OHSC, eGFP-positive granule cells were densely arranged in a compact layer with dendrites extending into the ML; Reelin-positive neurons were located at the HF and in the hilus (**Figures 2A,C,E**). In KA-treated OHSC, eGFP-positive granule cells appeared dispersed; there were still many Reelin-immunolabeled neurons at the HF, but almost all had disappeared from the hilus (**Figures 2B,D,F**). Densitometric evaluation revealed a slight reduction of Reelin immunofluorescence at the HF, but an almost complete, significant loss of the Reelin signal in the hilus (control: HF 25.46 ± 3:9; H 7.99 ± 2.4; KA: HF 14.83 ± 2.1; H 0.7 ± 0.18; **Figures 2G,H**). These results show that, like *in vivo*, Reelin-expressing neurons were lost in the hilus, whereas Reelin-synthetizing neurons at the HF survived.

Life Cell Imaging of eGFP-Positive Granule Cells in OHSC After Treatment with Increasing Doses of KA

As a prerequisite for later life cell imaging experiments, we first determined the minimal KA concentration needed to trigger migration. To this end, OHSC from Thy1-eGFP mice were exposed to 3 or 10 μM KA for 45 min, followed by incubation in fresh medium and life cell imaging for 8 h (**Figures 3A–C**). Afterward, the migration distances of individual eGFP-labeled granule cells were determined (**Figure 3D**). We found that 3 μM KA (4.43 ± 0.63 μm) increased the motility of eGFP-labeled granule cells only slightly above control values (3.68 ± 0.4 μm) during the observation period, whereas 10 μM KA (10.8 ± 1.4 μm) triggered the movement significantly. Hence, we used this KA concentration for all subsequent imaging experiments.

Migration Behavior of eGFP-Positive Granule Cells in OHSC After KA Treatment

Next, we aimed at investigating whether eGFP-labeled granule cells followed a specific migration pattern triggered by KA treatment. As described above, we performed life cell imaging after KA (10 μM) treatment and compared it to untreated controls. We randomly marked individual granule cells in the superficial and deep GCL (three cells per sublayer/OHSC) and tracked their position with respect to the border between the hilus and GCL (**Figure 4A**) over a period of 8 h. We quantified differences in motility by calculating (1) the total length of the traveled path over 8 h and (2) the effective distance of migration as the length of the vector between start ($t = 0$ min) and end point ($t = 450$ min; **Figure 4B**).

This analysis revealed that after KA treatment granule cells showed a significantly higher motility than under control conditions with an increased total path length (Ctrl = 5.10 ± 0.65 μm; KA = 13.86 ± 0.84 μm; $P < 1.73 \times 10^{-10}$) and an increased effective distance (Ctrl = 3.10 ± 0.36 μm; KA = 5.59 ± 0.49 μm; $P < 2.6 \times 10^{-4}$). Furthermore, we found that the total path length increased correspondingly with the length of the effective path (**Figure 4C**). In addition, we observed that the majority of granule cells from KA-treated slices (80%) migrated toward the hilus (**Figures 4D,E**), regardless of their initial position within the GCL (**Figure 4F**). Moreover, cells migrating toward the hilus traveled longer distances than those heading to the ML (changed distance to hilus, Ctrl = 0.76 ± 0.46 μm; KA = 3.17 ± 0.85 μm; $P < 0.02$; **Figure 4C**).

Influence of the Recombinant R3–6 Central Reelin Fragment on KA-Induced Motility of eGFP-Labeled Granule Cells

So far, our results showed that in KA-treated OHSC Reelin-expressing neurons are mainly lost in the hilus and that the majority of eGFP-positive granule cells migrate toward the hilus. Together, these findings point to a stop signal function of Reelin

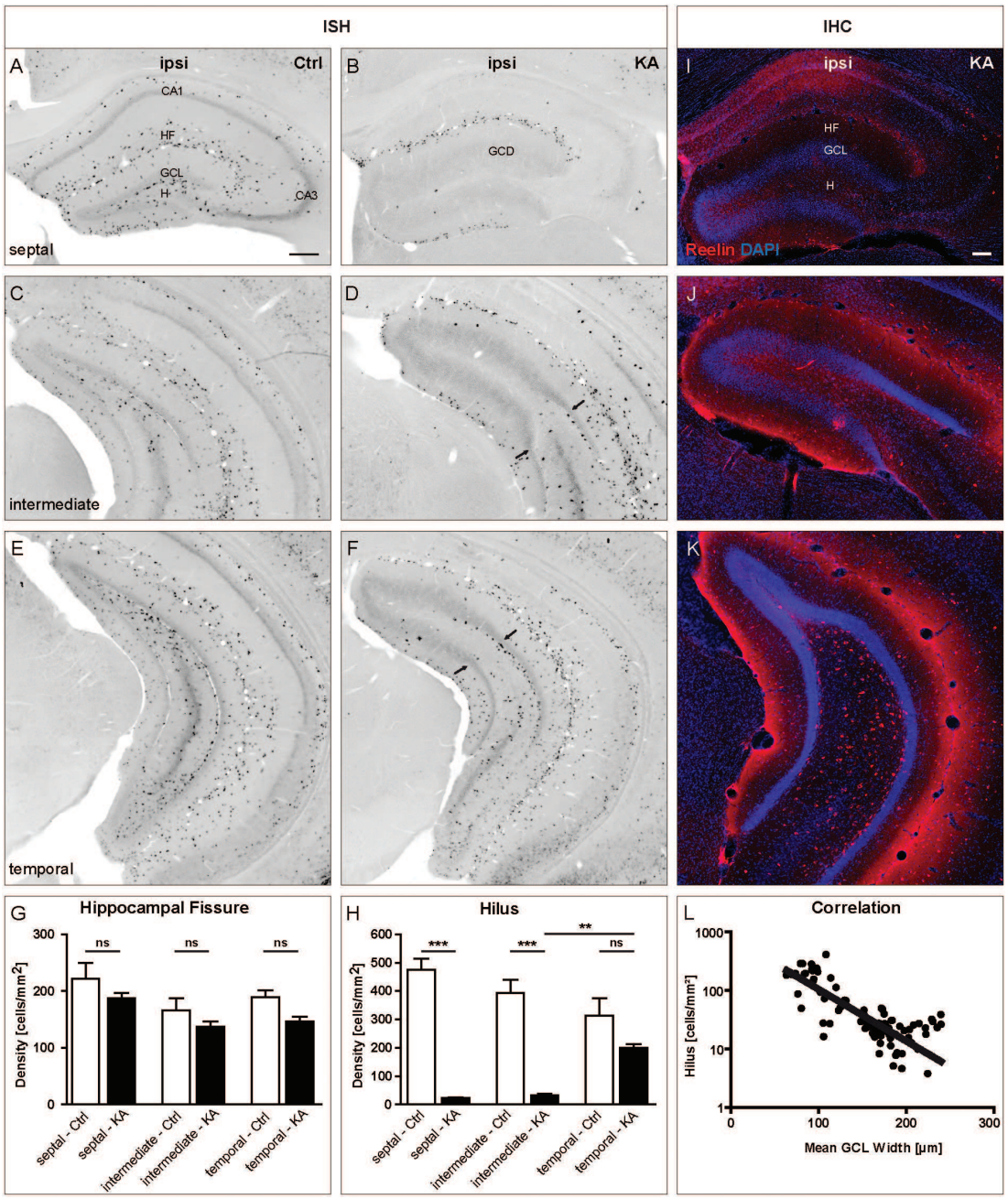

FIGURE 1 | Loss of reelin mRNA-expressing neurons coincides with GCD formation after unilateral intrahippocampal KA injection in adult mice. ISH for reelin mRNA was performed on tissue sections along the septotemporal axis at 21 days after KA injection. **(A–F)** Representative images showing the distribution of reelin mRNA-positive neurons in sections of the septal **(A**,**B)**, intermediate **(C**,**D)** and temporal **(E**,**F)** portions of the hippocampus of control animals **(A**,**C**,**E)** and ipsilateral to the KA injection **(B**,**D**,**F)**. **(A,C,E)** In controls, many reelin mRNA-expressing neurons are present in the *stratum oriens*, *stratum lacunosum moleculare*, along the HF and in the hilus at all three positions along the septotemporal axis of the hippocampus (septal, intermediate, and temporal). **(B)** At 21 days after intrahippocampal KA injection there is a drastic loss of reelin mRNA-expressing neurons all over the ipsilateral septal hippocampus, where GCD is present. Only at the HF, they are preserved. **(D,F)** A loss of reelin mRNA-positive neurons can also be observed in intermediate and temporal sections, however, being confined to the portion with GCD. Arrows indicate the transition from GCD to normal GCL, where reelin mRNA-positive neurons reappear. **(G,H)** Quantification of the number of reelin mRNA-expressing neurons in the HF **(G)** and the hilus **(H)** in controls ($n = 3$) and after KA injection ($n = 5$) at three different positions along the septotemporal axis of the hippocampus (septal, intermediate, and temporal). Note the significant loss of reelin mRNA-expressing neurons in the hilus at septal and intermediate positions. One-way ANOVA followed by Tukey's Multiple Comparison Test ($^{**}P < 0.01$; $^{***}P < 0.001$). Representative images of tissue sections immunolabeled for Reelin (red) counterstained with DAPI (blue) of the septal **(I)**, intermediate **(J),** and temporal **(K)** portions of the hippocampus ipsilateral to the KA injection. Like on the mRNA level, Reelin-expressing neurons are gone in the septal and intermediate hilus but are preserved at the HF. They reappear in the temporal hippocampus, mirroring the distribution of reelin mRNA expression. **(L)** Graph showing the correlation of the mean GCL width with the number of reelin mRNA-positive hilar neurons ($r^2 = 0.6112$). CA1, *cornu ammonis* 1; CA3, *cornu ammonis* 3; HF, hippocampal fissure; GCL, granule cell layer; H, hilus; ML, molecular layer. Scale bars: 200 µm.

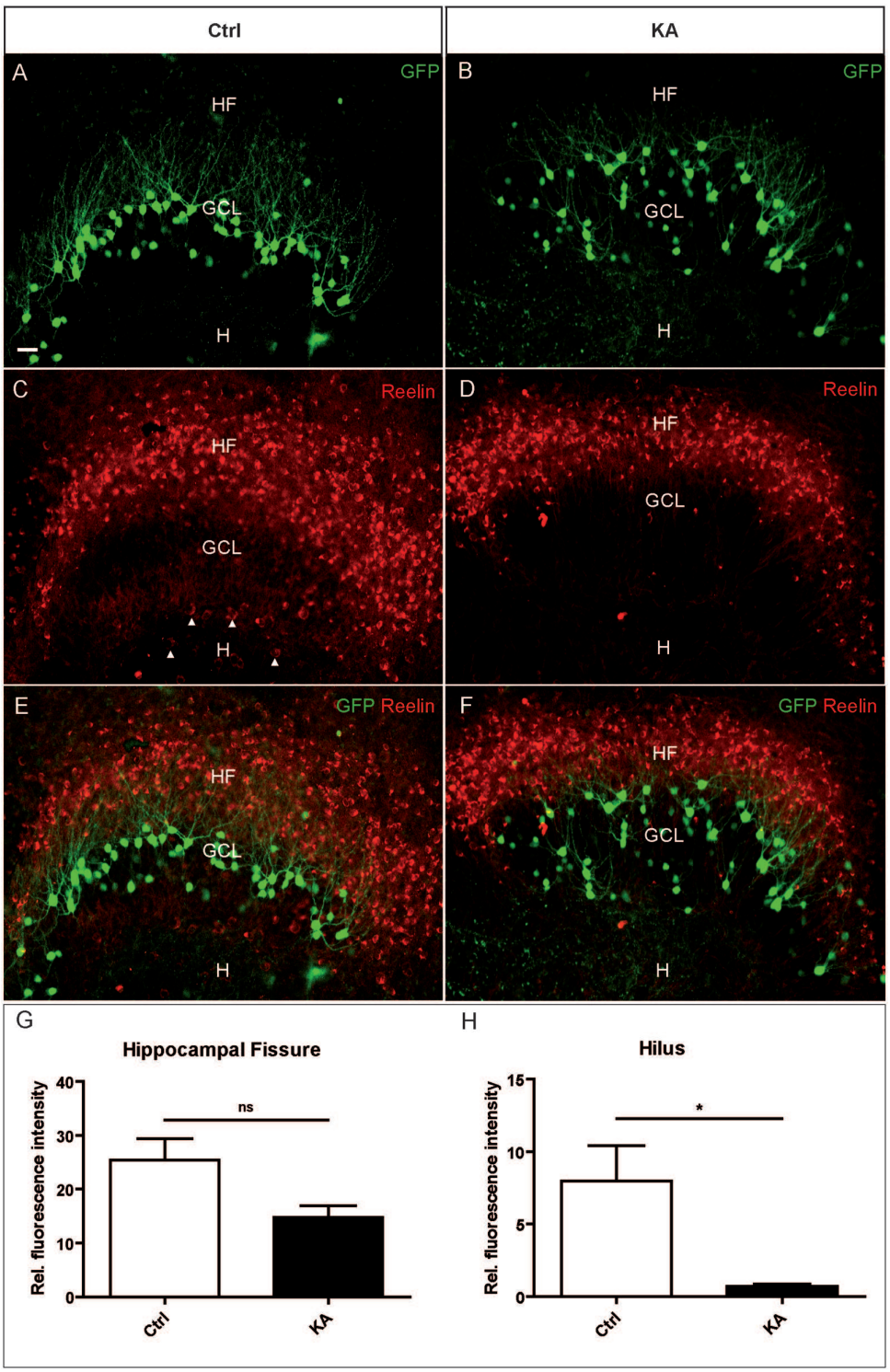

FIGURE 2 | Loss of Reelin-positive neurons in the hilus after KA treatment in OHSC. Representative confocal images of whole OHSC from Thy1-eGFP (enhanced green fluorescent protein; green) mice immunolabeled for Reelin (red). **(A,C,E)** Control. **(B,D,F)** OHSC, 8 h after KA application for 45 min. In the control, eGFP-positive granule cells are arranged in a compact layer **(A)** but appear dispersed after KA treatment **(B)**. **(C,D)** Immunolabeling for Reelin. Many strongly immunostained Reelin-expressing neurons are visible at the HF, and large Reelin-immunopositive neurons are located in the hilus (arrow heads, **C**). Note the loss of Reelin-positive hilar interneurons after KA treatment **(D)**. **(E,F)** Overlay of eGFP (green) and Reelin (red) signals. **(G,H)** Densitometric quantification of the Reelin signal at the HF **(G)** and in the hilus **(H)** in controls ($n = 4$) and after KA treatment ($n = 4$). Unpaired student's t-test ($^*P < 0.05$, ns with $P = 0.057$). GCL, granule cell layer; H, hilus; HF, hippocampal fissure. Scale bar: 10 μm.

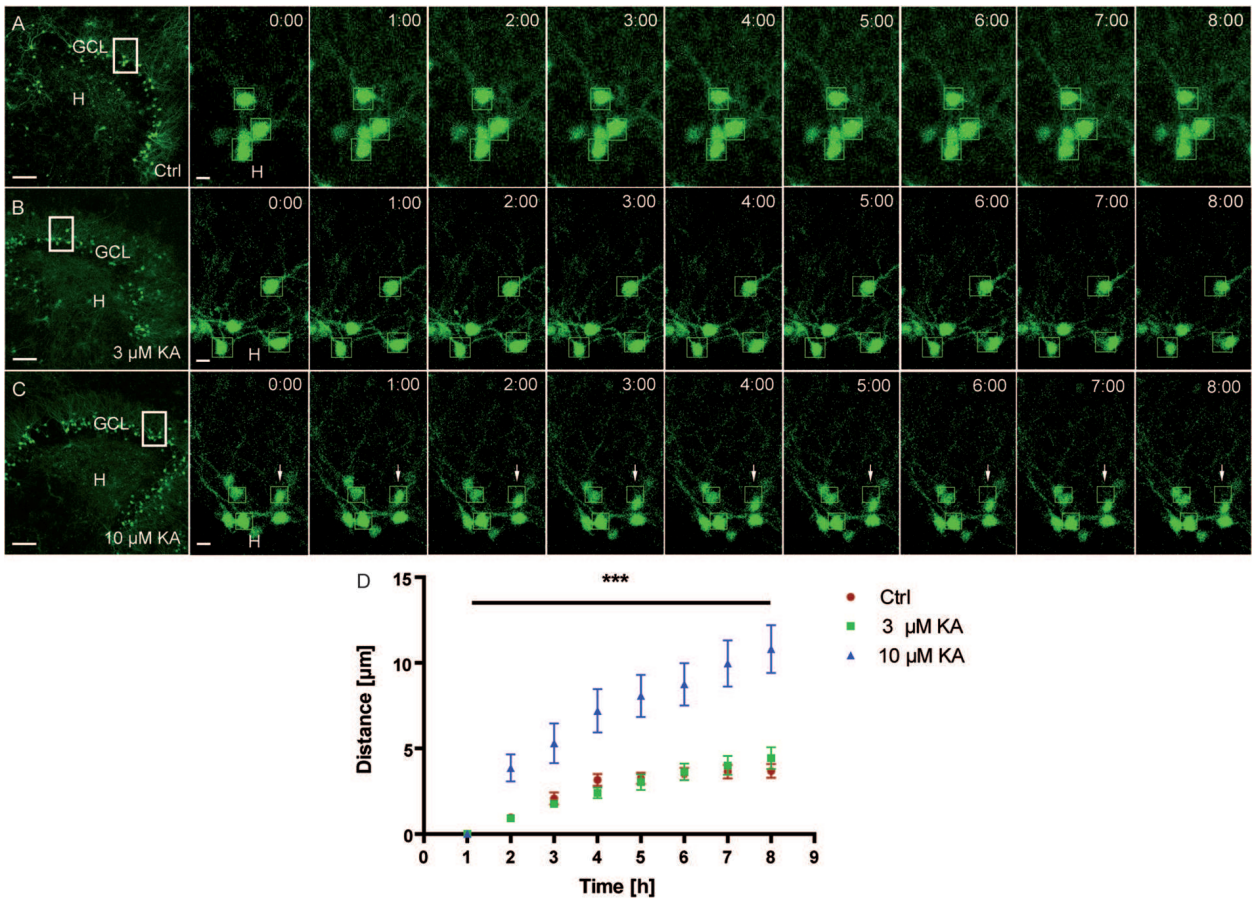

FIGURE 3 | Motility of granule cells depends on the KA concentration. Life cell imaging of individual eGFP-labeled granule cells was performed over a period of 8 h, and migration distances of randomly selected cells were assessed as described in Section "Materials and Methods." Representative confocal micrographs of nine imaging time points are shown for each condition. Left panel: overview; white frame indicates the area shown at high magnification on the right. Tracked cells, are marked by green frames. **(A)** Control ($n = 4$; 24 cells). Cells remain in place. **(B)** 3 µM KA ($n = 4$; 24 cells). Tracked cells behave similar to controls. **(C)** 10 µM KA ($n = 3$, 18 cells). Granule cells show increased motility (white arrows). **(D)** Statistical analysis of the motility of adult granule cells of control and KA-treated OHSC (3 µM KA, 10 µM). The migration behavior increases in a KA dose-dependent manner over the entire 8 h period. One-way ANOVA followed by Tukey's test with (***$P < 0.001$). GCL, granule cell layer; H, hilus. Scale bars: 80 µm for overview; 5 µm for high magnifications.

which might be lost in the presence of KA. Thus, we next investigated whether the R3–6 central Reelin fragment, known to be important for activation of the Reelin signaling cascade (Jossin et al., 2004), would be capable of preventing the observed KA-triggered movement of granule cells. For this purpose, OHSC were treated for 45 min with KA, followed by washout, addition of the recombinant R3–6 Reelin fragment to the medium and subsequent live cell imaging for 8 h.

As shown before, KA treatment alone caused an increased motility (10.4 ± 1.4 µm) of granule cells when compared to the controls (3.9 ± 0.4 µm; **Figures 5A,B**). In contrast, the presence of the R3–6 Reelin fragment significantly prevented the movement of granule cells (4.2 ± 0.5 µm) when compared with KA-treated OHSC (**Figures 5C–E**). As a control, we showed that the application of KA and the R3–6 Reelin fragment did not prevent the KA-mediated loss of Reelin-positive hilar neurons (see Supplementary Figure S4). These results indicate that the central Reelin fragment was able to stop the KA-mediated migration of eGFP-positive granule cells and support the role for Reelin as a positional signal for granule cells in the adult hippocampus.

DISCUSSION

In this study, we show that reelin-producing neurons are mainly lost in the hilus after intrahippocampal KA injection in mice and that this loss correlates precisely with the occurrence of GCD. Also in OHSC, KA treatment causes a rapid and complete loss of hilar, Reelin-producing neurons. Using life cell microscopy, we provide evidence that differentiated eGFP-positive granule cells actively migrate toward the Reelin-free hilar region, and that this migration process can be prevented by application of the recombinant R3–6 Reelin fragment. Thus, the development of the GCD seems to depend on the loss of functionally active Reelin.

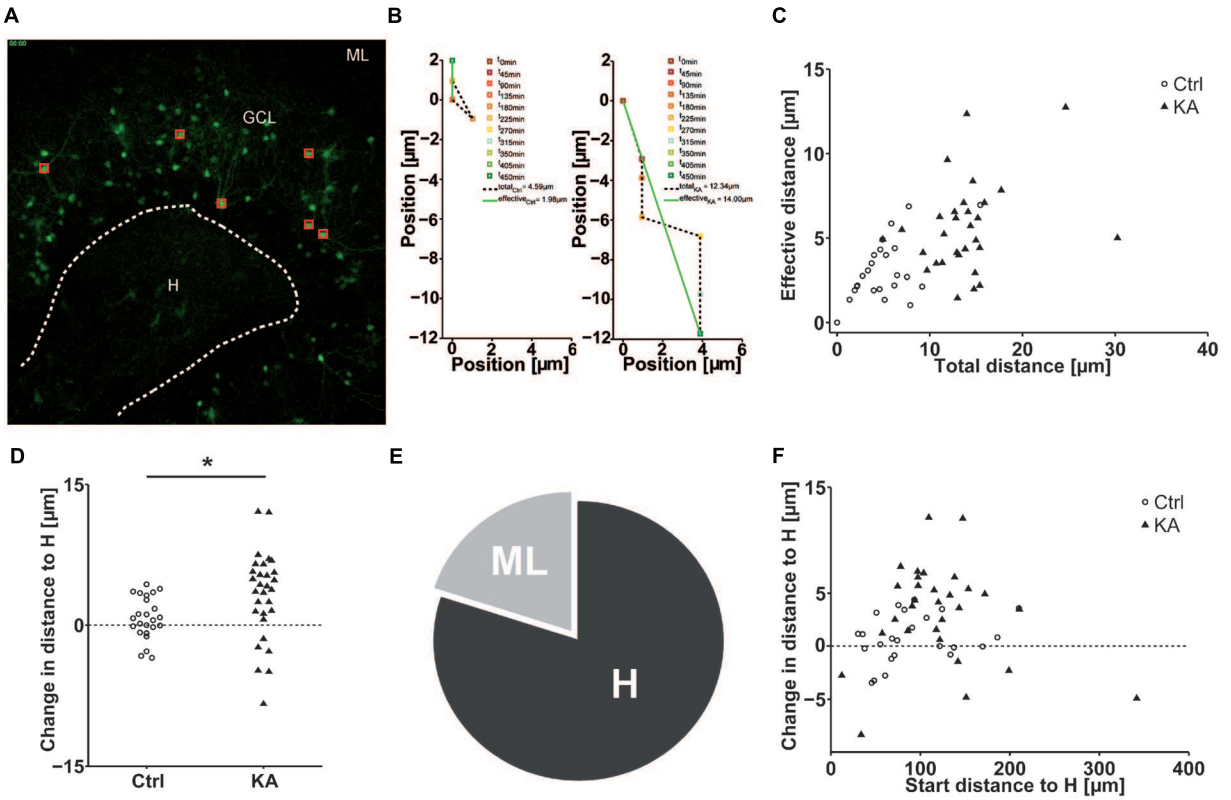

FIGURE 4 | EGFP-positive granule cells migrate preferentially to the hilus. Life cell imaging of individual eGFP-labeled granule cells in the superficial and deep granule cell layer after exposure to KA and in controls over a period of 8 h. **(A)** Representative photomicrograph of a KA-treated OHSC from a Thy-1 eGFP mouse ($t = 0$ min). Red squares indicate the initial position of monitored cells. White dashed line marks the border between the hilus and the GCL, taken as reference to calculate the relative migration distances of individual eGFP-labeled cells. **(B)** Representative graphs showing the motility of one tracked neuron in a control (left graph) or a KA-treated OHSC (right graph). Cell motility was assessed by calculating both, the total length of the traveled path over 8 h (black dashed line = total distance) and the effective distance of the migration as the length of the resulting vector (green solid line) between start (at $x = 0$; $t = 0$ min) and end point ($t = 450$ min). **(C)** Diagram showing the relation between total and effective migration distance of all tracked granule cells. In contrast to controls, all cells change their position after KA treatment. **(D)** Migration distances of all tracked individual granule cells with respect to the border between hilus and GCL in control ($n = 4$, 24 cells) and KA-treated OHSC ($n = 5$, 30 cells). To differentiate the migration direction, we marked the border between the hilus and GCL (**A**, white dashed line) and calculated the distance of the start and end point of the cells to this border. Cells that reduce their distance to the border over time [$H_{distance}$ ($t = 0$ min) – $H_{distance}$ ($t = 450$ min), positive values] are considered migrating toward the hilus. Cells showing an increase in distance (negative values) moved toward the ML. Cells in KA-treated OHSC (black triangles) travel longer distances in both directions, toward ML and hilus, than controls (black circles). Two-sample Student's *t*-test (*$P < 0.05$). Cells which migrate toward the hilus travel longer distances than those heading toward the ML. The majority of cells progresses toward the hilus. **(E)** Relative distribution of cells traveling toward the hilus (80%) or molecular layer (20%) in KA-treated OHSC. **(F)** Diagram showing the relation between initial distance to the hilus-GCL border and change in distance to the border of all tracked granule cells. In contrast to controls, all cells change their position after KA treatment and travel longer distances regardless of their position in the deep or superficial granule cell layer. ML, molecular layer; GCL, granule cell layer; H, hilus.

Loss of Reelin mRNA-Expressing Neurons Correlates Spatially with the Occurrence of GCD in the Epileptic Mouse Hippocampus

In the hippocampus reelin mRNA and protein is mainly expressed by GABAergic interneurons located primarily in *stratum oriens* and *radiatum* of *cornu ammonis* (CA) 1 and CA3 and in the hilus of the dentate gyrus but also by CR cells located along the HF (Alcántara et al., 1998; Pesold et al., 1998; Ramos-Moreno et al., 2006). In the present study, we show by detailed quantification of reelin mRNA expression along the septotemporal axis of the epileptic mouse hippocampus that reelin mRNA-synthetizing CR cells at the HF survive, whereas in the hilus reelin mRNA-expressing interneurons are lost. In fact, this loss is confined to the septal hippocampus and precisely matches spatially with the occurrence of GCD, as demonstrated by correlating GCL width and numbers of reelin-synthesizing neurons. These results confirm and extend previous studies in the same epilepsy model, showing that Reelin-producing CR cells are preserved at the HF but lost in the hilus (Heinrich et al., 2006; Antonucci et al., 2008; Duveau et al., 2011). However, all these reports focused only on the area close to the KA injection site. Here, we demonstrate that the loss of Reelin synthesis precisely mirrors the septotemporal gradient of GCD described for this epilepsy model (Häussler et al., 2012) and highlight a link between Reelin loss and GCD formation.

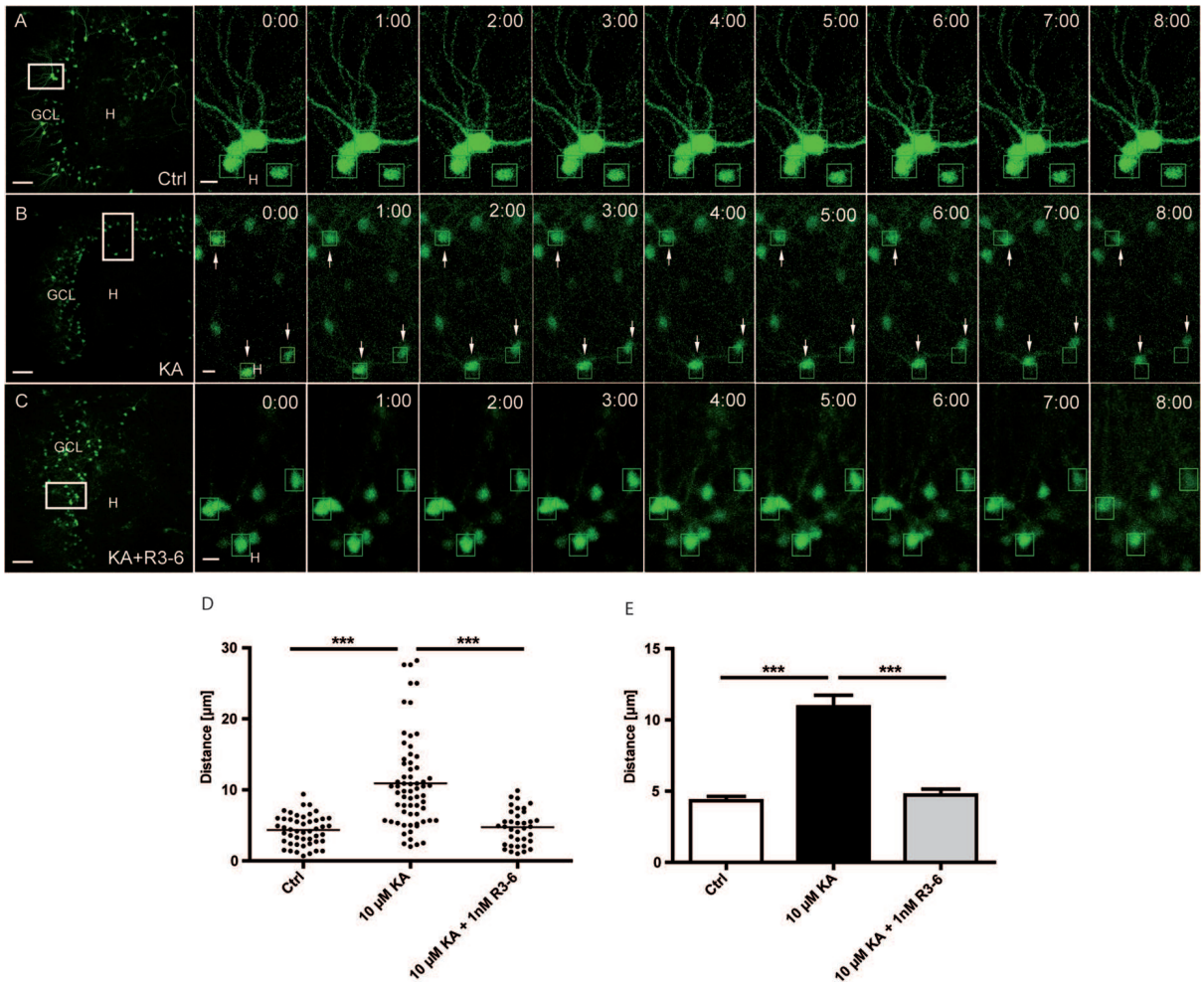

FIGURE 5 | Kainate-induced migration of differentiated granule cells is prevented by the central R3–6 Reelin fragment. Live cell imaging of individual eGFP-positive granule cells was performed over a period of 8 h, and migration distances of randomly selected cells were assessed as described in Section "Materials and Methods." Representative confocal micrographs of nine imaging time points are shown for each condition. Left panel: overview; white frame indicates the area shown at high magnification on the right. Tracked cells are marked by green frames. **(A)** Untreated control ($n = 8$; 48 cells). Cells do not change position. **(B)** Treatment of OHSC with 10 μM KA for 45 min ($n = 10$; 60 cells). Granule cells (GCs) with high motility are indicated by arrows. **(C)** Treatment of OHSC with 10 μM KA for 45 min, followed by incubation with fresh medium and subsequent application of recombinant R3–6 Reelin fragment (1 nM; $n = 6$; 36 cells). Like controls, cells do not change position. **(D,E)** Quantitative evaluation of migration distances of individual GCs. KA-treated GCs migrate, but not in the presence of the recombinant R3–6 Reelin fragment. One-way ANOVA, followed by Tukey's Multiple Comparison Test (***$P < 0.001$; Ctrl vs. 10 μM KA + 1 nM R3–6 shows no significance). GCL, granule cell layer; H, hilus. Scale bars: 80 μm for overview; 5 μm for high magnifications.

KA Treatment of OHSC Causes a Rapid Loss of Reelin-Producing Neurons in the Hilus

Organotypic hippocampal slice cultures have been shown to be a suitable model to study neuronal changes induced by epileptiform activity. Challenged by treatment with the glutamate receptor agonist KA, OHSC develop histopathological features similar to AHS such as cell death, mossy fiber sprouting, GCD, and epileptic activity (Routbort et al., 1999; Tinnes et al., 2011; Chai et al., 2014). Here we used OHSC obtained from Thy1-eGFP mice, known to express eGFP primarily in a subset of differentiated granule cells as shown previously (Feng et al., 2000; Chai et al., 2014) and by double labeling with Prox1, a marker for differentiated granule cells (Supplementary Figure S5). When OHSC were exposed to KA for 45 min, a rapid (within 8 h) and selective loss of Reelin-positive interneurons was observed in the hilus, whereas Reelin-positive CR cells persisted. This observation confirms the high vulnerability of Reelin-producing interneurons to KA-mediated excitotoxicity seen *in vivo* and reported in OHSC previously (Tinnes et al., 2011; Chai et al., 2014), resulting in a Reelin loss confined to the hilar region. The observed difference in survival rate between interneurons and CR cells is most likely due to differential expression of glutamate receptors, since only hilar interneurons, but not CR cells, up-regulate c-Fos after KA treatment (Tinnes et al., 2011).

eGFP-Positive Granule Cells Preferentially Migrate toward the Reelin-Poor Hilus

In the adult dentate gyrus, granule cells form a densely packed layer. Under epileptic conditions, the lamination can dissolve and result in GCD as observed, in MTLE patients (Houser, 1990; Haas et al., 2002) and in our MTLE mouse model (Bouilleret et al., 1999; Heinrich et al., 2006; Häussler et al., 2012). GCD formation is a process affecting differentiating granule cells, since GCD develops in the absence of neurogenesis as shown previously after intrahippocampal KA injection (Kralic et al., 2005; Heinrich et al., 2006; Nitta et al., 2008). Recent *in vitro* studies reported that this migration process is based on somal translocation (Murphy and Danzer, 2011; Chai et al., 2014). Somatic translocation is a principal migratory mechanism of neurons during brain development and occurs when the nucleus and perisomatic cytoplasm are displaced into a leading process (Rakic, 1972; Métin et al., 2008). Here, we show by life cell microscopy that eGFP-labeled, differentiated granule cells become motile in response to KA challenge. By tracking the path of individual neurons during the whole observation period, we found that the majority (80%) moved in the direction of the hilus, whereas only 20% traveled to the opposite direction toward the ML. The migration pattern appeared rather complex. The neurons did not move straight into one direction but traveled erroneously in different directions before they reached a position closer to the hilar (or ML) area. It is tempting to speculate that most of the granule cells moved specifically to the direction of the Reelin-free hilar area. We cannot exclude, however, that a functional inactivation of Reelin by impaired proteolytic processing, known to occur under epileptic conditions (Tinnes et al., 2011, 2013), might play a role in the movement of neurons toward the ML.

KA-Induced Motility of Adult Granule Cells is Prevented by Application of the Central R3–6 Reelin Fragment

Reelin acts as a positional cue for dentate granule cells during development, since rescue of granule cell lamination in Reelin-deficient *reeler* mice could be achieved when Reelin was present in normotopic position, provided by a wild-type co-culture (Zhao et al., 2004). Conversely, infusion of Reelin-blocking antibodies (CR-50) into the hippocampus of normal adult mice induced GCD locally (Heinrich et al., 2006). These findings established a role for Reelin in stabilizing the lamination of the dentate gyrus. Accordingly, we hypothesized that the KA-triggered motility of dentate granule cells toward the Reelin-free hilar area might be caused by a loss of the positional cue. Granule cells constitutively express the Reelin receptors ApoER2 and VLDLR (Haas et al., 2002) and have been shown to maintain their expression after KA injection (Müller et al., 2009). Here, we demonstrated that addition of recombinant R3–6 Reelin fragment (1 nM) was able to prevent the movement of granule cells observed after KA application alone. The R3–6 Reelin fragment has been shown to be sufficient for activating the Reelin signal transduction cascade on target cells (Jossin et al., 2004). Despite the existence of several Reelin isoforms *in vivo*, only fragments containing R3–6 are capable of binding ApoER2 and VLDLR, and both receptors alone are capable of binding Reelin with similar affinity. The residues Lys-2360 and Lys-2467, found in Reelin R3–6, are directly responsible for coordinated binding of Reelin to the conserved ligand binding domains of ApoER2/VLDLR (Yasui et al., 2007, 2010). In agreement with these findings, application of Reelin fragments containing R5–6 to *reeler* cortical explants is sufficient to induce Dab1 phosphorylation and to normalize cortical lamination (Jossin et al., 2004).

So far, our findings point to a stop signal function of Reelin, which might be lost in the presence of KA. In contrast, a recent study did not detect the formation of GCD after conditional Reelin knockout in the adult dentate gyrus (Lane-Donovan et al., 2015). In these mice, however, Reelin was not ablated completely; they still exhibited around 5% of the initial Reelin concentration. Since very low Reelin concentrations (1 nM) were sufficient to obtain significant effects in our hands and also in other studies (Leemhuis et al., 2010), the incomplete Reelin knockout may explain the controversy.

Thus, our results indicate that the central Reelin fragment is able to prevent the KA-mediated migration of eGFP-positive granule cells and support the role for Reelin as a positional signal for granule cells in the adult hippocampus.

AUTHOR CONTRIBUTIONS

CO: performed experiments, data analysis, manuscript writing; GM: performed experiments, data analysis; JG: performed experiments, data analysis; AK: data analysis and Matlab® calculations; MF: confocal imaging and data analysis; UE: data analysis; CH: conception, supervision, manuscript writing.

FUNDING

This work was financially supported by the German Research Council (DFG) as part of the Cluster of Excellence 'BrainLinks-BrainTools' (EXC1086), by the German Federal Ministry of Education and Research (BMBF) (Bernstein Focus Neurotechnology Freiburg*Tuebingen, FKZ 01GQ0830), by ERA-Net NEURON II CIPRESS and by a fellowship of the German Academic Exchange Service (DAAD) to CO. The article processing charge was funded by the German Research Foundation (DFG) and the University of Freiburg in the funding programme Open Access Publishing.

ACKNOWLEDGMENTS

We are grateful to S. Huber and A. Djie-Maletz for excellent technical assistance and to Dr. H. Bock, University of Düsseldorf, for plasmids and reagents.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2016.00183

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 25 May 2016
doi: 10.3389/fncel.2016.00141

Alterations of Cell Proliferation and Apoptosis in the Hypoplastic *Reeler* Cerebellum

Carolina Cocito[1], Adalberto Merighi[1], Mario Giacobini[2] and Laura Lossi[1]**

[1] Laboratory of Neuroscience, Department of Veterinary Sciences, University of Turin, Grugliasco, Italy, [2] Laboratory of Dynamical Systems and Epidemiology, Department of Veterinary Sciences, University of Turin, Grugliasco, Italy

A mutation of the *reln* gene gives rise to the *Reeler* mouse (*reln*$^{-/-}$) displaying an ataxic phenotype and cerebellar hypoplasia. We have characterized the neurochemistry of postnatal (P0–P60) *reln*$^{-/-}$ mouse cerebella with specific attention to the intervention of cell proliferation and apoptosis in the P0–P25 interval. Homozygous *reln*$^{-/-}$ mice and age-matched controls were analyzed by immunofluorescence using primary antibodies against NeuN, calbindin, GFAP, vimentin, SMI32, and GAD67. Proliferation and apoptosis were detected after a single intraperitoneal BrdU injection and by the TUNEL assay with anti-digoxigenin rhodamine-conjugated antibodies. Quantitative analysis with descriptive and predictive statistics was used to calculate cell densities (number/mm^2) after fluorescent nuclear stain (TCD, total cell density), labeling with BrdU (PrCD, proliferating cell density), or TUNEL (ApoCD, apoptotic cell density). By this approach we first have shown that the temporal pattern of expression of neuronal/glial markers in postnatal cerebellum is not affected by the *Reeler* mutation. Then, we have demonstrated that the hypoplasia in the *Reeler* mouse cerebellum is consequent to reduction of cortical size and cellularity (TCD), and that TCD is, in turn, linked to quantitative differences in the extent of cell proliferation and apoptosis, as well as derangements in their temporal trends during postnatal maturation. Finally, we have calculated that PrCD is the most important predictive factor to determine TCD in the cerebellar cortex of the mutants. These results support the notion that, beside the well-known consequences onto the migration of the cerebellar neurons, the lack of Reelin results in a measurable deficit in neural proliferation.

Keywords: *Reeler*, cerebellum, mouse, cell proliferation, apoptosis, programmed cell death, development, neurochemistry

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Edited by:
Marco Martina,
Northwestern University, USA

Reviewed by:
Rafael Linden,
Federal University of Rio de Janeiro, Brazil
Roy Vincent Sillitoe,
Baylor College of Medicine, USA

***Correspondence:**
Adalberto Merighi
adalberto.merighi@unito.it;
Laura Lossi
laura.lossi@unito.it

***Received:** 23 March 2016*
***Accepted:** 10 May 2016*
***Published:** 25 May 2016*

Citation:
Cocito C, Merighi A, Giacobini M and Lossi L (2016) Alterations of Cell Proliferation and Apoptosis in the Hypoplastic Reeler Cerebellum. Front. Cell. Neurosci. 10:141. doi: 10.3389/fncel.2016.00141

INTRODUCTION

The cerebellum is one of the main parts of the brain involved in motor coordination. It consists of a highly ordered layered cortex made of gray matter at its surface, and a deep central medullary body made of white matter that spreads radially to form a typical arborization in reaching the axis of individual cerebellar laminae (folia). Embedded into the medullary body are three (four in humans) nuclei of neurons giving rise to the majority of the cerebellar efferent fibers. In mice and other altricial mammals—such as humans—most of cerebellar development occurs postnatally (Altman and Bayer, 1997). Postnatal development of the cerebellar cortex consists of a very complex series

of tightly regulated events leading to the generation of the granule cells and the GABAergic cortical interneurons. These cells, as well as the other cortical neurons and the glia, have to perform very long distance migration from their progenitor niches, and neurons extend their axons for making highly precise synaptic connections with targets (Marzban et al., 2015). Until the second week after birth in rodents, extensive neurogenesis within a temporary external granular layer (EGL) gives rise to the granule cells that migrate through an increasingly thick molecular layer (ML) and the forming Purkinje cell layer. The granule cells finally reach the internal granular layer (IGL), which, eventually, will be the only granular layer of the mature cerebellar cortex (Altman, 1972a,b; Hatten, 1990). Notably, postnatal neurogenesis in cerebellum accompanies with an intense programmed cell death to attain a numerical match between cortical neurons, mainly the granule cells, and the Purkinje neurons. Several types of death may affect neurons, and among them apoptosis (Kerr et al., 1972), whose key features are DNA fragmentation, chromatin condensation, cell shrinkage and/or fragmentation, and activation of specific cellular proteases (Yamaguchi and Miura, 2015). The granule cells undergo apoptosis in two different phases of their differentiation, maturation, and migration: one phase occurs in the EGL and is independent from synapse formation, the latter takes place in the IGL, and depends on the classical mechanisms of neuron-to-target interaction at synapses. The first phase of granule cell apoptosis is rapid, and necessary for a gross regulation of the expanding pre-mitotic population of these neurons; the second occurs in a longer interval of time, and corresponds to the period of synaptic sculpting of the cerebellar cortex (Lossi et al., 2002). Wiring of the connections between the granule cells and the Purkinje neurons is fundamental to cerebellar function (Hirai and Launey, 2000; Cesa and Strata, 2009), and it is unanimously agreed that these two neuronal populations exert reciprocal influences that are fundamental in the regulation of their survival or death (Altman, 1992). Therefore, the maintenance of a correct balance between neurogenesis and apoptosis is essential for proper cerebellar maturation, to the point that such a balance is deeply altered in several spontaneous mutations affecting the murine cerebellum and leading to ataxias (Cendelin, 2014).

Reeler is the first described mouse cerebellar mutation (Falconer, 1951). The *Reeler* phenotype is characterized by typical alterations in gait ("reeling") and was named thereafter. The recessive homozygous mouse ($reln^{-/-}$) displays a total absence of Reelin, a protein discovered several decades after the initial description of the mutant (D'Arcangelo et al., 1995). Reelin is necessary for brain development as it plays a prominent role in neuronal migration. During cerebellar development, granule cells in the EGL secrete the protein, which is fundamental for the proper migration and positioning of the Purkinje neurons (D'Arcangelo et al., 1995). The role of Reelin in the adulthood is still under debate, but many authors demonstrated, among others, that it intervenes in the growth of apical dendrites, the formation of dendritic spines, and the regulation of synaptic function and plasticity (D'Arcangelo, 2014). In $reln^{-/-}$ mice, the lack of Reelin leads to a severe hypoplasia of cerebellum. Histologically, $reln^{-/-}$ mice display a thinner ML, a reduction in the density of the granule cells in the IGL, and a misplacement of the Purkinje neurons. Just 5% of these neurons align into their normal position, 10% are located in the IGL, and the remaining ones are scattered in a central deeper mass inside the white matter of the medullary body (Mariani et al., 1977; Heckroth et al., 1989; Yuasa et al., 1993). Despite these obvious alterations, part of the cerebellar circuitry is maintained, but, as demonstrated by several studies, at the expenses of a drastic reduction in the number of synapses between the Purkinje neurons and the parallel fibers, and of an altered pruning of the climbing fibers-Purkinje neurons synapses (Mariani et al., 1977; Qiao et al., 2013; Castagna et al., 2014). In keeping with these observations, other studies highlighted how the lack of Reelin also results in impaired hippocampal postnatal neurogenesis and functionality (Won et al., 2006; Pujadas et al., 2010).

Work on the *Reeler* mutation has focused onto the intracellular pathways of signal transduction mediated by Reelin and the cellular and molecular mechanisms that are affected by its deficiency (see D'Arcangelo, 2014 for a recent review). Surprisingly, little or no attention was devoted to the relationship between neurogenesis and programmed cell death in the course of postnatal cerebellar development. In this study, we have investigated such a relationship and quantitatively demonstrated an impairment of postnatal neurogenesis in $reln^{-/-}$ mice, in parallel with an increase in apoptosis. In addition, as previous ultrastructural observations from our group showed a high degree of immaturity in $reln^{-/-}$ Purkinje neurons' synaptic circuitry (Castagna et al., 2014) we have compared the timing of expression of several markers of neurochemical differentiation across the mutants and the normal mice. This had the purpose to exclude that substantial temporal differences in the acquisition of specific phenotypic features of the different cerebellar neural cells biased our analysis. As no obvious neurochemical differences emerged between the two genotypes, our data show, for the first time, the occurrence of precise imbalances in the ratio of neurogenesis to apoptosis in the *Reeler* mouse, and further prove the contribution of Reelin in neuronal survival.

MATERIALS AND METHODS

Animals

Mouse cerebella at postnatal (P) day 0, 5, 10, 15, 20, 25, 30, and 60 from $reln^{+/+}$ and $reln^{-/-}$ mice were compared in this study (n = three each). The number of animals was kept to a minimum, and all efforts were made to minimize their suffering. The Italian Ministry of Health (#65.2016.PR) and the Bioethics Committee of the University of Turin authorized all experiments. Animal procedures were carried out according to the guidelines and recommendations of the European Union (Directive 2010/63/UE) as implemented by current Italian regulations on animal welfare (DL n. 26-04/03/2014).

Genotyping

All animals were genotyped according to current protocols to ascertain their genetic background. Briefly, a small sample was

cut from the tail tip and incubated overnight with continuous shaking in a solution containing 50 μg/mL proteinase K (Sigma) in lysis buffer (Tween 20, NonIDET P40, 1% gelatin, KCl 1M, Tris 1M pH 8.5, MgCl2 0.5M). On the following morning, samples were centrifuged at 14,000 g for 5 min and the DNA-containing surnatant used for PCR amplification. The gene mutation ($reln^{-/-}$) in the *Reeler* phenotype is responsible for deletion of part of an intron having control over correct gene expression and stability of the mRNA (D'Arcangelo et al., 1996). To identify the three possible genotypes in littermates ($reln^{+/+}$, $reln^{+/-}$, and $reln^{-/-}$), a sequence containing the potentially deleted one is amplified by PCR using three different primers. Primers were a common forward primer within the conserved protein sequence upstream the mutation, and two different reverse primers, one of which is specific for the wild type sequence and the other for a sequence downstream the mutation. By this procedure, the band obtained from amplification of the cDNA for the wild type Reelin has a molecular weight of 266 bp, and that obtained for the mutated Reelin weighs 363 bp. After electrophoresis and ethidium bromide staining in 1.5% agarose gel, mice were classified as follows: i. Wild type controls ($reln^{+/+}$) when displaying a single 266 bp band, ii. *Reeler* ($reln^{-/-}$) when displaying a single 363 bp band, and iii. Heterozygous ($reln^{+/-}$) when displaying both bands.

Histology

All procedures were carried out at room temperature, unless otherwise stated. Two hours before the sacrifice, mice were injected with 5-bromo-2′-deoxyuridine (BrdU) intraperitoneally (0.1 mg/g body weight). Under deep anesthesia (sodium pentobarbital 30 mg/kg), animals then were perfused through the left ventricle with cold Ringer solution (0.01 M phosphate buffer pH 7.4–7.6, 0.8% NaCl, 0.025% KCl, 0.05% $NaHCO_3$) followed by fixative (4% paraformaldehyde in phosphate buffer 0.2 M pH 7.4). Cerebella were removed and post-fixed for 2 h in the same fixative. Tissues were then dehydrated through a graded ethanol series and embedded in paraffin wax. The entire cerebellum was serially cut in parasagittal sections (7 μm) that were collected and mounted on poly-L-lysine (PLL; Sigma Aldrich) pre-coated slides (5 sections/slide).

For immunohistochemistry, sections were rehydrated and subjected to microwave antigen retrieval (9 min at 95–99°C in sodium citrate buffer pH 6). After microwave treatment, sections where washed 5 min in phosphate buffered saline 0.01 M pH 7.4 (PBS) and blocked for 1 h in PBS containing 1% ovoalbumin (Sigma Aldrich) and 0.3% Triton X-100 (Sigma Aldrich). Sections were incubated overnight in primary antibodies at optimal titer, with the exception of BrdU immunostaining where incubation was carried out for 1 h only. After being rinsed 3 × 5 min in PBS, sections were incubated for 1 h with fluorescent secondary antibody conjugates (anti-rabbit or anti-mouse Alexa Fluor® 488 or Alexa Fluor® 594, Molecular Probes, Life Technology) diluted 1:800 in the same diluent used for primary antibodies. When necessary, nuclear counterstaining with propidium iodide (PI, Sigma Aldrich) was applied (1 μg/mL in PBS containing 0.3% Triton X100, 2 min), and slices were eventually mounted in antifade medium (Sigma Aldrich).

Primary Antibodies

Primary antibodies were: mouse anti-NeuN (Millipore, 1:200); rabbit anti-vimentin (Cell Signaling, 1:100); mouse anti-calbindin (ABCAM, 1:300); rabbit anti-GFAP (ABCAM, 1:2000); mouse anti-Smi32 (ABCAM, 1:1500); rabbit anti-GAD67 (Ana Spec, 1:100); mouse anti-BrdU (GE Healthcare, prediluted in nuclease solution). Primary antibodies were diluted in PBS containing 2% bovine serum albumin (BSA; Sigma Aldrich) and 2% PLL. Routine specificity and method controls were performed by omission of primary antibodies, their substitution with normal serum, omission of secondary antibodies or their substitution with inappropriate species-specific conjugates.

Analysis of Apoptosis

In situ labeling of cells with fragmented DNA was performed following a modification of the original terminal dUTP nick end labeling (TUNEL) procedure (Gavrieli et al., 1992). In brief, after being brought to double distilled water (ddH_2O), sections were pretreated with proteinase K (Sigma Aldrich) for 15 min at 37°C in a humid atmosphere. The proteinase K working solution (20 μg/mL) was prepared from a stock solution of proteinase K (1 mg/mL in 10 mM Tris pH 7.7) by mixing together 80% Tris 10 mM pH 7.7, 18% 1 mM $CaCl_2$, and 2% proteinase K stock solution. After 4 × 2 min washings in ddH_2O, sections were immersed for 10 min in TdT buffer (30 mM Tris-HCl, pH 7, 140 mM sodium cacodylate, 1 mM cobalt chloride) and subsequently incubated in TdT buffer supplemented with 0.05 U/μL terminal transferase and 10 μM digoxigenin-dUTP at 37°C for two and half hours in a humid atmosphere. The reaction was stopped by transferring the sections to terminal buffer (300 mM sodium chloride, 30 mM sodium citrate in ddH_2O) for 15 min. Sections were subsequently rinsed in ddH_2O, blocked in 2.5% BSA in ddH_2O for 10 min, and incubated overnight at 4°C with a rabbit anti-digoxigenin antibody conjugated to rhodamine (Molecular Probes, Life Technology) diluted 1:75 in PBS/PLL/BSA. After extensive washing in PBS, sections were counterstained with 1:1000 4′, 6-diamidino-2-phenylindole (DAPI—Sigma Aldrich) in PBS for 2 min and finally mounted in antifade medium.

Quantitative Analysis of Proliferating and Apoptotic Cells

Sampling Strategy

The whole series of sections cut through the entire latero-lateral (transversal) axis of cerebellum was divided into ten sampling units. The total number of sections for each sampling unit then was related to the overall size of the cerebellum. Following a systematic random sampling procedure (Geuna, 2000), two slides were arbitrarily selected within each sampling unit, one to be processed for BrdU immunostaining ($n = 5$ sections) and the other according to the TUNEL protocol ($n = 5$ sections). Therefore, fifty randomly selected sections were processed to gather quantitative data on BrdU or TUNEL labeled cells. Five additional sections were selected at random, stained with PI and used for cell density studies as described in Section Calculation of Total Cell Density (TCD) in Single Layers of Cerebellar Cortex and Medullary Body.

Image Acquisition

A 20x lens and a transmitted/fluorescence light microscope equipped with appropriate filter combinations to detect rhodamine and DAPI (DM6000B, Leica) were used to collect wide-field fluorescence microscopy images (1392 × 1040 pixels). Laser scanning confocal images (1024 × 1024 pixels) were collected with 40x dry lens and a laser scanning confocal microscope (LSCM—SP5, Leica) using the laser excitation lines required by Alexa Fluor® 488, Alexa Fluor® 594, or PI.

Identification of Cortical Layers and Medullary Body

The well-known alterations in neuronal migration that characterize the *Reeler* histological phenotype were taken into account to subdivide cerebellar sections into discrete areas when moving from the pial surface to the medullary body. Therefore, on cytoarchitectonic features, we could easily distinguish the EGL and the ML that displayed similar features in *reln*$^{+/+}$ and *reln*$^{-/-}$ mice. However, in the *reln*$^{-/-}$ genotype, impaired migration of the Purkinje neurons in the course of the physiological process of their alignment to a cell monolayer results in the lack of a true Purkinje cell layer in the mutants. Therefore, the Purkinje cell layer (only present in *reln*$^{+/+}$ mice) and the IGL were considered together and simply referred to as the IGL in the following. The term medullary body was used in its *bona fide* significance for *reln*$^{+/+}$ animals, and to indicate the inner central mass of non-migrated neurons intermingled with the white matter that characterizes the histology of the deep cerebellum in *reln*$^{-/-}$ mutants (**Figure 1**).

Calculation of Total Cell Density (TCD) in Single Layers of Cerebellar Cortex and Medullary Body

Five randomly selected areas from each of the three cortical layers and the medullary body were sampled from PI stained sections (**Figures 1A,B**). Confocal images were acquired and used to calculate TCD (n. cells/mm^2) with the Count Particle command of the Image J software (http://imagej.nih.gov/ij/) after appropriate scale and threshold setting.

Calculation of the Density of BrdU$^+$ and TUNEL$^+$ Cells in Single Layers of Cerebellar Cortex and Medullary Body

BrdU$^+$ cell

In each cortical layer and the medullary body, we have calculated the density of BrdU$^+$ cells (n. cells/mm^2) as an index of cell proliferation, and thus herein referred to it as PrCD = proliferating cell density. To do so, we first calculated the relative percentages of the areas of each cortical layer and of the medullary body vs. the total area of the section after staining with DAPI (**Figures 1C,D**) and observation in the wide-field fluorescence microscope. Then, we randomly acquired five LSCM snapshot images from each of the BrdU-immunostained sections (sampled as described in Section Sampling Strategy). As exemplified in **Figures 1E,F**, the relatively high number of IR cells permitted an easy identification of the three cortical layers and the medullary body by simple histological landmarks and in the absence of nuclear counterstain. For each digital image, PrCD was measured in the four cerebellar subdivisions with the Image J software, as described in Section Calculation of Total Cell Density (TCD) in Single Layers of Cerebellar Cortex and Medullary Body. Finally, measures were corrected according to the ratio between each of the areas and the total area of the section, exemplified as $\mathrm{PrCD}_{\mathrm{EGL}} = \mathrm{PrCD}^{*}_{(\mathrm{BrdU})}[\mathrm{EGL\ area}_{(\mathrm{DAPI})}/\mathrm{total\ section\ area}_{(\mathrm{DAPI})}]$.

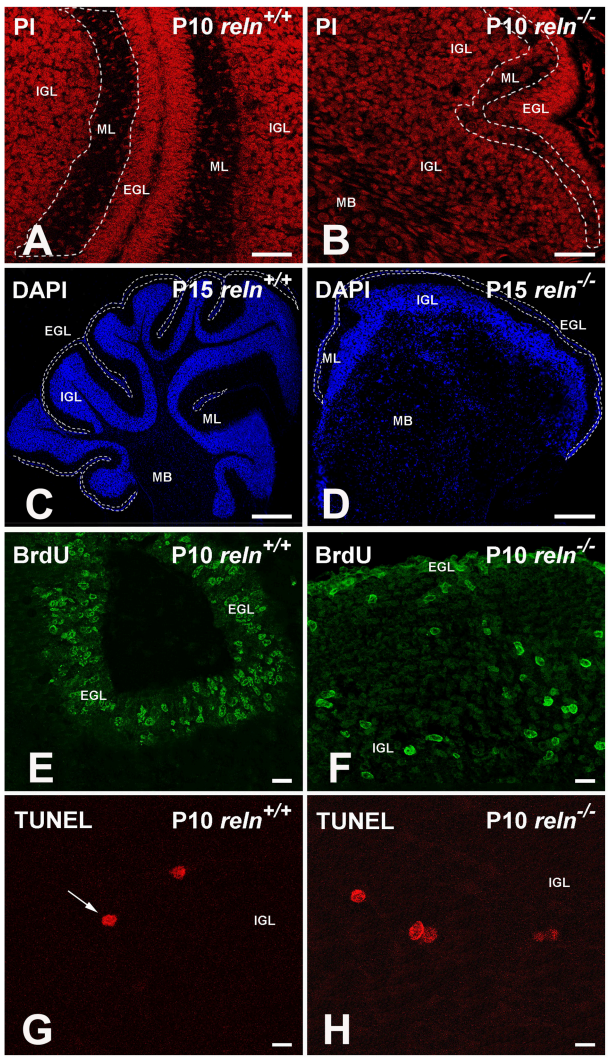

FIGURE 1 | Exemplificative images to explain the histological procedures described in Material and methods. (A,B) Propidium iodide (PI) staining was used to calculate TCD in EGL, ML, IGL and medullary body. The ML is delimited by the white dashed lines; **(C,D)** Reconstruction of cerebellar slices. DAPI staining allowed the identification and the measurement of the area of each cortical layer and of the medullary body. As an example of the procedure, the borders of the EGL were traced with a white dashed line. Compare the two images to easily appreciate the difference in the cytoarchitecture between the two genotypes. **(E,F)** BrdU immunostaining. Mice were sacrificed 2 h after the BrdU injection. The EGL has the higher density of proliferating cells in both genotypes, but in *reln* $^{-/-}$ mice its extension is clearly reduced. **(G,H)** TUNEL$^+$ nuclei in the IGL are intensely fluorescent and display different staining intensities. A condensed nucleus in G is indicated by the arrow. EGL, external granular layer; IGL, internal granular layer; MB, medullary body; ML, molecular layer; P, postnatal age. Scale bars: **(A,B)** = 25 μm; **(C,D)** = 50 μm; **(E,F)** = 10 μm; **(G,H)** = 100 μm.

TUNEL$^+$ cell

In each cortical layer and the medullary body we have calculated the density of TUNEL$^+$ cells (n. cells/mm^2) as an index of apoptosis, and thus herein referred to it as ApoCD = apoptotic cell density. As TUNEL$^+$ cells were by far less abundant than BrdU$^+$ cells (**Figures 1G,H**), the procedure described in Section BrdU$^+$ cell was prone to introduce an error too high. Therefore, to calculate ApoCD, we directly counted *all* TUNEL$^+$ cells in cerebellar sections that were reconstructed from individual wide-field fluorescence images after DAPI nuclear counterstain. Reconstructions were made with the Adobe Photoshop CS6 software (Adobe Systems). At the same time, in reconstructed images, we measured the areas of each of the three cortical layers and the medullary body, and cell densities were calculated directly.

Normalization of BrdU$^+$ and TUNEL$^+$ cell densities according to genotype

Significant differences emerged from the comparison of the areas of cortical layers and medullary body between the two genotypes. Therefore, densities of each compartment in *reln*$^{-/-}$ mice were normalized to values in normal mice by correcting per area ratios (*reln*$^{-/-}$ areas/*reln*$^{+/+}$ areas).

Cell Percentages

The percentages of BrdU$^+$ and TUNEL$^+$ cells were obtained by calculating the ratios of PrCDs or ApoCDs and TCDs for each of the cerebellar subdivisions defined in Section Identification of Cortical Layers and Medullary Body.

Statistics

The role of raw TCD, PrCD, and Apo CD with respect to the *reln* $^{+/+}$ and *reln*$^{-/-}$ groups was investigated by within-subjects, i.e., repeated-measures, analysis of variance (ANOVA) with the R open-source software (R version 3.2.0, http://www.R-project.org/). Each feature was considered as an independent categorical variable, with the animal ID as within-subject factor for the observation grouping. Observed independent variables were considered significantly correlated to the outcome when the *P*-value associated to the F statistics was found to be smaller than 0.05.

With GraphPad Prism (GraphPad Software), areas and percentages of BrdU$^+$ and TUNEL$^+$ cells were compared with parametric Student T Test and nonparametric Mann Whitney test. Normality was ascertained with D'Agostino-Pearson omnibus normality test. Using a one-way ANOVA with Tukey correction for multiple comparisons, normalized TCD, PrCD, and ApoCD were compared at different postnatal ages within each genotype.

Mean values of TCD, PrCD, and ApoCD in spreadsheet were finally used to calculate Pearson's correlation curves and polynomial models for time effects, by plotting time (independent variable) vs. TCD, PrCD, or Apo CD (dependent variables) with the Microsoft Excel graph function. TCD, PrCD, and Apo CD mean values were also used for multiple regression analysis and statistics using the Microsoft Excel data analysis tool pack.

RESULTS

The Temporal Pattern of Expression of the Main Neuronal/Glial Markers in Postnatal Cerebellum is Not Affected by the *Reeler* Mutation

We first wanted to know if the lack of *Reelin* had direct consequences on the expression of the more general markers of neuronal and glial differentiation, as it is often assumed that development of the cerebellum in *Reeler* recapitulates—but with considerable retard—the sequences of events occurring in normal mouse. Therefore, we have analyzed the time of expression of some neurochemical markers of neural differentiation in the postnatal *Reeler* cerebellum and compared it with the pattern of expression in *reln*$^{+/+}$ mice (**Figure 2**)[1].

The first markers to be expressed were calbindin and vimentin that could be detected at all developmental stages from birth onward. Calbindin is a specific marker of the Purkinje neurons (Schwaller et al., 2002). Calbindin$^+$ Purkinje neurons in P0 *reln*$^{+/+}$ mice were arranged in a multi-stratified immature layer immediately below the ML. In *reln*$^{-/-}$ mice, calbindin$^+$ Purkinje neurons were positioned instead deeply in cerebellar cortex and medullary body. Vimentin was expressed by glia (Schnitzer et al., 1981): it labels the Bergmann glia of the ML, and the glial cells of the white matter. In *reln*$^{-/-}$ mice, vimentin-immunoreactive (IR) Bergmann glia displayed an oblique orientation rather than being perpendicular to the pial surface of the cerebellar laminae, and were irregularly scattered in the ML.

Three other markers, NeuN, SMI32, and 67 kDa glutamic acid decarboxylase (GAD 67), tagged different populations of neurons, started to be expressed from P5, and could be subsequently detected at all other ages. Within the cerebellum, NeuN is a specific marker of post-mitotic granule cells, and its expression in these neurons positively correlates with neuritogenesis (Weyer and Schilling, 2003). SMI32, is a non-phosphorylated neurofilament protein belonging to a group of cytoskeletal proteins (Lee et al., 1988). Antibodies to SMI32 label the Purkinje neurons and the deep nuclei neurons (Milosevic and Zecevic, 1998; Leto et al., 2006). GAD 67 is a general marker of the GABAergic neurons (Greif et al., 1991). Irrespective of the genotype, NeuN positive granule cells were located in the inner part of the EGL, which harbors the pre-migratory post-mitotic subpopulation of these neurons. They were also well visible in the IGL, which becomes progressively populated by the post-migratory granule cells at the end of their migration from EGL, and in a subpopulation of neurons of the deep nuclei, which were not GAD 67-IR (see below) and, hence, use glutamate as their main neurotransmitter (Leto et al., 2006). SMI32-immmunoreactive deep nuclei neurons were embedded in the medullary body, which consisted of *bona fide* white matter in *reln*$^{+/+}$ mice and, in *reln*$^{-/-}$ mutants, of a central mass made of glial cells, myelinated fibers, granule cells, and Purkinje neurons that failed to properly migrate to the cortex. SMI32-IR

[1]Outside the cerebellum, expression of these markers can change substantially as regarding to the type of labeled cells and temporal appearance. Thus, this description should be considered strictly in relation to cerebellum.

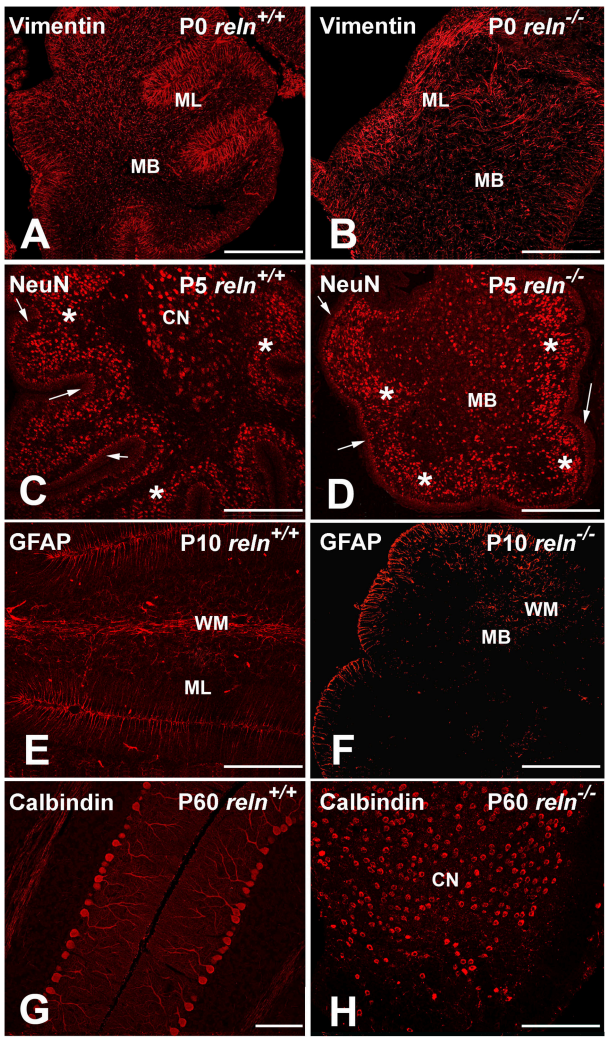

FIGURE 2 | Exemplificative images of the neurochemistry of the postnatal cerebellar cortex in normal and mutant mice. Immunocytochemistry shows that the timing of expression of the main neuronal/glial markers in cerebellum is not affected by the *reln* mutation. **(A,B)** From P0, vimentin starts to be expressed by the Bergmann glial and the glial cells in the white matter. In the *reln*$^{-/-}$ ML, the Bergmann glia is poorly oriented along radial planes in comparison with controls (arrows). **(C,D)** NeuN is expressed, starting from P5, by the granule cells of the deep post-mitotic portion of the EGL (marked by arrows), the post-migratory granule cells in the IGL (asterisks) and the deep nuclei neurons (white dashed lines); **(E,F)** starting from P10, GFAP is detected in the Bergmann glia and white matter glial cells. **(G,H)** show calbindin immunoreactive Purkinje neurons in young adult mice (P60). The two images highlight the aberrant positioning of these neurons at the end of cerebellar development in *reln*$^{-/-}$ mice, when compared to littermate controls. In mutants, positive Purkinje neurons in the medullary body are intermingled with immunoreactive neurons of the cerebellar nuclei. BG, Bergmann glia; CN, cerebellar nucleus; EGL, external granular layer; IGL, internal granular layer; MB, medullary body; ML, molecular layer; P, postnatal age; PNs, Purkinje neurons. Scale bars: **(A–F,H)** = 100μm; **(G)** = 30 μm.

deep nuclei neurons partly co-expressed NeuN (glutamatergic subgroup) or GAD 67 (GABAergic subgroup). In *reln*$^{-/-}$ medullary body, we could easily differentiate the SMI32-IR neurons of the deep nuclei from the Purkinje neurons using a double staining with calbindin; in *reln*$^{+/+}$ mice there was no need to employ SMI32+calbindin immunostaining as the two cellular populations displayed well recognizable locations in the deep gray matter and cerebellar cortex, respectively. At P5, besides to the GABAergic subgroup of the deep nuclei neurons, also the Purkinje neurons and the GABAergic interneurons in the expanding ML were GAD 67-IR.

Glial cells expressed GFAP from P10 onward. GFAP totally co-localized with vimentin in the Bergmann glia and in the white matter of the medullary body.

Remarkably, the time of expression of all markers did not show appreciable differences between *Reeler* and normal mice, and the differences that we here observed were simply related to the well-known cell mispositioning in the mutants. As marker expression is obviously linked to the differentiation status of individual cells, we could conclude that there was not a delay in the acquisition of specific cell fate markers in *reln*$^{-/-}$ mice. Therefore, cerebella from age-matched normal and mutant mice could be properly compared to assess the effects of the lack of Reelin.

Hypoplasia in the *Reeler* Mouse Cerebellum is Consequent to Reduction of Cortical Size and Cellularity, and is Linked to Altered Temporal Trends of TCD

That *Reeler* mice have a hypoplastic cerebellum almost completely devoid of folia is an established fact. What remains to be established in full are the causes of hypoplasia, as the mere impairment of neuronal migration, i.e., the primary effect of the Reelin absence, can alone hardly explain the reduction of cerebellar volume. Such a reduction can be either consequent to a diminution in the absolute numbers of neural cells and/or their density in the cerebellar cortex and nuclei (gray matter) and/or in the medullary body (white matter). In rat, the volume of the cerebellar gray matter is 3.76-fold that of the white matter (Bush and Allman, 2003) and, based on data in the cerebral cortex (Zhang and Sejnowski, 2000), ratio should reach 4.5-fold in mouse. Thus, in *Reeler* cerebellum, a reduction of TCD in the gray matter can be the primary consequence of the mutation. If such a reduction accompanies with a decrease in the area of the gray matter, then one can infer that the cerebellar hypoplasia of the *Reeler* mouse also follows the diminution in number of the cortical neurons. Another point of attention is that not only the *Reeler* mutation is responsible for hypoplasia and lack of foliation, but also it profoundly affects the cerebellar cytoarchitecture. Whether deficits are uniformly distributed or rather preferentially hit specific lobes or lobules remains to be fully ascertained. In addition, no statistical data are available to demonstrate whether the cerebellar hypoplasia specifically affects one or more layers of the forming cerebellar cortex and/or the medullary body. In the following sections, we report the results of the experiments aiming to clarifying these issues.

Size

Table 1 reports data on the areas of cortical layers and the medullary body in normal and *Reeler* mice in the P0–P25

TABLE 1 | Statistical analysis of the areas of cortical layers and medullary body.

Age	Genotype	Cerebellar cortex			Medullary body
		EGL	ML	IGL	
P0	*reln*$^{+/+}$	0.07±0.007	0.07 ± 0.008	0.26 ± 0.035	0.31 ± 0.055
	reln$^{-/-}$	0.06 ± 0.014	0.03 ± 0.004	0.19 ± 0.013	0.31 ± 0.021
P5	*reln*$^{+/+}$	0.34 ± 0.041	0.25 ± 0.029	0.54 ± 0.043	0.30 ± 0.025
	reln$^{-/-}$	0.09 ± 0.007	0.05 ± 0.005	0.44 ± 0.026	0.42 ± 0.030
P10	*reln*$^{+/+}$	0.48 ± 0.019	0.80 ± 0.064	1.32 ± 0.101	0.50 ± 0.028
	reln$^{-/-}$	0.07 ± 0.006	0.07 ± 0.005	0.50 ± 0.053	0.48 ± 0.035
P15	*reln*$^{+/+}$	0.14 ± 0.026	1.40 ± 0.112	1.61 ± 0.099	0.62 ± 0.043
	reln$^{-/-}$	0.07 ± 0.008	0.14 ± 0.016	0.51 ± 0.032	0.64 ± 0.036
P20	*reln*$^{+/+}$	N/A	1.19 ± 0.102	1.13 ± 0.099	0.51 ± 0.059
	reln$^{-/-}$	N/A	0.23 ± 0.020	0.33 ± 0.025	1.09 ± 0.104
P25	*reln*$^{+/+}$	N/A	1.81 ± 0.169	1.63 ± 0.147	0.58 ± 0.072
	reln$^{-/-}$	N/A	0.18 ± 0.030	0.31 ± 0.050	1.09 ± 0.144

The Table reports the statistics of area measurements in the cerebellum of normal and Reeler mice (mean values in mm^2 ± SEM). Statistically significant differences between genotypes ($P < 0.05$) have been indicated with gray background. EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; N/A, not applicable.

time interval. From these data, *reln*$^{-/-}$ mice displayed a dimensionally reduced cerebellar cortex than their age-matched controls, and such a reduction was particularly prominent in the ML and IGL. When the cerebellum matures, the ML becomes populated (for the most) by the parallel fibers (i.e., the axons of the granule cells): notably, the increase in size of the ML was 16.86-fold in *reln*$^{+/+}$ mice, but only six-fold in the mutants. In parallel, post-migratory granule cells populate the IGL during normal development, but, from P0 to P10, the IGL increased in size of 5.1-folds in *reln* $^{+/+}$ and only 2.6-fold in *reln*$^{-/-}$. After P10, this cortical layer only increased slightly (1.23-fold) in *reln*$^{+/+}$ mice, but drastically reduced its size (to 0.62-fold) in *reln*$^{-/-}$ mutants. Differently from the cortex, the medullary body was larger in *reln*$^{-/-}$ mice than in normal animals. In normal cerebellar development, the size of the medullary body mainly reflects the progressive myelination of the axons of the Purkinje neurons that leave the cortex traveling across the white matter and reach the cerebellar nuclei, as well as the development of the afferent and efferent fibers entering or exiting the cerebellum. The size of the medullary body increased in parallel with postnatal age in both genotypes (*reln*$^{-/-}$ 2.59, *reln*$^{+/+}$ 1.93-fold), but, at P25, *Reeler* mice resulted to have a larger medullary body than their normal counterparts (1.88-fold). Thus, *Reeler* mice had a smaller cerebellar cortex but a larger medullary body than their normal littermates.

Cellularity and Its Temporal Variations

We then moved to consider whether and how cellularity (TCD) varied in the two groups of animals. With the Excel spreadsheet, we displayed the trend of TCD over time in line charts (**Figure 3** solid lines); then, using the parametric Student T test and the non-parametric Mann Whitney test, we compared the mean values of TCD in the three cortical layers and in the medullary body between the two genotypes at corresponding postnatal ages (asterisks in **Figure 3**). With a One way ANOVA we have also studied TCD separately in *reln*$^{+/+}$ and *reln*$^{-/-}$ mice and compared data in relation to developmental age (**Figure 4**). In the EGL (**Figure 3A**), TCD was significantly higher in *reln*$^{-/-}$ mice at P0, but drastically declined at P5 and P10 to reach similar values to those of *reln*$^{+/+}$ mice at P15. In the ML (**Figure 3B**), TCD was not different across genotypes at P0, but a drastic drop occurred at P5 and P10 in *reln*$^{-/-}$ mice, and thus mutants displayed significantly lower values than age-matched normal mice from P5 onward. In the IGL (**Figure 3C**), TCD was not different among genotypes at P0. However, from P5, there was a progressive increase of cellularity in normal mice, but not in mutants. In the medullary body (**Figure 3D**), TCD displayed very different trends in *reln*$^{+/+}$ and *reln*$^{-/-}$ mice: in the first there was a tendency to a progressive reduction, whereas in mutants cellularity was relatively high at birth, dropped to its lowest value at P10, and increased thereafter to reach statistically significant differences with the *reln*$^{+/+}$ mice at P20 and P25. **Figure 4** shows the results of statistical analysis comparing animals of the same genotype grouped in relation to age. This type of analysis showed that some age-related differences in TCD need to be considered with attention. For example, in the EGL of normal mice differences at P0, P5, and P10 were not statistically significant between them, as well as those at P5 and P10 in the mutants. Notably, in both genotypes TCD was lower at P15 than at birth, but in *reln*$^{-/-}$ mice there was a statistically significant drop at P10 (**Figure 4A**). As at P15 there was not a statistically significant difference in TCD between *reln*$^{+/+}$ and *reln*$^{-/-}$ mice (**Figure 3A**) we concluded that temporal variations in cellularity were worthy to be investigated. A similar conclusion was also drawn for the ML, IGL and medullary body after carefully inspecting the graphs in **Figure 4**. Therefore, we studied the temporal trend of TCD by correlation statistics.

By Pearson's correlation, we calculated the functions that best fitted our data plotting TCD against time (**Table 2**) and drew their graphs (**Figure 3** dashed lines). Functions found to provide the best fit ($R^2 = 1$) were 5th degree polynomial (quintic) functions. Therefore, TCD was neither constant nor varied linearly with time in the two genotypes of mice. Polynomial functions have a number of turning points at most equal to n-1, where n is the degree of the function, and each turning point is a local or global maximum (positive peak) or minimum (negative peak). Obviously, maxima and minima alternate with one another, after the curve displays an inflection point. **Figure 3** clearly shows the good fit of the descriptive graphs (solid lines) with the graphs of the corresponding regression functions (dashed lines). Regression analysis allowed predicting the values of TCD continuously over time, besides the six time points of sampling. Thus, it was possible to locate temporally the turning points (i.e., the maxima and minima—indicated in **Figure 3** by upwards or downwards arrows, respectively) anywhere between sampling range (interpolation) or outside this range (extrapolation). Interestingly, Pearson's correlation not only showed that relevant TCD maxima occurred earlier in the EGL and ML of *reln*$^{-/-}$ mice, but also that, in the P0–P5 interval, the trend of TCD was

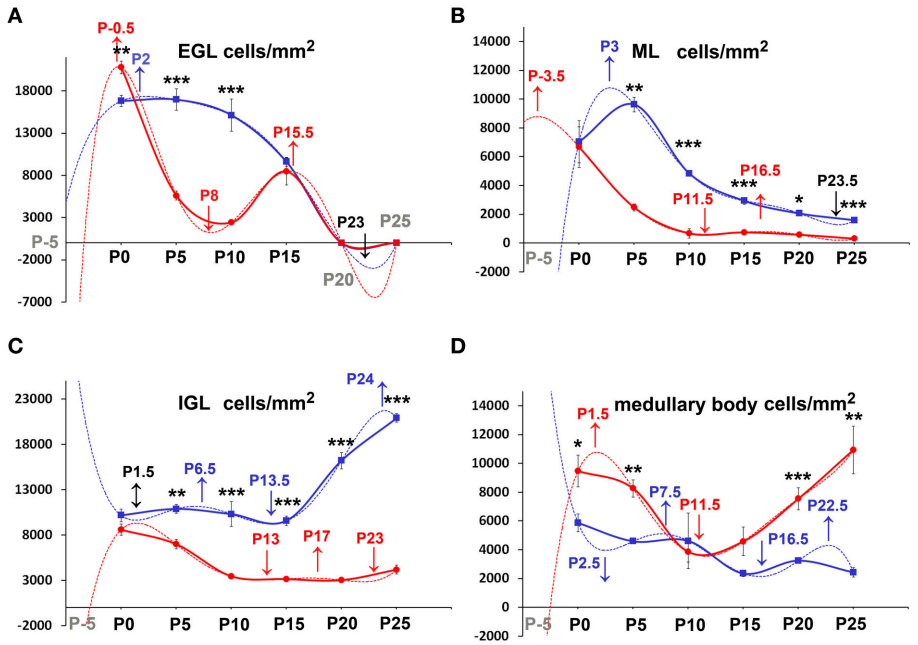

FIGURE 3 | Descriptive graphs (solid lines) and correlation curves (dashed lines) of normalized TCDs in cortical layers and the medullary body. Graphs highlight the trends and differences *across* genotypes of normalized TCDs in the four cerebellar compartments defined in this study. Postnatal ages corresponding to the minima and maxima of correlation curves are indicated with colored letters and upwards or downwards arrows. Blue and red lines or letters/arrows refer to *reln*$^{+/+}$ and to *reln*$^{-/-}$ mice, respectively. Black letters for postnatal ages and arrows **(A–D)** refer to both genotypes. Postnatal ages in gray (x axis) indicate the time points of correlation curves outside the sampling interval (extrapolations). In *reln*$^{+/+}$ mice, TCD decreased in function of time after an interpolated positive peak at P2 in the EGL (**A**, blue dashed line) and at P3 in the ML (**B**, blue dashed line), whereas it increased in the IGL with an interpolated peak at P24 (**C**, red lines), and displayed an oscillatory trend in the medullary body (**D**, red lines). Although a decrease also occurred in the *reln*$^{-/-}$ EGL (**A**, red lines) and ML (**B**, red lines), descriptive and correlation curves were notably different when compared to those calculated for *reln*$^{+/+}$ mice. Extrapolated peaks at P0.5 (EGL) and P3.5 (ML) occurred earlier than in normal mice (**A**,**B**, dashed red and blue lines respectively). At P15, TCD in *Reeler* mouse EGL displayed a positive peak (**A**, solid red line), which did not have a counterpart in normal mice. The negative peaks (extrapolations) of the regression curves between P20 and P25 (in black) are predictive of the factual disappearance of the EGL in both groups of mice. In the IGL, TCD in normal *reln*$^{+/+}$ mice was progressively increasing (**C**, blue lines), while in *reln*$^{-/-}$ mice it displayed a totally opposite temporal trend, dropping dramatically over time from P5 onward (**C**, red lines). Note that, after an extrapolated minimum in *reln*$^{+/+}$ (blue dashed line) or maximum in *reln*$^{-/-}$ (red dashed line) at P1.5, a temporal switch of interpolated peaks occurs in the correlation curves of the two genotypes. Also in the medullary body **(D)** regression curves describing the correlation of TCD with time were very different in *reln*$^{+/+}$ (blue lines) and *reln*$^{-/-}$ animals (red lines). EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; P, postnatal age. One-way ANOVA with multiple comparisons; error bars indicate SEM; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

opposite to that of normal mice in the IGL and medullary body of the mutants.

Significance

Collectively, the results reported in Sections Size and Cellularity and its Temporal Variations demonstrated the existence of important genotype-related differences in the size and cellularity (TCD) of the cerebellar cortex and the medullary body during the course of postnatal development, in parallel with profound derangements of the temporal evolution of TCD in the *Reeler* cerebellum. Specifically, the hypoplastic *reln*$^{-/-}$ cerebellum showed a reduction in size and cellularity of the cortex, and an increase in the medullary body. The overgrowth of the medullary body in the *Reeler* mouse is obviously a consequence of the well-known migratory defects that follow the lack of Reelin. We here quantitatively showed that such an overgrowth was insufficient to compensate the cortical hypoplasia. In addition, the concurrent reduction of the size and cellularity of the cerebellar cortex led to conclude that cortical neurons were less numerous in the mutants, as they did not display discernable differences in size. We have in fact used TEM to measure the size of the granule cells, which are—by several orders of magnitude—the most numerous cells in cerebellum, without detecting notable variations between mice of different genotypes (data not shown).

In *Reeler* mice, the alterations in size and cellularity leading to the hypoplasia of the cerebellar cortex were not uniform among layers, and the deficit in cortical growth substantially depended on reduction of the size of the ML and cellularity of the IGL. The granule cell precursors and the pre-migratory granule cells are tightly packed spheroid cells with no or very little intermingled neuropil in the EGL. Previous TEM studies in our laboratory did not show obvious ultrastructural differences in the EGL of normal and mutant mice (Castagna et al., 2014). Therefore, the higher value of TCD in the *reln*$^{-/-}$ EGL at birth indeed reflected the existence of a larger population of granule neurons in the mutants. The lower TCD and number of granule cells at P5–P10 (when the EGL area was also significantly reduced in *reln*$^{-/-}$ mice) indicated that the population of granule cell precursors

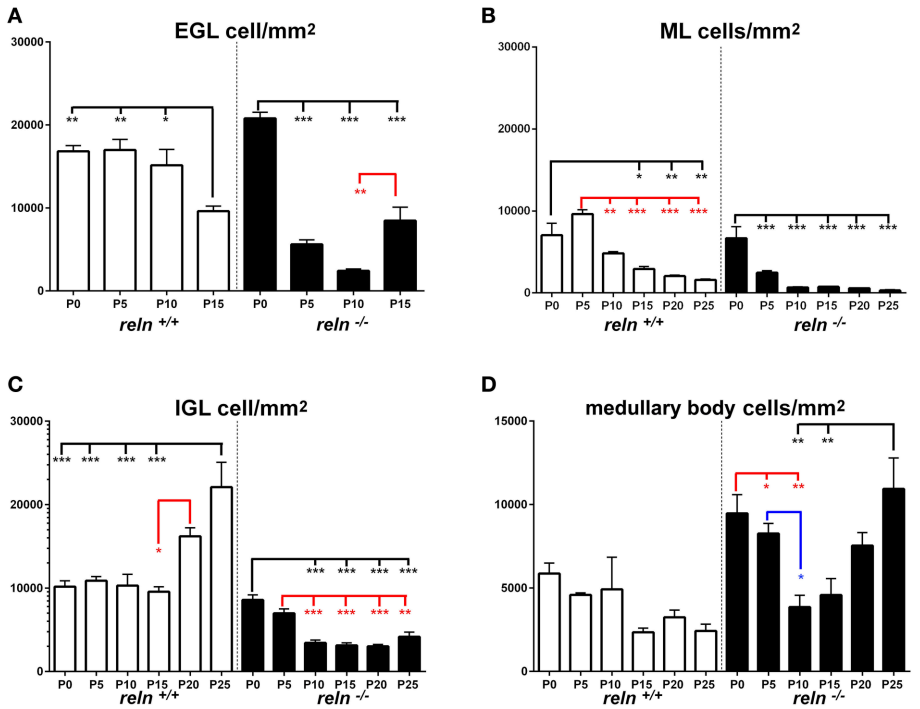

FIGURE 4 | Comparison of TCD *within* genotypes. Cortical layers **(A–C)**, medullary body **(D)**. EGL, external granular layer; ML, molecular layer; P, postnatal age; TCD, total cell density after nuclear stain. One-way ANOVA with multiple comparisons; error bars indicate SEM; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

was more slowly expanding in *Reeler* mice than in age-matched control animals.

Differently from the EGL, the ML is for the most occupied by an expanding neuropil that consists of the parallel fibers (the axons of the granule cells), the climbing fibers, the mossy fibers and the dendrites of the Purkinje neurons. At the same time, as the neuropil increases its size, inhibitory interneurons, i.e., the basket and the stellate cells, migrate in an outward direction from the medullary body to populate the forming ML (Miale and Sidman, 1961). Not surprisingly, at birth, when the neuropil is still poorly developed, TCD was not different across genotypes. However, as the area of the ML was smaller in *reln*$^{-/-}$ mice, the number of cells should be lower in the mutants, this reflecting the well documented defects in the migration of granule cells across this layer. It was also not surprising that TCD decreased with time in both genotypes but remained significantly lower in *Reeler* mice, as this reflected the drastic reduction of synaptic contacts between the Purkinje neurons and the parallel or the climbing fibers in the mutants (Mariani et al., 1977; Castagna et al., 2014). Another possible explanation of hypo cellularity in the *Reeler* ML can be advanced from the results of a study on Reelin signaling in the cerebral cortex, where interneuron laminar positions depended, at least in part, by interactions with projection neurons born on the same day in neurogenesis (Hevner et al., 2004). If this holds for cerebellum, the low cellularity of ML in *reln*$^{-/-}$ mice could also be linked to impairment of the migration of inhibitory interneurons as a consequence of the ectopy of the Purkinje neurons.

Post-migratory granule cells progressively populate the IGL, which will eventually become the only granule cell layer of the mature cerebellum. In *reln*$^{-/-}$ mice, this layer also contains some of the ectopic Purkinje neurons that failed to migrate outwardly to form the Purkinje cell layer. The area of the IGL increased with time in both genotypes, although much less in the mutants. TCD, instead, increased in *reln*$^{+/+}$ mice and decreased in *reln*$^{-/-}$ mice. Therefore, the statistically significant differences in TCD across the two genotypes also demonstrated a numerical reduction of the granule cells population in this cortical layer. These observations are fully consistent with the well-documented action of Reelin on granule cells' migration, and the previously reported reduction in their density when the *Reeler* cerebellum was analyzed *in toto* (Mariani et al., 1977; Mikoshiba et al., 1980; Heckroth et al., 1989; Yuasa et al., 1993; D'Arcangelo et al., 1995; Katsuyama and Terashima, 2009).

Finally, we observed variations in the medullary body area and TCD all along the P0–P25 interval. In *reln*$^{-/-}$ mice, temporal variations in TCD displayed an oscillatory trend without any discernible tendency. Therefore, our observations did not permit to assess whether there were more cells and/or they were more tightly packed in the deep central mass. Both possibilities are fully consistent with the impairment of cell migration in *Reeler* mice. No clear data are available in the literature as to the eventuality that, in the mutants, there is a volumetric reduction of the white matter. However, some forms of lissencephaly with cerebellar hypoplasia in humans have been linked to a *Reln*$^{-/-}$ mutated genotype, and, in these pathologies, several alterations of the

TABLE 2 | Pearson's correlation equations in cortical layers and medullary body.

Genotype	*f*(x)		Correlation equation
reln$^{+/+}$	EGL TCD	$R^2 = 1$	$y = 106.52x^5 - 1543.7x^4 + 8232.5x^3 - 21391x^2 + 26560x + 4858$
	Turning points	2	relative max: x = 1.4; relative min: x = 5.6
	EGL PrCD	$R^2 = 1$	$y = 17.342x^5 - 210.5x^4 + 794.46x^3 - 1149x^2 + 932.7x + 212$
	Turning points	3	relative max: x = 2.8; relative min: x = 5.6
	EGL ApoCD	$R^2 = 1$	$y = -3.7833x^5 + 70.728x^4 - 494.73x^3 + 1585.2x^2 - 2279.7x + 1226.7$
	Turning points	3	relative max: x = 2.9; x = 5.7; relative min: x = 1.6; x = 4.9
reln$^{-/-}$	EGL TCD	$R^2 = 1$	$y = 564.52x^5 - 9342.8x^4 + 56276x^3 - 148897x^2 + 160219x - 38022$
	Turning points	3	relative max: x = 0.9; x = 4.1; relative min: x = 2.6; relative min x = 5.6
	EGL PrCD	$R^2 = 1$	$y = 48.712x^5 - 783.33x^4 + 4517.5x^3 - 11103x^2 + 10094x - 224.5$
	Turning points	4	relative max: x = 0.7; x = 4; relative min: x = 2.6; x = 5.6
	EGL ApoCD	$R^2 = 1$	$y = 4.6211x^5 - 77.385x^4 + 476.4x^3 - 1319.6x^2 + 1595.4x - 613.48$
	Turning points	4	relative max: x = 1.2; x = 4.1; relative min: x = 2.5; x = 5.6
reln$^{+/+}$	ML TCD	$R^2 = 1$	$y = 111.18x^5 - 2172.5x^4 + 16208x^3 - 56631x^2 + 88166x - 38639$
	Turning points	2	relative max: x = 1.6; relative min: x = 5.7
	ML PrCD	$R^2 = 1$	$y = -0.1843x^5 + 2.2804x^4 - 9.606x^3 + 32.317x^2 - 154.4x + 322.69$
	Turning points	2	relative max: x = 5.6; relative min: x = 4.3
	ML ApoCD	$R^2 = 1$	$y = -0.2909x^5 + 6.577x^4 - 55.16x^3 + 210.39x^2 - 356.96x + 224.03$
	Turning points	3	relative max: x = 3.4; relative min: x = 1.8; x = 5.6
reln$^{-/-}$	ML TCD	$R^2 = 1$	$y = 31.762x^5 - 541.92x^4 + 3266x^3 - 7706.6x^2 + 3205.2x + 8417.6$
	Turning points	3	relative max: x = 0.2; x = 4.3; relative min: x = 3.3; x = 5.7
	ML PrCD	$R^2 = 1$	$y = 0.1389x^4 - 3.8335x^3 + 36.252x^2 - 143.28x + 202.93$
	Turning points	2	relative max: x = 5.5; relative min: x = 4.3
	ML ApoCD	$R^2 = 1$	$y = 0.456x^5 - 8.0257x^4 + 53.04x^3 - 162.72x^2 + 228.34x - 110.73$
	Turning points	4	relative max: x = 1.5; x = 4.1; relative min: x = 2.8; x = 5.6
reln$^{+/+}$	IGL TCD	$R^2 = 1$	$y = -193.22x^5 + 3164.2x^4 - 18895x^3 + 51014x^2 - 61543x + 36625$
	Turning points	4	relative max: x = 2.3; x = 5.8; relative min: x = 1.3; x = 3.7
	IGL PrCD	$R^2 = 1$	$y = 6.7819x^5 - 126.46x^4 + 877.65x^3 - 2705.6x^2 + 3209.6x - 265.66$
	Turning points	4	relative max: x = 1; x = 4.3; relative min: x = 4; x = 5.6
	IGL ApoCD	$R^2 = 1$	$y = 0.1916x^5 - 4.9915x^4 + 47.961x^3 - 210.6x^2 + 403.61x - 215.11$
	Turning points	1	relative max: x = 1.9
reln$^{-/-}$	IGL TCD	$R^2 = 1$	$y = 102.78x^5 - 1884.1x^4 + 13023x^3 - 41254x^2 + 56085x - 17495$
	turning points	4	relative max: x = 1.3; x = 4.4; relative min: x = 3.6; x = 5.4
	IGL PrCD	$R^2 = 1$	$y = 14.191x^5 - 264.7x^4 + 1870.6x^3 - 6130.6x^2 + 8869.9x - 3812$
	Turning points	4	relative max: x = 1.5; x = 4.2; relative min: x = 3.7; x = 5.6
	IGL ApoCD	$R^2 = 1$	$y = 1.1118x^5 - 19.114x^4 + 123.54x^3 - 374.31x^2 + 526.24x - 237.61$
	Turning points	4	relative max: x = 1.6; x = 3.7; relative min: x = 2.9; x = 5.6
reln$^{+/+}$	MB TCD	$R^2 = 1$	$y = -161.66x^5 + 2802.7x^4 - 18122x^3 + 53867x^2 - 73062x + 40544$
	Turning points	4	relative max: x = 2.5; x = 5.5; relative min: x = 1.5; x = 4.3
	MB PrCD	$R^2 = 1$	$y = -11.207x^5 + 193.02x^4 - 1204x^3 + 3257.2x^2 - 3668.2x + 1819.1$
	Turning points	4	relative max: x = 2.4; x = 5.6; relative min: x = 1; x = 4.8
	MB ApoCD	$R^2 = 1$	$y = 1.4747x^5 - 27.548x^4 + 193.73x^3 - 630.95x^2 + 924.49x - 431.46$
		4	relative max: x = 1.5; x = 4.4; relative min: x = 3.2; x = 5.8
reln$^{-/-}$	MB TCD	$R^2 = 1$	$y = 102.34x^5 - 2002.2x^4 + 14758x^3 - 49305x^2 + 70263x - 24345$
		2	relative max: x = 1.3; relative min: x = 3.3
	MB PrCD	$R^2 = 1$	$y = 6.0922x^5 - 102.44x^4 + 666.11x^3 - 2152.6x^2 + 3392.1x - 1347.1$
		2	relative max: x = 1.9; relative min: x = 5.7
	MB ApoCD	$R^2 = 1$	$y = 3.2606x^5 - 56.109x^4 + 357.6x^3 - 1039.4x^2 + 1365x - 610.27$
		4	relative max: x = 1.4; x = 4.2; relative min: x = 2.5; x = 5.6

The Table reports statistically data of correlation analysis in the three layers of the cerebellar cortex and the medullary body. The quartic describing the relation of PrCD with time is highlighted with a lighter color. All other functions are quintic. ApoCD, density of apoptotic cells; EGL, external granular layer; IGL, internal granular layer; MB, medullary body; ML, molecular layer; PrCD, density of proliferating cells; TCD, total cell density.

white matter tracts have been reported (Ross et al., 2001; Miyata et al., 2003).

Reeler Mice Display Quantitative Differences in the Extent of Cell Proliferation and Apoptosis, as well as a Derangements in Their Temporal Trends during Postnatal Cerebellar Maturation

During the course of normal postnatal development, a complex interplay of cell proliferation and death is ultimately responsible for proper cerebellar maturation (Marzban et al., 2015). Therefore, we hypothesized that an imbalance between proliferation and programmed cell death could be one of the main factors to explain the cortical hypoplasia in *Reeler* mice. As apoptosis is the commonest form of programmed cell death in cerebellum (Yamaguchi and Miura, 2015), we devised a set of experiments to quantitatively investigate cell proliferation and apoptosis in our material. After a single BrdU administration, we detected high numbers of BrdU-IR nuclei in animals of both genotypes (**Figures 1E,F**). The number of apoptotic TUNEL$^+$ nuclei was by far lower (**Figures 1G,H**). We have calculated the densities of the cells displaying BrdU$^+$ or TUNEL$^+$ nuclei and statistically analyzed data (**Figures 5–8**). To prove or disprove our hypothesis it was necessary to establish whether, above all, genotype had an influence on PrCD or ApoCD, and if additional factors, i.e., developmental age, localization in cortical layers or the medullary body, and sampling position were influential. In doing so, we first have used a 2-way repeated measures ANOVA for related, not independent groups—a type of analysis suitable for investigating changes in mean scores under three or more different conditions—to assess the influence of all these factors on PrCD or ApoCD (Section Genotype, Age, and Localization in Cortical Layers or Medullary Body—but Not Sampling Position—Affect PrC and ApoCD in the Whole Cerebellum). Then, we performed a 1-way ANOVA with multiple comparisons to establish the existence of time-related differences within each genotype and across the two genotypes at corresponding developmental ages. Finally, we used single and multiple regression analysis to model the trends of PrCD and ApoCD with time (Section Temporal Variations of PrCD and ApoCD Display a Non-Linear Relationship with Time, with Differences Across Genotypes), their reciprocal dependence, and their combined effects in determining the cellularity (TCD) of the cerebellar cortex and the medullary body (Section Regression Analysis Shows Different Non-Linear Relationships of PrCD and Apo CD Across Genotypes).

Genotype, Age and Localization in Cortical Layers or Medullary Body—but Not Sampling Position—Affect PrC and ApoCD in the Whole Cerebellum

According to the sampling design described in Materials and Methods, we first aimed to establish whether genotype, age, position along the latero-lateral axis of cerebellum, and localization in the cerebellar cortex or the medullary body influenced cell proliferation and/or death in the cerebellum considered in its entirety.

After a two-way ANOVA for repeated measures we found that genotype ($P < 0.0001$; $F = 36.57$; $Df = 1$), age and localization in individual cortical layers or the medullary body (age $P < 0.0001$, $F = 25.36$, $Df = 5$; cerebellar layer $P < 0.0001$, $F = 439.89$, $Df = 3$) had significant effect on PrCD values. ApoCD was also subjected to the two-way ANOVA for repeated measures and significant effects of genotype ($P < 0.0001$; $F = 38.5$, $Df = 1$), age ($P < 0.0001$, $F = 67.54$, $Df = 5$), and layer ($P < 0.0001$, $F = 162.73$, $Df = 3$) were observed. Notably, position of sampled slices along the latero-lateral axis of cerebellum had no effect on PrCD and ApoCD.

The cerebellum has a remarkably conserved architecture. With the exception of the unipolar brush cells in the vestibulocerebellum (Nunzi et al., 2001), all the other neuronal cortical types are found throughout its three major functional subdivisions, i.e., the vestibulocerebellum (cortex of the vermis+fastigial nucleus), the spinocerebellum (paravermian cortex of the hemispheres+nucleus interpositus), and the pontocerebellum (lateral hemispheric cortex+cerebellar lateral nucleus). Such a functional subdivision follows a latero-lateral axis, along which a banding pattern also occurs in relation to the expression of zebrin-II by the Purkinje neurons and the topographical distribution of the climbing and mossy fibers (Ebner et al., 2012). *Reeler* mice are ataxic, and, remarkably, in a transgenic mouse model of spinocerebellar ataxia type 8 the cerebellar banding pattern is lost, thereby contributing to the motor phenotype (Moseley et al., 2006). Therefore, absence of statistically significant difference in PrCD and ApoCD in parasagittal sections randomly sampled all along the latero-lateral axis is of biological relevance, as it demonstrated that the effect of the mutation was not related to the banding pattern of the normal mature cerebellum, but, instead, it concerned the entire organ. The spinocerebellar and vestibulocerebellar afferent projections in the *Reeler* mouse do not distribute randomly, but have specific target regions, and the position of these regions, relative to each other, is conserved in the mutants (Vig et al., 2005). The distribution of the Purkinje neurons and of the neurons of the cerebellar nuclei is not random either (Vig et al., 2005). *Reelin* binds to two high affinity extracellular receptors on the Purkinje neurons—the very low density lipoprotein receptor (Vldlr) and apolipoprotein E receptor 2 (Apoer2). In *Reeler* mice or double-null mice for Vldlr and Apoer2, Purkinje neurons' clusters failed to disperse, but animals null for either Vldlr *or* Apoer2 individually exhibited specific and parasagittally-restricted Purkinje neurons ectopias (Larouche et al., 2008). Altogether, these observations support the results of our investigation as regarding the absence of parasagittal positional differences in PrCD and ApoCD in *Reeler* mutants, as the lack of Reelin *per se* had no locally patterned influences on neuronal migration.

Differences in PrCD and ApoCD in Relation to Cerebellar Architecture

Once established that sampling position was irrelevant, we proceeded to analyze the differences of PrCD (**Figures 5**, **6**) and ApoCD (**Figures 7**, **8**) across genotypes and within genotypes. Overall our observations demonstrated that PrCD and ApoCD

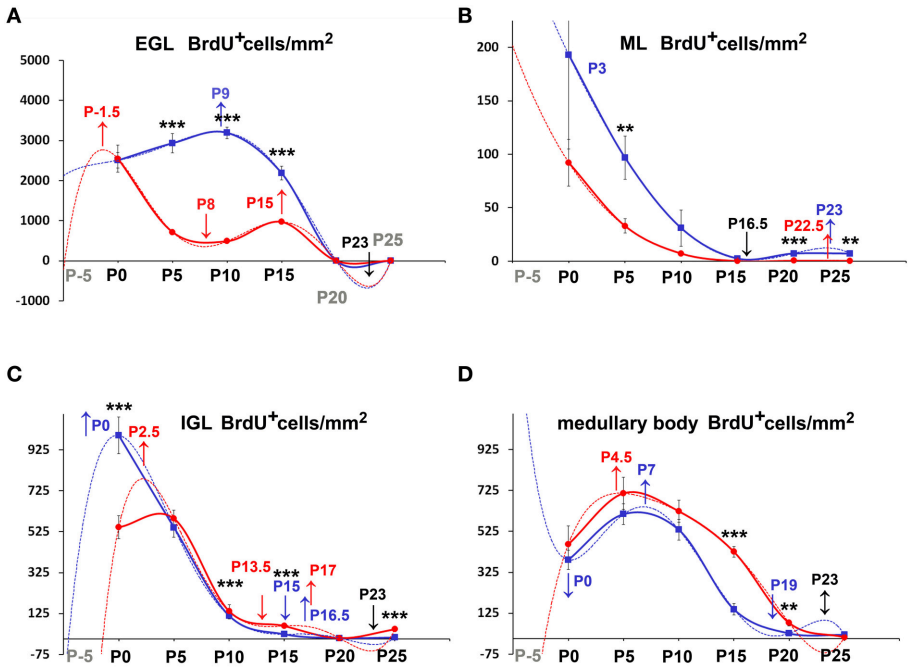

FIGURE 5 | Descriptive graphs (solid lines) and correlation curves (dashed lines) of normalized PrCDs. Graphs highlight the trends and differences *across* genotypes of normalized PrCD ($BrdU^+$ cells/mm^2) in the four cerebellar compartments defined in this study (EGL, ML, IGL, and medullary body). Postnatal ages corresponding to the minima and maxima of correlation curves are indicated with colored letters and upwards or downwards arrows. Blue and red lines or letters/arrows refer to $reln^{+/+}$ and to $reln^{-/-}$ mice, respectively. Black letters for postnatal ages and arrows indicate reference to both genotypes. Postnatal ages in gray (x axis) indicate the time points of correlation curves outside the sampling interval (extrapolations). In the EGL, there were no significant differences across the two genotypes at birth, but, in the P5–P15 interval, PrCD was lower in the mutants (**A**, red lines). Note the temporal switch of the positive/negative peaks in the regression curves of proliferating cells in $reln^{+/+}$ (blue dashed lines) and $reln^{-/-}$ (red dashed lines) mice. In the ML **(B)**, $reln^{+/+}$ mice displayed higher PrCD (blue lines). Notably, $reln^{+/+}$ mice had a higher percentage of proliferating cells at P25 ($reln^{+/+}$ 7.16±0.01, $reln^{-/-}$ 0.25 ± 0.06, $P < 0.01$). Trends and related differences were more complex in the IGL **(C)**. PrCD was higher in $reln^{+/+}$ mice at birth, but $reln^{-/-}$ mice displayed higher values at P10, P15, and P25. Notably, in both genotypes there was a drop at P10. The percentages of $BrdU^+$ cells were higher in $reln^{+/+}$ mice at P0 ($reln^{+/+}$ 9.07±0.34, $reln^{-/-}$ 6.27±0.36, $P < 0.01$), in $reln^{-/-}$ mice at P10 ($reln^{+/+}$ 1.07 ± 0.25, $reln^{-/-}$ 7.12 ± 1.41, $P < 0.05$) and P15 ($reln^{+/+}$ 0.26 ± 0.16, $reln^{-/-}$ 2.17 ± 0.41, $P < 0.05$). In the medullary body **(D)**, there were no differences in PrCD in the P0–P15 interval, and at P25. At P15 and P20, *Reeler* mice (red lines) displayed significantly higher values than $reln^{+/+}$ mice. There were not statistically significant differences in the percentages of $BrdU^+$ cell. EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; P, postnatal age. One-way ANOVA with multiple comparisons; error bars indicate SEM; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

were lower in the three cortical layers but higher in the medullary body of the $reln^{-/-}$ mice. A correlation analysis was then performed to study the reciprocal influences of the two parameters over time.

Temporal Variations of PrCD and ApoCD Display a Non-Linear Relationship with Time, with Differences Across Genotypes

Figures 5, **7** show the trends of PrCD and Apo CD over time (dashed lines) and **Table 2** reports their regression functions together with maxima and minima after Pearson's correlation analysis. The best fit functions ($R^2 = 1$) were 5th degree polynomials, with the sole exception of that calculated for the ML in *Reeler*, where a 4th degree polynomial (quartic) function model was instead appropriate to describe the correlation between time and PrCD. Therefore, as it was the case for TCD (Section Cellularity and Its Temporal Variations), also PrCD and ApoCD were neither constant nor varied linearly with time, in both genotypes. It was of interest that when the regression curves describing the correlation of time with cell proliferation or apoptosis were compared in the two genotypes the following generalizations could be made: i. Proliferation/apoptotic minima and maxima were not coincident in the two genotypes; ii. Genotype-related differences in correlation curves were more evident for ApoCD; and iii. Proliferation/apoptotic maxima in *Reeler* mice generally preceded those in normal mice.

Regression Analysis Shows Different Non-Linear Relationships of PrCD and Apo CD Across Genotypes

There is a wide debate as to the possibility that proliferation and apoptosis are tightly interconnected during neurogenesis, as well as regarding an initial activation of mitosis as a preliminary step to apoptotic cell death (Wang et al., 2009). Therefore, we have applied regression analysis to investigate these issues. This type of analysis allows demonstrating whether a variable (predictor) is likely to be a meaningful addition to a prediction model. If this is the case ($P < 0.05$), changes in the predictor's value are related to changes in the response variable. Here we have

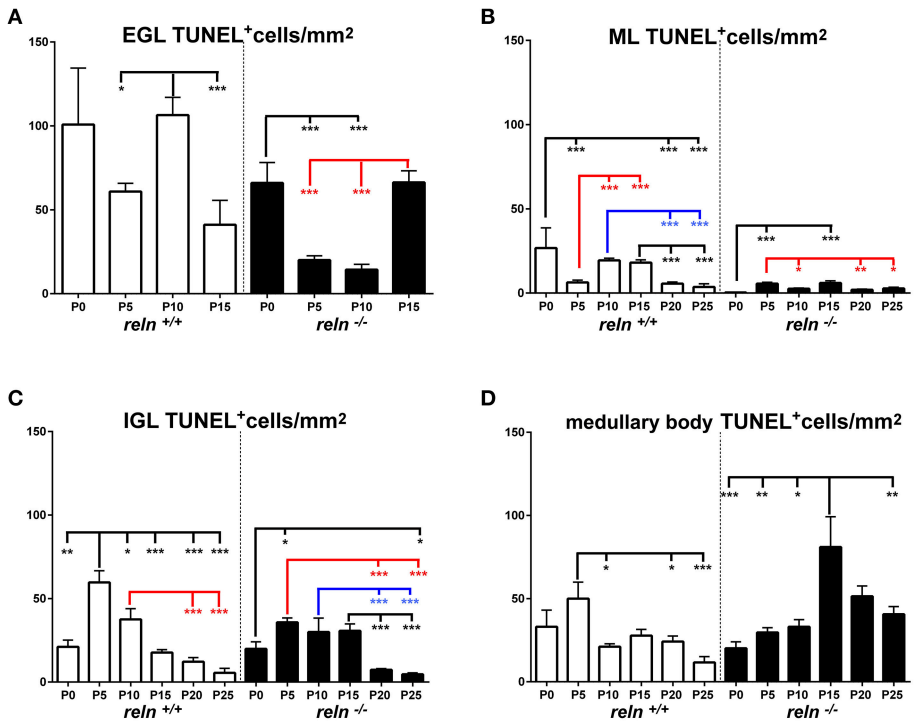

FIGURE 6 | Comparison of PrCD *within* genotypes. Cortical layers **(A–C)**, medullary body **(D)**. EGL, external granular layer; ML, molecular layer; P, postnatal age; One-way ANOVA with multiple comparisons; error bars indicate SEM; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

postulated that PrCD depended on ApoCD or *vice versa*. In $reln^{+/+}$ mice, we notably found that ApoCD depended on PrCD in EGL and IGL. In $reln^{-/-}$ mice, instead, ApoCD depended on PrCD in EGL only, and PrCD depended on ApoCD in EGL and ML. Specifically, in $reln^{+/+}$ EGL ApoCD depended on $(PrCD)^4$ (adjusted $R^2 = 1$; $P = 1{,}00168E\text{-}16$; $Df = 4$; $F = 5{,}60616E{+}31$). In $reln^{-/-}$ EGL, ApoCD depended on $(PrCD)^4$ (adjusted $R^2 = 1$; $P = 1.93625E\text{-}17$; $Df = 4$; $F = 1.50037E{+}33$) and PrCD depended on $(ApoCD)^4$ (adjusted $R^2 = 1$; $P = 8.065E\text{-}17$; $Df = 4$; $F = 8.64797E{+}31$). In $reln^{+/+}$ IGL, ApoCD depended on $(PrCD)^2$ (adjusted $R^2 = 0.91$; $P = 0.012248952$; $Df = 2$; $F = 26.7287703$). In $reln^{-/-}$ ML, PrCD depended on $(ApoCD)^3$ (adjusted $R^2 = 0.98$; $P = 0.011565762$; $Df = 3$; $F = 85.6282253$). Collectively these observations demonstrated that predictors were different in the two genotypes, reinforcing the notion that the interdependence of PrCD and ApoCD was disturbed in the mutants.

Multiple Regression Predicts the Relationship of TCD with PrCD, ApoCD and Time in Connection with Genotype

When we studied the variations of TCD with time using Pearson's correlation (Section Cellularity and Its Temporal Variations), relationships were non-linear. Therefore, we performed polynomial regression analysis to calculate the best fit of TCD with PrCD, ApoCD or time, singularly. **Table 3** reports statistically significant data. Finally, we run a multiple regression analysis modeling the relationship of TCD (response variable) simultaneously with PrCD, ApoCD and time (predictor variables). In this type of analysis, we have postulated that changes in TCD were dependent on changes in one or more of the three predictor variables: if regression was statistically significant, then it was reasonable to infer a dependency of TCD on predictor variables.

In the EGL, we were unable to find a suitable model for $reln^{+/+}$ mice, although linear regression yielded data very close to statistical significance (adjusted $R^2 = 0.91$; $P = 0.051$; $Df = 1$; $F = 18.46307681$). In mutant mice, instead, regression with PrCD (1st degree) and ApoCD (4th degree) was statistically significant (adjusted $R^2 = 0.99$; $P = 0.0003$; $Df = 2$; $F = 321.8028354$), and coefficient statistics showed that TCD was significantly related to PrCD (P = 0.0007). In the ML, regression with PrCD (2nd degree) and time (linear) was statistically significant in $reln^{+/+}$ mice (adjusted $R^2 = 0.99$; $P = 0.0002$; $Df = 2$; $F = 395.3623104$) and coefficient analysis showed that TCD was significantly related to both variables ($PrCD^2$, $P = 0.0009$; time, $P = 0.0138$). In $reln^{-/-}$ mice linear regression statistics gave the best fit (adjusted $R^2 = 0.98$; $P = 0.0091$; $Df = 3$; $F = 108.4326361$), and coefficient statistics showed that TCD was significantly related to PrCD ($P = 0.0155$). In the IGL, multiple regression analysis in $reln^{+/+}$ mice did not yield a suitable model to correlate TCD simultaneously with the three predictor variables of this study. In mutants, on the other hand, both linear regression (adjusted $R^2 = 0.97$; $P = 0.0173$; $Df = 3$; $F = 56.7676746$) and polynomial regression (adjusted $R^2 = 0.93$; $P = 0.043$; $Df = 3$; $F = 22.52511229$) were statistically significant. However, the linear

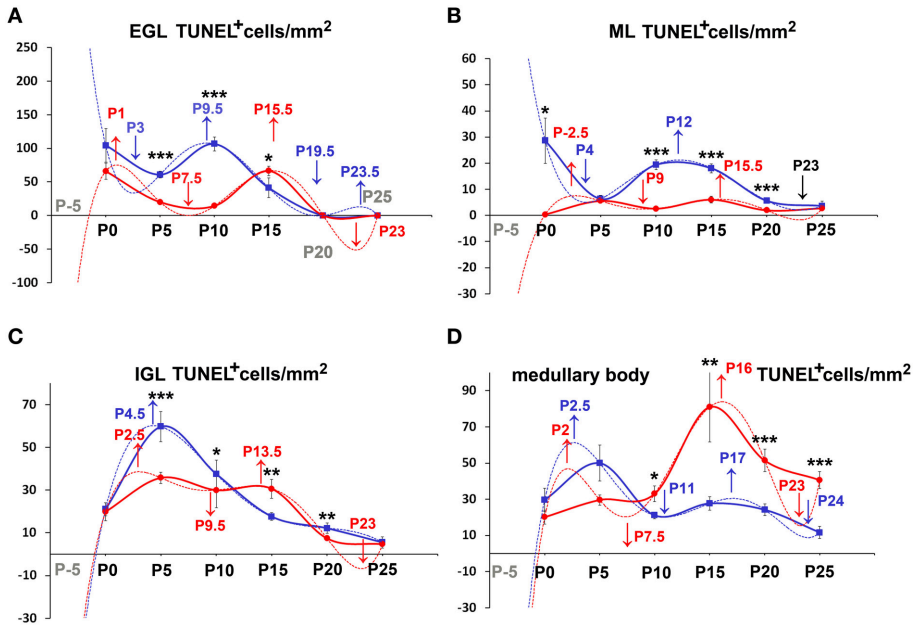

FIGURE 7 | Descriptive graphs (solid lines) and correlation curves (dashed lines) of normalized ApoCD. Graphs highlight the trends and differences *across* genotypes of normalized ApoCD (TUNEL$^+$ cells/mm^2) in the four cerebellar compartments defined in this study (EGL, ML, IGL, and medullary body). Postnatal ages corresponding to the minima and maxima of correlation curves are indicated with colored letters and upwards or downwards arrows. Blue and red lines or letters/arrows refer to *reln*$^{+/+}$ and to *reln*$^{-/-}$ mice, respectively. Black letters for postnatal ages and arrows indicate reference to both genotypes **(B)**. Postnatal ages in gray (x axis) indicate the time points of correlation curves outside the sampling interval (extrapolations). In the EGL **(A)**, ApoCD was higher in normal mice at P5 and P10, but was then overtaken by that in *reln*$^{-/-}$ mice at P15, i.e., immediately before the complete disappearance of this temporary cortical layer. The percentages of apoptotic cells were not different between genotypes at any developmental age. In the ML **(B)**, *reln*$^{+/+}$ mice displayed higher ApoCD at P0, P10, and P20. ApoCD was not statistically different at P5 and P25, but *reln*$^{-/-}$ mice had a higher percentage of TUNEL$^+$ cells at P5 (*reln*$^{+/+}$ 0.06 ± 0.02, *reln*$^{-/-}$ 0.23 ± 0.02, $P < 0.001$). In the IGL **(C)**, ApoCD was higher in *reln*$^{+/+}$ mice at P5, P10 and P20 (blue solid line), but surpassed by that calculated in mutants at P15 (red solid line). At P25, differences among genotypes were not statistically significant. The percentages of TUNEL$^+$ cells were significantly higher in *reln*$^{-/-}$ mice only at P15 (*reln*$^{+/+}$ 0.18 ± 0.02, *reln*$^{-/-}$ 1 ± 0.15, $P < 0.05$) and P20 (*reln*$^{+/+}$ 0.08 ± 0.02, *reln*$^{-/-}$ 0.24 ± 0.03, $P < 0.01$). In the medullary body **(D)**, Apo CD was higher in *reln*$^{-/-}$ mice from P10 onward. There were not statistically significant differences in the percentages of TUNEL$^+$ cells. In all graphs **(A–D)**, note the profound differences in the maxima/minima of correlation curves in the two genotypes. One-way ANOVA with multiple comparisons; error bars indicate SEM; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

model gave the worst match and coefficient statistics was not significant, whereas the polynomial regression gave a statistically significant coefficient for PrCD ($P = 0.0098$), and a value not far from significance for ApoCD[4] ($P = 0.07402$). In the medullary body, linear and polynomial regressions were not suitable to model the relationship of TCD with PrCD, ApoCD, and time in both genotypes. Quartic functions made the interpretation less intuitive than linear or quadratic regression functions, because the effect of changing one predictor varies depending on the value of that predictor: in the temporal range of variation of this study these functions, in fact, display up to three turning points. However, these data confirmed that proliferative and apoptotic events primarily affected the cellularity of the cerebellar cortex rather than that of the medullary body, also on a predictive basis.

Significance

Collectively, the results reported in Sections Genotype, Age, and Localization in Cortical Layers or Medullary Body—but Not Sampling Position—Affect PrC and ApoCD in the Whole Cerebellum and Multiple Regression Predicts the Relationship of TCD with PrCD, ApoCD and Time in Connection with Genotype converged to prove that differences in cell proliferation and apoptosis in the *Reeler* mouse were more prominent in the cerebellar cortex, differently affected cortical layers, statistically influenced TCD (cellularity), and, hence, concurred to explain cerebellar hypoplasia.

Cerebellar cortex

The observation of important differences in PrCD and ApoCD among the layers of the forming cortex and the medullary body in normal mice was fundamental to understand that a layer-related analysis was mandatory to appreciate in full the importance of cell proliferation and apoptosis in the genesis of the mature cerebellum in *reln*$^{-/-}$ animals. Broadly speaking, cell proliferation, as measured by calculating PrCD, was higher in the EGL of *reln*$^{+/+}$ mice (**Figure 5A**). Therefore, taking into account the well-known impairment of granule cell migration in the mutants, it was somewhat surprising that proliferating granule cells—blocked in the EGL by the lack of Reelin—failed to increase PrCD to values higher than those calculated in *reln*$^{+/+}$ mice. At P5-10, migration failure of the granule cells in the mutants should theoretically lead to values of TCD higher than those recorded in normal mice; however, this was not the case (**Figure 3A**). Therefore, our observations demonstrated that a

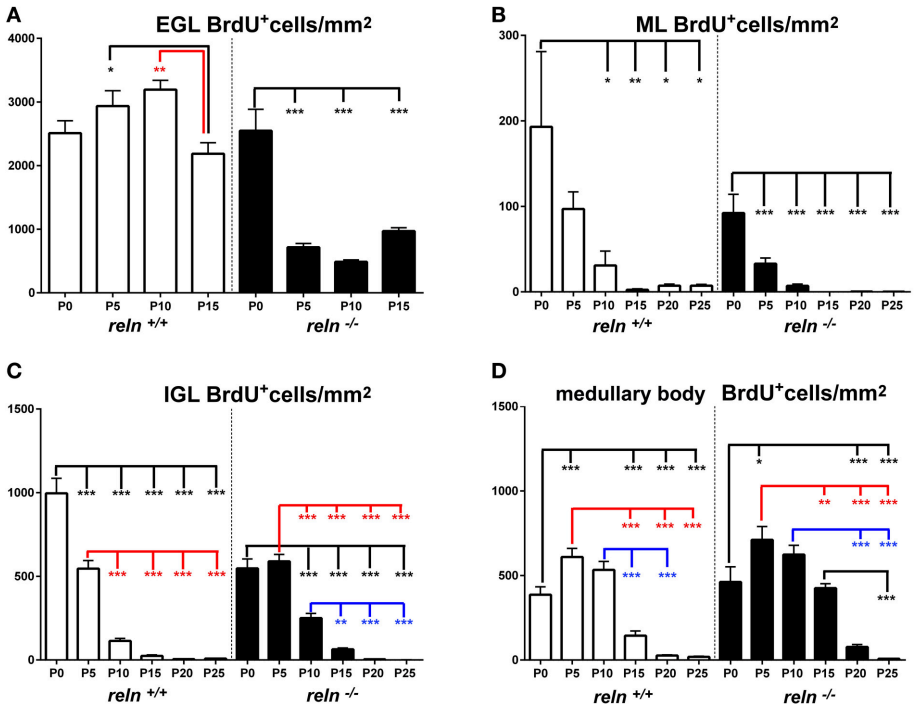

FIGURE 8 | Comparison of ApoCD *within* genotypes. Cortical layers **(A–C)**, medullary body **(D)**. EGL, external granular layer; ML, molecular layer; P, postnatal age. One-way ANOVA with multiple comparisons; error bars indicate SEM; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

TABLE 3 | Regression analysis of TCD with PrCD, ApoCD or time.

Cerebellar division	Gen	Variables		Type of function	Regression statistics			
		y	*x*		Adj R^2	*P*	*Df*	*F*
EGL	*reln*$^{+/+}$	TCD	PrCD	4th deg polynomial	1	7.60734E-17	4	9.71978E+31
		TCD	ApoCD	4th deg polynomial	1	2.37729E-18	4	9.95306E+34
		TCD	Time	3rd deg polynomial	0.93	0.042	3	22.91
	reln$^{-/-}$	TCD	PrCD	Linear	0.99	2.39816E-05	1	496.860073
		TCD	ApoCD	4th deg polynomial	1	9.36107E+31	4	7.75173E-17
ML	*reln*$^{+/+}$	TCD	PrCD	2nd deg polynomial	0.92	0.009	2	32.05
		TCD	Time	Linear	0.72	0.02	1	13. 8285904
	reln$^{-/-}$	TCD	PrCD	Linear	0.99	1.86916E-05	1	563.237209
		TCD	PrCD	3rd deg polynomial	0.98	0.008	3	120.545224
		TCD	PrCD	3rd deg polynomial	0.99	0.0036	3	273.093488
IGL	*reln*$^{+/+}$	TCD	Time	2nd deg polynomial	0.87	0.021	2	17.8175611
	reln$^{-/-}$	TCD	PrCD	Linear	0.85	0.005131047	1	30.877
		TCD	ApoCD	4th deg polynomial	0.99	0.022776642	4	1083.86757
		TCD	Time	2nd deg polynomial	0.90	0.012	2	26.2066774
Medullary body	*reln*$^{+/+}$	TCD	Time	Linear	0.75	0.015	1	16.0907388
	reln$^{-/-}$	TCD	Time	2nd deg polynomial	0.79	0.044	2	10.4717317

The Table reports statistically significant data of regression analysis in the three layers of the cerebellar cortex and the medullary body. ApoCD, density of apoptotic cells; EGL, external granular layer; IGL, internal granular layer; Gen, genotype; ML, molecular layer; PrCD, density of proliferating cells; TCD, total cell density.

severely impaired cell proliferation, rather than the granule cell migratory deficit, was responsible for the differences in TCD observed in the EGL. In the ML, PrCD was also higher in normal mice (**Figure 5B**), but, in this case, ApoCD was higher too (**Figure 7B**). In normal mice, the basket/stellate cell are generated in the medullary body during the P0–P15 interval; in the same temporal window, these neurons migrate to the ML, their numerical size being regulated by apoptosis (Yamanaka et al., 2004). Therefore, lower values in the mutants likely reflected a Reelin-dependent impairment of the migration of the GABAergic interneurons. In the IGL, PrCD, and ApoCD followed a complex pattern (**Figures 5C**, **7C**). At the end of postnatal development, PrCD was higher in the mutants, in parallel with a lower ApoCD. The GABAergic interneurons of the forming cerebellar cortex are the proliferating cells normally populating the postnatal IGL (Miale and Sidman, 1961). At birth, when the stellate and the basket cells start to be generated, PrCD was higher in $reln^{+/+}$ mice (about two-fold than in mutants). In normal mice, around P2–P7, these neurons start migrating to the ML (Miale and Sidman, 1961). We believe that migration failure explains why mutants had higher PrCD from P5. Although differences in PrCD were statistically significant, it was reasonable to suppose that the migratory deficit of the relatively small population of the GABAergic interneurons minimally reflected onto TCD. A major event was, instated, that the population of the post-mitotic post-migratory granule cells in the IGL of $reln^{-/-}$ mice underwent late apoptosis (P15 and P25 vs. P5–P10 in $reln^{+/+}$ mice) to a degree higher than in normal animals, as demonstrated by the values of ApoCD. Notably, apoptosis of mature granule cells is consequent to a failure of making proper synaptic contacts in the forming ML, and, in normal mice, programmed cell death affects IGL-migrated granule cells when synapses between the parallel fibers and the Purkinje neurons are established (Lossi et al., 2002). There are much less contacts in the mutants (Castagna et al., 2014), this explaining the higher ApoCD recorded here at P25 in $reln^{-/-}$ mice.

Medullary body

In the medullary body, from the second postnatal week, *Reeler* mice had higher PrCD and ApoCD (**Figures 5D**, **7D**). However, predictive statistics failed to model TCD as a function of PrCD and ApoCD (Section Multiple Regression Predicts the Relationship of TCD with PrCD, ApoCD, and Time in Connection with Genotype). To understand the significance of these results, one needs to consider that the medullary body of the *Reeler* mouse contains the ectopic Purkinje neurons, the neurons of the cerebellar nuclei, and different types of glial cells. Apart from the Purkinje neurons, it is unclear whether the mutation also affects the migration of the other two groups of cells. Previous observations demonstrated that neurons of the cerebellar nuclei, after having been generated in the rhombic lip, migrate rostrally in a subpial stream to the nuclear transitory zone, and that a subset of rhombic lip-derived cells express Reelin (Fink et al., 2006). In later stages of development, the EGL replaces the subpial stream, and the nuclear transitory zone organizes into distinct cerebellar nuclei. It is worth noting that, in *Reeler* mice, rhombic lip-derived cells migrated normally to the nuclear transitory zone (Fink et al., 2006). Therefore, it may well be possible that the lack of Reelin does not interfere with the migration of the neurons of cerebellar nuclei. Birth-dating studies using a single pulse of BrdU (Miale and Sidman, 1961; Altman and Bayer, 1997; Sekerkova et al., 2004) indicated that most cerebellar astrocytes are generated during late embryonic and postnatal development in the prospective white matter, to which we have referred to as the medullary body in this study. Fate-restricted precursors of the astrocyte lineage reside in the postnatal prospective white matter (Cai et al., 2011), and express several markers of the juvenile astrocytes among which vimentin (Silbereis et al., 2009) that we localized in this study with overlapping patterns in the two genotypes. Whereas the interactions of Reelin with the Bergmann glia are widely documented, we were unable to find information on the intervention, if any, of the protein on the migration of immature astrocytes from the prospective white matter. It should be mentioned that some of these cells remain in the medullary body, giving rise to the white matter fibrous astrocytes, whereas others migrate to the IGL and differentiate into the velate protoplasmic astrocytes, including the bushy cells of the mature granular layer and the Bergmann glia (Palay and Chan-Palay, 1974). Thus, the higher TCD that we recorded in the $reln^{-/-}$ medullary body (**Figure 3D**) could also be a consequence of the entrapment of the velate protoplasmic astrocytes into the white matter because of the lack of Reelin.

DISCUSSION

Several spontaneous mutations primarily affecting the mouse cerebellum have been long ago discovered. They are of high interest to the neuroscientists not simply as suitable models of human ataxias (Cendelin, 2014), but also as valuable tools to study the normal development of cerebellum. Since their initial discovery, most mutants such as *Lurcher*, Purkinje cell degeneration, nervous, *Weaver*, and *Staggerer* mice were widely investigated and resulted phenotypically characterized by high levels (up to 100%) of cell death affecting the granule cells and/or the Purkinje neurons (see Table 4 in Castagna et al., 2016). Thus, mutant mice were very useful in understanding the mutual relationship between these two types of cortical neurons as regarding their reciprocal maintenance or death during the course of cerebellar maturation.

The *Reeler* mouse is somehow an exception, because programmed cell death was seldom investigated. The relative lack of interest for programmed cell death in $reln^{-/-}$ mice is not so much surprising, as the mutation was immediately recognized to be a disorder of cellular migration during the course of CNS development. The subsequent discovery of Reelin boosted a wide number of studies aiming to elucidate the cellular and molecular mechanisms that could explain its function in the normal and pathological brain (D'Arcangelo, 2014). In subsequent years, several studies described the cerebellar atrophy/hypoplasia, the disorganization of cerebellar architecture, the reduction in the number of the Purkinje neurons and density of the granule cells (Falconer, 1951; Mariani et al., 1977; Mikoshiba et al., 1980;

Heckroth et al., 1989; Yuasa et al., 1993; D'Arcangelo et al., 1995; Katsuyama and Terashima, 2009). Unexpectedly, however, the only study (to our knowledge) using the TUNEL technique reported negative results (Herrup and Busser, 1995).

Here, descriptive statistics and predictive models using regression analysis proved to be useful in understanding the relationship of cellularity with proliferation, apoptosis, and time during the course of postnatal cerebellar development, disclosing the existence of notable differences between normal mice and the *Reeler* mutants. Therefore, as we will discuss below, differences in cell proliferation and apoptosis do explain, at least in considerable part, the phenotypic alterations that led to the generation of a hypoplastic cerebellum in $reln^{-/-}$ mice.

The Cerebellar Cortex of the *Reeler* Mouse Displays Altered Relationship between Cell Proliferation and Apoptosis

Especially in the EGL and IGL, $reln^{-/-}$ mice lose the link between cell proliferation and apoptosis that characterizes the normal mice; the two events are, instead, less clearly dysregulated in the ML.

In the $reln^{+/+}$ EGL, we observed a bidirectional dependence of PrCD and ApoCD that could be modeled by a 4th degree polynomial in both directions. Existence of biological phenomena that could be modeled by nonlinear regressions in the course of central neuron development is not a novel finding. For example, a 3rd degree polynomial best described pyramidal cell differentiation in layer II of the piriform cortex (Sarma et al., 2011), and hippocampal neurogenesis was adequately modeled by non-linear equations in (among others) neuronal progenitor cells and immature neurons (Cacao and Cucinotta, 2016). In addition, mathematic modeling led to better understand the complex dynamics of apoptotic regulation during brain development (Spencer and Sorger, 2011; Lavrik, 2014). Our regression statistics confirmed that cell proliferation and apoptosis of the cerebellar granule cells were tightly interconnected in normal mice, the present data being in full accordance with previous observations demonstrating that some of the newly generated granule cells in postnatal rabbits died very soon thereafter their birth, and before starting their migration to the ML/IGL (Lossi et al., 2002). Notably, such a relationship was totally lost in *Reeler* mice. In the $reln^{+/+}$ IGL, ApoCD depended on $(PrCD)^2$ after regression analysis. This observation is, again, consistent with findings obtained directly by time-window labeling of proliferating rabbit granule cells that underwent a second, delayed phase of apoptotic programmed cell death after their migration to the IGL (Lossi et al., 2002). Remarkably, also this relationship was lost in $reln^{-/-}$ mice. That in $reln^{-/-}$ ML PrCD was predictively linked to $(ApoCD)^3$ is more difficult to explain considering that there is not local neurogenesis in this layer. It seems possible that some granule cells in transit to the IGL remained entrapped in the ML failing to properly migrate. However, due to the high speed of migration of these cells (Zheng et al., 1996), a sequential BrdU injection protocol would be required to fully prove or disprove such a possibility. Results of predictive statistics (Section PrCD is the Most Important Predictive Factor to Determine TCD in Cortical Layers of the *Reeler* Mouse below) were also supportive of the above hypothesis.

PrcD is the Most Important Predictive Factor to Determine TCD in Cortical Layers of the *Reeler* Mouse

After multiple regression statistics with cell proliferation, apoptosis and time as independent variables to predict TCD (cellularity), significant differences were observed among cortical layers across genotypes. However, in the EGL we were unable to find a suitable model for $reln^{+/+}$ mice, although linear regression yielded statistic data very close to significance (adjusted $R^2 = 0.91$; $P = 0.051$). As we have only examined six time points in this study, and only four of them applied to this temporary layer of the cerebellar cortex, it seemed reasonable to hypothesize that increasing temporal sampling would yield a statistically significant model of regression. In mutant mice, multiple regression considering PrCD and ApoCD as independent variables confirmed the interdependence of the two phenomena, and coefficient statistics showed that TCD was significantly related to PrCD ($P = 0.0007$), but not to ApoCD ($P = 0.237651133$). In the ML, after coefficient analysis, TCD was significantly correlated with PrCD and time in $reln^{+/+}$ mice, but with PrCD only in $reln^{-/-}$ mice. Migration of the granule cells along the Bergmann glia is relatively rapid, as video microscopy studies *in vitro* have shown that these neurons travel at speeds between 20 and 50 μm/hour (Zheng et al., 1996). Thus, timing in migration of the granule cells may be altered in the mutants because the ML was hypoplastic, and/or migration was, itself, impaired. Finally it was remarkable that in the $reln^{+/+}$ IGL, we did not find a suitable model to correlate TCD simultaneously with cell proliferation, apoptosis and time. We interpret this finding considering the error introduced by the impossibility to quantitate cell migration according to the experimental design of this study, as the proper migration of the granule cells is of paramount importance to correctly populate the IGL. In keeping with this interpretation, in mutants, where cell migration is highly impaired, the error introduced by the regression equation was smaller and a statistically significant coefficient was calculated for PrCD ($P = 0.0098$), whereas $ApoCD^4$ was not far from significance ($P = 0.07402$). Therefore, in $reln^{+/+}$ mice, the impossibility to predict the cellularity of the IGL by the predictive analysis employed in this study gave a reduction ad absurdum of the primary influence of granule cell migration in the normal development of the granular layer of the mature cerebellum.

Collectively, these predictive data confirmed the profoundly altered relationship between cell proliferation and apoptosis demonstrated after descriptive statistics in the *Reeler* mutants.

Reelin and Cell Proliferation/Apoptosis

The biology of Reelin in relation to positioning, growth and maturation of neurons during brain development and to synaptic activity in the adult brain has been recently and very authoritatively reviewed (D'Arcangelo, 2014). In recapitulating the history of discovery of the importance of Reelin in

neuronal migration, D'Arcangelo writes *"...the layer organization of Purkinje cells in the cerebellar cortex represented the step that was directly affected by the absence of Reelin. Thus, the failure of granule cells to proliferate, which ultimately leads to the lack of foliation and cerebellar hypoplasia in reeler mice, was recognized as a secondary defect due to the malposition of Purkinje cells, which failed to enter the cerebellar cortex after leaving the ventricular zone and remained localized in a deep cerebellar mass."* This explains why, as already mentioned in the Introduction to this paper, limited attention has been paid to the possible effects of Reelin on proliferation, and, even less, apoptosis. This work shows the existence of a deficit in granule cell proliferation as a consequence of the lack of Reelin: specifically, the very rapid temporal switch from proliferation to death in the EGL of normal mice excludes a role of mal-positioned Purkinje neurons in the target-independent apoptosis of the granule cells (Lossi et al., 2002). It seems therefore possible that the lack of Reelin directly or indirectly interferes with the proliferation program of these neurons. In keeping with this possibility, evidence is accumulating to suggest a role of Reelin in regulating cell proliferation *in vitro* (Ohkubo et al., 2007; Massalini et al., 2009) and in hippocampus *in vivo* (Duan et al., 2007; Zhao et al., 2007; Fournier et al., 2010; Teixeira et al., 2012; Sibbe et al., 2015). Our present findings are also consistent with an intervention of Reelin and/or its downstream signaling molecule Dab1 in cell death (Zhao et al., 2007; Teixeira et al., 2012). They are also in line with very recent ultrastructural findings demonstrating higher numbers of granule cells and Purkinje neurons undergoing programmed cell death in the cerebellar vermis of postnatal *reln*$^{-/-}$ mice in comparison with age-matched controls (Castagna et al., 2016). Current knowledge about programmed cell death has indeed accumulated toward the recognition of various different mechanisms and forms, only part of which may be subject to detection using the TUNEL technique, as we have very recently reviewed (Lossi et al., 2015). In such a scenario, it is worth mentioning that Castagna et al. (2016) have demonstrated that apoptosis is not the only type of programmed cell death occurring during postnatal cerebellar development in normal and *Reeler* mice, as autophagic neurons and neurons undergoing non-canonical forms of cell death and dark degeneration were additionally observed in both genotypes after TEM examination. To date, few data are available as regarding the possible intervention of forms of cell death other than apoptosis in cerebellar development (Marzban et al., 2015), and reported observations are often contradictory as regarding the possibility that autophagy is protective rather than harmful to cerebellar neurons. For example, the autophagy-related *Unc51.1* murine gene signals the program of gene expression leading to the formation of the granule cell axons (Tomoda et al., 1999), and the selective ablation of the *Atg5* or *Atg7* autophagy-related genes leads to behavioral deficits associated with severe neuronal loss in the cerebellar cortex (Komatsu et al., 2006, 2007). On the other hand, autophagy may be a preliminary step to granule cell apoptosis (Canu et al., 2005), and dysfunction of endosomal sorting complex required for transport 3 (ESCRT-3) causes autophagosome accumulation and neurodegeneration of the Purkinje neurons (Lee et al., 2007). Notably, the density of apoptotic neurons, irrespective of the genotype and age, was consistently higher than that of autophagic neurons in the cerebellar vermis after TEM observations (see Table 3 in Castagna et al., 2016). These observations reinforce the notion that apoptosis is the commonest form of programmed cell death not only in normal cerebellar development, also as far as the *Reeler* mouse is concerned. We might have somewhat underestimated the extent of programmed cell death in this study—if indeed autophagy of the granule cells and/or the Purkinje neurons is injurious to these neurons—and/or a switch among alternative cell death programs occurs in postnatal cerebellar development *in vivo*. Nonetheless, our observations provide a sound basis for further investigations on the intervention of Reelin in the regulation of cell proliferation in the course of (cerebellar) neurogenesis. Under this perspective, it is of relevance that the ectopic expression of Reelin in *Reeler* mice rescued animals from cerebellar ataxia, and supported a substantial recovery in granule cell proliferation (Magdaleno et al., 2002).

AUTHOR CONTRIBUTIONS

CC performed ICC and microscopy studies, animal genotyping, descriptive statistics, and helped to draft the manuscript; AM participated in the design and coordination of the study, performed predictive statistics, critically revised statistical analysis, and drafted the manuscript; MG performed descriptive statistics; LL conceived the study, participated in its design and coordination, participated in experiments to assess cell proliferation and apoptosis, critically reviewed the manuscript draft. All authors read and approved the final manuscript.

FUNDING

This work was supported by local grants of the University of Turin (Fondi ex60%).

ACKNOWLEDGMENTS

We wish to thank Prof. Luigi Bertolotti for help with some descriptive statistics procedures.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 11 March 2016
doi: 10.3389/fncel.2016.00062

Reelin Signaling in the Migration of Ventral Brain Stem and Spinal Cord Neurons

*Ankita R. Vaswani and Sandra Blaess**

Neurodevelopmental Genetics, Institute of Reconstructive Neurobiology, Life and Brain Center, University of Bonn, Bonn, Germany

The extracellular matrix protein Reelin is an important orchestrator of neuronal migration during the development of the central nervous system. While its role and mechanism of action have been extensively studied and reviewed in the formation of dorsal laminar brain structures like the cerebral cortex, hippocampus, and cerebellum, its functions during the neuronal migration events that result in the nuclear organization of the ventral central nervous system are less well understood. In an attempt to delineate an underlying pattern of Reelin action in the formation of neuronal cell clusters, this review highlights the role of Reelin signaling in the migration of neuronal populations that originate in the ventral brain stem and the spinal cord.

Keywords: dopaminergic neurons, cranial motor neurons, somatic motor neurons, Dab1, preganglionic neurons, midbrain, hindbrain, mouse models

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers, The State Univesity of New Jersey, USA

Reviewed by:
Toshio Terashima, Kobe University School of Medicine, Japan
Björn Spittau, Albert-Ludwigs-University Freiburg, Germany

****Correspondence:***
Sandra Blaess
sblaess@uni-bonn.de

Received: *20 December 2015*
Accepted: *26 February 2016*
Published: *11 March 2016*

Citation:
Vaswani AR and Blaess S (2016) Reelin Signaling in the Migration of Ventral Brain Stem and Spinal Cord Neurons. Front. Cell. Neurosci. 10:62. doi: 10.3389/fncel.2016.00062

INTRODUCTION

The first indication for the role of Reelin in neuronal migration during brain development came from studies in *reeler* mice (Falconer, 1951; D'Arcangelo et al., 1995). In addition to a reeling, ataxic gait, to which these mutants owe their name, *reeler* mice exhibit improper cortical and hippocampal layering, cerebellar atrophy and ectopia of several neuronal populations (Falconer, 1951; Caviness and Sidman, 1973; Caviness, 1982; Goffinet, 1984b; Sheppard and Pearlman, 1997). Hence, this mutant strain was studied as a model for disrupted neuronal lamination long before the gene product responsible for its characteristic phenotype was identified, and it was established that defective neuronal migration is the primary cause of the *reeler* phenotype (Caviness and Sidman, 1973; Caviness, 1982). The discovery of the gene *Reelin*, as being the site of a deletion in *reeler* mice, paved way for its identification as an important regulator of neuronal migration and heightened interest in its exact role and mechanism of action (D'Arcangelo et al., 1995).

In what is described as the canonical signaling pathway, the product of the *Reelin* gene, a large extracellular matrix molecule, binds apolipoprotein E receptor 2 (ApoER2), also known as low-density lipoprotein receptor-related protein 8 (LRP8) or very low density lipoprotein receptor (VLDLR; Trommsdorff et al., 1999). This binding event results in the phosphorylation of the intracellular downstream effector disabled homolog 1 (Dab1) through the Src-family (rous sarcoma oncogene) tyrosine kinases Fyn (Fyn proto-oncogene) and Src (Howell et al., 1997; Hiesberger et al., 1999; Arnaud et al., 2003; Ballif et al., 2003). While downstream signaling events are not completely understood, the phosphorylated Dab1 molecule has been shown to be capable of recruiting several signaling pathways such as the Crk/CrkL-C3G-Rap1 pathway (Crk: adapter molecule crk; CrkL: Crk-like; C3G: Rap guanine nucleotide exchange factor 1; Rap1: Ras-proximate-1) to promote

cell adhesion, or the LimK1-Cofilin1 pathway (LimK1: LIM domain kinase 1) that stabilizes the cytoskeleton (Park and Curran, 2008; Voss et al., 2008; Chai et al., 2009).

Most of the work on Reelin signaling and function has focused on its role during development of dorsal, laminar brain structures such as the cerebral cortex, hippocampus and cerebellum. From these studies, the following functions for Reelin signaling in neuronal migration emerge: stabilization of the leading process of migrating neurons, regulation of neuronal cell orientation or polarity, function as a stop signal for migrating neurons and indirect effects on neuronal migration e.g., by regulating the morphology and maturation of radial glia (for detailed recent reviews, refer to Sekine et al., 2004; D'Arcangelo, 2014; Förster, 2014).

Reelin also regulates radial and tangential migration of neurons that settle in the ventral brain stem and spinal cord; these cells are primarily organized into cell clusters. The underlying principles of Reelin function are less well understood in the development of these neuronal populations. Here, we will review the proposed roles of Reelin in the migration of neurons in the ventral brain stem and highlight the common themes in this regulation. We will focus in particular on the neuronal populations that are generated in the ventral progenitor domain of the brain stem and spinal cord. Nuclei derived from dorsal progenitor domains such as the pontine nucleus, dorsal cochlear nucleus, lateral reticular nucleus and inferior olivary complex will not be discussed.

VENTRAL MIDBRAIN

The ventral midbrain contains a number of nuclei derived from the ventricular zone in the ventral midbrain including the oculomotor nucleus, the red nucleus and midbrain dopaminergic (mDA) neurons. mDA neurons are arranged in three distinct anatomical clusters, the substantia nigra pars compacta (SNc), the ventral tegmental area (VTA) and the retrorubral field (RRF). The final position of the red nucleus and oculomotor neurons suggests that they undergo a one-step radial migration, while mDA neurons migrate first radially followed by a tangential migration step of SNc-mDA neurons (Prakash et al., 2009; Bodea et al., 2014).

Expression Pattern of Reelin and Its Downstream Pathway Components in the Ventral Midbrain

Both *Reelin* and *Dab1* expression are observed in the lateral ventral midbrain at E (embryonic day) 11.5 (Allen Institute for Brain Science, 2015). From E13.5 to early postnatal stages, *Reelin* expression is localized to the red nucleus (Bodea et al., 2014; Allen Institute for Brain Science, 2015; **Figures 1A–C**). After E15.5, its expression extends to additional regions in the ventral midbrain (Ikeda and Terashima, 1997; Allen Institute for Brain Science, 2015). *Reelin* mRNA is not expressed in mDA neurons at embryonic stages (Bodea et al., 2014), but there are conflicting reports on the presence of Reelin protein in the area where mDA neurons are located. Sharaf et al. (2014) report no immunoreactivity for Reelin (using a monoclonal antibody, clone G10; de Bergeyck et al., 1998) in mDA neurons at E16.5, P (postnatal day) 15 and P90. However, the authors found evidence for intra- and extracellular Reelin expression in mDA neurons at P0 (Sharaf et al., 2014). In contrast, immunostaining with the CR-50 antibody (Miyata et al., 1996), detects Reelin protein in the extracellular space surrounding mDA neurons (but not in mDA neurons), both at E15.5 and P0 (Nishikawa et al., 2003; **Figures 1B,C**). Based on the lack of Reelin *mRNA* in mDA neurons, Nishikawa et al. (2003) propose that Reelin might be deposited in the ventral midbrain through axonal transport in projections from the striatum to the SNc. *Dab1* is expressed in laterally positioned mDA neurons (presumptive mDA neurons of the SNc) at E13.5 and expression is maintained in a subset of mDA neurons at least up to P15 (Bodea et al., 2014; Sharaf et al., 2014; **Figures 1B,C**). High levels *of Dab1* expression have also been detected in the forming of substanta nigra pars reticulata (SNr; Bodea et al., 2014; Allen Institute for Brain Science, 2015). Beginning at E13.5, Reelin receptor*s ApoER2* and *VLDLR* are both weakly expressed throughout the ventral midbrain (Bodea et al., 2014; **Figures 1B,C**). This widespread expression seems to be maintained at subsequent embryonic stages. At E16.5, VLDLR is expressed in and adjacent to mDA neurons, while ApoER2 expression is reported to be weaker and more specific to mDA neurons (Sharaf et al., 2014). A similar expression pattern has been documented at P15 (Sharaf et al., 2014).

Function of Reelin Signaling in the Ventral Midbrain

Despite the broad expression of Reelin, Dab1 and the Reelin receptors in the ventral midbrain, the impact of Reelin signaling has only been studied in the context of mDA neuronal migration in the ventral midbrain and we will summarize these findings in the following section.

Midbrain Dopaminergic (mDA) Neurons

mDA neurons in the SNc project to the dorsal striatum and form the nigrostriatal pathway; they are involved in the regulation of motor control. VTA-mDA neurons innervate the ventral striatum and prefrontal cortex, and modulate cognitive and reward behaviors. Neurons of the RRF have been reported to project to the dorsal striatum and prefrontal cortex, but their innervation targets and functions are incompletely understood (Björklund and Dunnett, 2007).

mDA neurons are generated from progenitors in the floor plate of the ventral midbrain between E10.5 and E13.5. From their progenitor zone, mDA neurons first migrate radially, towards the pial surface. Subsequently, neurons of the SNc (but not the VTA) undergo tangential migration to take up their final lateral position (Bodea et al., 2014; **Figure 1D**). In the absence of Reelin signaling, the SNc and RRF are disorganized, a phenotype that is first obvious at E16.5. The defect is particularly severe at intermediate anteroposterior levels in the midbrain where the lateral SNc essentially fails to

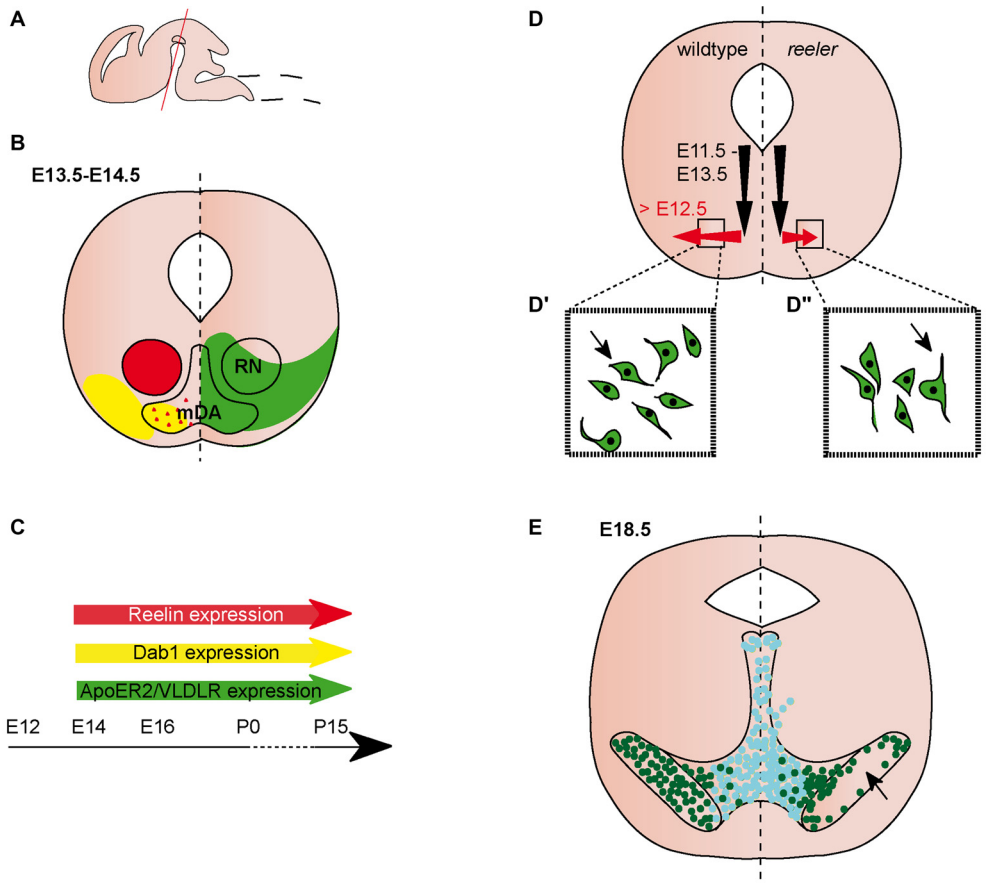

FIGURE 1 | Migration of midbrain dopaminergic (mDA) neurons. (A) Sagittal view of embryonic brain. Red line indicates level of coronal sections in **(B,D,E)**. **(B)** Expression patterns of Reelin (red), Dab1 (yellow), ApoER2 (green) and very low density lipoprotein receptor (VLDLR; green) at the indicated time point. Red triangles in the mDA neuron region indicate Reelin secreted by striatal projections. Reelin and Dab1 expression are only presented in the left half of the brain, ApoER2 and VLDLR expression are only presented in the right half of the brain. Expression in the dorsal midbrain is not included in the schematic. **(C)** Expression of Reelin, Dab1, ApoER2 and VLDLR in the midbrain is maintained into adulthood. **(D)** Schematic of migratory paths of mDA neurons in wildtype (left half of the brain) and *reeler* mice (right half of the brain). Black arrows indicate radial migration, red arrows indicate tangential migration. Tangential migration is truncated in *reeler* mutants **(D′,D″)**. In wildtype mice **(D′)**, processes of migrating mDA neurons are oriented tangentially, in *reeler* mice **(D″)** processes are oriented radially. **(E)** mDA neuron distribution in E18.5 wildtype mice (left half of the brain) and *reeler* mutants (right half of the brain). Turquoise dots represent Calbindin positive mDA neurons of the ventral tegmental area (VTA); dark green dots represent Girk2 positive mDA neurons of the substantia nigra (SN). SN-mDA neurons separate from VTA-mDA neurons but fail to reach their normal lateral location in *reeler* mice (arrow).

form (Nishikawa et al., 2003; Kang et al., 2010; Sharaf et al., 2013; Bodea et al., 2014; **Figure 1E**). Consequently, there is a drastic decrease in the number of mDA neurons in the SNc at P0 compared to heterozygous controls, while there is no significant change in the overall number of mDA neurons (Nishikawa et al., 2003; Kang et al., 2010). In addition, the number of mDA neurons is significantly increased in the VTA of *reeler* mice (Kang et al., 2010). Analysis of *yotari* mice, which are homozygous for an autosomal recessive mutation in the *Dab1* gene (Sheldon et al., 1997), shows an abnormal organization of the SNc comparable to the one observed in *reeler* mutants.

Since radial and tangential fiber tracts might serve as guides for migrating mDA neurons, two studies examined the effect of the loss of Reelin signaling on these fibers. Radial glia fibers appear to be normal at E14.5 and E15.5, but are reduced at E16.5 in *reeler* mice (Nishikawa et al., 2003; Kang et al., 2010). Nishikawa et al. (2003) report unaltered tangential fiber formation (potential axonal tracts) in E15.5 *reeler* mutants, while Kang et al. (2010) show that tangential fibers are already reduced at E14.5, at least at posterior midbrain levels. These data indicate that Reelin might regulate migration of SNc-mDA neurons both in a direct manner and indirectly through regulating the normal development of guidance structures for these neurons.

The analysis of *ApoER2* and *VLDLR* single and double knockout mice demonstrates that Reelin signaling in the ventral midbrain is primarily transmitted through these canonical Reelin receptors (Sharaf et al., 2013; Bodea et al., 2014). Sharaf et al. (2013) analyzed *ApoER2* and *VLDLR* single

knockout mutants, as well as *ApoER2/VLDLR* double knockout mice at P25. *ApoER2/VLDLR* double knockout show a severe reduction in the number of mDA neurons in the SNc, while *ApoER2* and *VLDLR* single knockout mice both show a mild reduction of SNc-mDA neurons, indicating that the two receptors cannot fully compensate for each other's function. The total number of mDA neurons remains unchanged in *VLDLR* knockout mice at P25 (the numbers have not been assessed for the *ApoER2* single or *ApoER2/VLDLR* double knockout mice), in agreement with what has been reported for *reeler* and *yotari* mice (Kang et al., 2010). Consistent with the normal number of mDA neurons, cell death is not increased in mDA neurons of postnatal *ApoER2/VLDLR* double knockouts or single receptor mutants. Based on the analysis of *ApoER2/VLDLR* double knockouts at E18 and P15, Sharaf et al. (2013) report the phenotype in these mice as similar to that of the *yotari* mice (Kang et al., 2010; Sharaf et al., 2013). In contrast, the direct comparison of midbrain sections of *reeler*, *Dab1* knockout and *ApoER2/VLDLR* double knockout mice suggests that the disorganization of mDA neurons in the receptor knockout mice is less severe than in *reeler* or *Dab1* mutant mice (Bodea et al., 2014). Thus, additional, non-canonical Reelin receptors might be involved in transducing the signal.

Calbindin and Girk2 (G protein-gated inwardly rectifying potassium channel 2 also known as Kcnj6) label two distinct mDA neuronal subpopulations; Calbindin positive cells are primarily located in the VTA, Girk2 positive cells predominantly in the SNc. Girk2 positive mDA neurons are still located adjacent to the Calbindin-positive VTA-mDA neurons at P25 in *reeler*, *Dab1* and *ApoER2/VLDLR* knockout mice (Bodea et al., 2014; **Figure 1E**). Similarly, Calbindin negative mDA neurons are mostly found lateral to Calbindin positive mDA neurons in the VTA of *VLDLR* single knockout mice (Sharaf et al., 2013). Hence, Reelin signaling does not affect the segregation of SNc and VTA neurons. Together, these data indicate that in the absence of Reelin signaling SNc-mDA neurons fail to migrate out to their normal lateral positions and remain clustered medially, adjacent to the VTA. Despite the disorganization of SNc- and RRF-mDA neurons, nigrostriatal projections show no obvious alterations in *reeler* mutant, *Dab1* knockout or *ApoER2/VLDLR* double knock-out mice (Nishikawa et al., 2003; Sharaf et al., 2013).

Given the abnormal distribution of SNc-mDA neurons in mice in which the Reelin signaling pathway is inactivated, Bodea et al. (2014) studied how Reelin signaling alters the migratory behavior of SNc-mDA neurons. Migrating mDA neurons were monitored with time-lapse imaging in an organotypic slice culture system. Blocking Reelin signaling with a function-blocking antibody for Reelin in these slices results in decreased speed of tangentially migrating neurons, while speed of radially migrating neurons is not affected. In addition, inhibiting Reelin signaling in slices causes a significant deviation of migrating neurons from tangential trajectories as compared to untreated slices. Accordingly, analysis of laterally positioned mDA neurons in E13.5 *reeler* mice show that these mDA neurons fail to orient tangentially and are instead oriented perpendicular to their direction of migration (**Figures 1D–D″**).

Despite these advances, it remains to be elucidated whether Reelin is required directly by migrating mDA neurons or influences mDA neuronal migration by altering guidance scaffolds. While it is clear that Reelin is involved in the later part of tangential migration of SNc-mDA neurons, the mechanism by which the Reelin signal regulates this process, and the downstream factors involved are yet to be unraveled.

VENTRAL HINDBRAIN

The ventral hindbrain consists of a large array of anatomically and functionally distinct neuronal clusters. The embryonic hindbrain is divided into eight rhombomeres (r1–r8) along its rostrocaudal axis. Hindbrain neurons are generated in the ventricular zone and a specialized germinal zone at the dorsal tip of the hindbrain, the rhombic lip. These neurons undergo complex migration events along the dorsoventral and rostrocaudal axis that occur over several days during embryonic development (Ray and Dymecki, 2009; Wanner et al., 2013). Thus, the final location of a particular neuronal cluster along the rostrocaudal or dorsoventral axis of the hindbrain is not necessarily indicative of its origin. As mentioned in the introduction, we focus our discussion on the hindbrain cranial motor neurons since they originate from the ventral progenitor domain in the hindbrain.

Expression Pattern of Reelin and Its Downstream Pathway Components in the Ventral Hindbrain

Reelin expression is observed in the lateroventral hindbrain at E12.5 (Ikeda and Terashima, 1997; Rossel et al., 2005; Allen Institute for Brain Science, 2015). Expression appears in patches, suggesting that Reelin is expressed in specific clusters of cells. These patches are mostly juxtaposed to *Dab1* positive areas, but there also appears to be some overlap of the two expression patterns (analyzed in rhombomere r4–r6; Rossel et al., 2005; **Figures 2A–E**). At E14.5, *Reelin* is widely expressed in the ventrolateral hindbrain, but appears to be excluded from certain cell populations, including the facial motor neurons (Ikeda and Terashima, 1997; Carroll et al., 2001; Allen Institute for Brain Science, 2015). This broad expression pattern appears to be maintained at later embryonic stages (Allen Institute for Brain Science, 2015). *Dab1* expression can be detected at E11.5 (Allen Institute for Brain Science, 2015). Starting at E12.5, it is expressed in several cell clusters in the ventral hindbrain, including the facial branchial motor (FBM) nucleus and the facial visceral motor (FVM) nucleus (Carroll et al., 2001; Rossel et al., 2005; Allen Institute for Brain Science, 2015; **Figures 2A–E**). *ApoER2* is also widely distributed in the developing hindbrain, but it remains unclear whether it is expressed in the neuronal populations (see below) that are altered in the absence of Reelin signaling. The expression onset is after E11.5

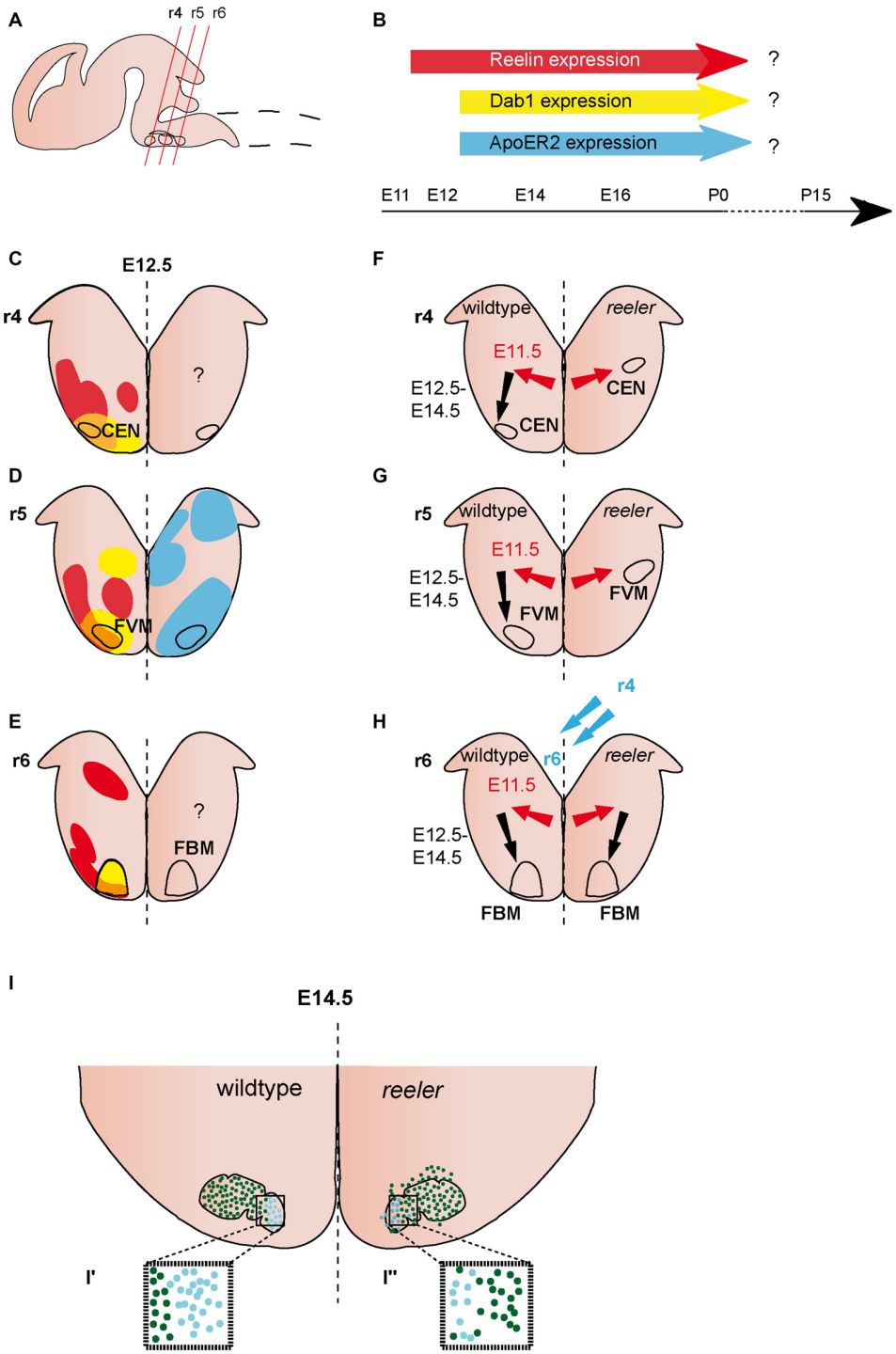

FIGURE 2 | Migration of ventrally derived hindbrain neurons. (A) Sagittal view of embryonic brain. Red lines indicate level of coronal section in **(C–H)**. **(B)** Expression of Reelin, Dab1 and ApoER2 over time. **(C–E)** Expression patterns of Reelin (red), Dab1 (yellow) and ApoER2 (blue) at the indicated time point. Reelin and Dab1 expression are only presented in the left half of the brain, ApoER2 expression is only presented in the right half of the brain. Question mark indicates a lack of expression data. The VLDLR expression pattern is not well characterized in the hindbrain, thus it is not included in the schematics in **(B–E)**. **(F–H)** Schematics of migratory paths of cochlear efferent nucleus (CEN) neurons, facial visceral motor (FVM) neurons and facial branchial motor (FBM) neurons in the wildtype (left half of the brain) and *reeler* mutants (right half of the brain). Red arrows indicate dorsolateral tangential migration, black arrows indicate ventral radial migration, and blue arrows indicate rostrocaudal migration. **(F,G)** Ventral migration of CEN and FVM neurons is truncated in *reeler* mutants. **(H,I)** The majority of FBM neurons reach their final superficial position in r6 of the hindbrain, but the nuclei shows subtle disorganizations. **(I′,I′′)** The medial lobe (Lhx4+) is reduced (turquoise dots), the lateral lobe (Er81+) is disorganized (dark green dots) in *reeler* mutants compared to the wildtype.

(Rossel et al., 2005; Allen Institute for Brain Science, 2015; **Figures 2A–E**). The expression of *VLDLR* has essentially not been assessed in the hindbrain, except for the r5 level, where it is not or only very weakly expressed at E12.5 (Rossel et al., 2005).

Function of Reelin Signaling in the Ventral Hindbrain

Trigeminal Motor Nucleus

The trigeminal motor nucleus (motor nucleus of V) in r2 and r3 extends projections to the muscles of the first branchial arch that control the jaw jerk reflex. Trigeminal branchiomotor neurons are generated in the ventricular zone of ventral r2 and r3 between E9.5 and E10.5. Upon exiting the cell cycle they migrate dorsolaterally to their final position in the trigeminal motor nucleus (Pattyn et al., 2003; Ohsawa et al., 2005). Terashima et al. (1994) used horseradish peroxidase (HRP) injections into jaw-opening or jaw-closing muscles of wildtype and *reeler* mice to retrogradely label the subsets of trigeminal motor neurons that control these different muscles. They demonstrated that the motor neurons that control jaw-opening muscles are more scattered in *reeler* mice compared to wild-type. Whether this defect is caused by aberrant neuronal migration in the absence of Reelin signaling has not been investigated.

Facial Motor Neurons

The facial nucleus (nucleus VII) is comprised of branchial and visceral motor neurons (FBM and FVM neurons) that innervate muscles controlling facial expressions and parasympathetic ganglia, respectively. The FBM neurons are generated in the ventral ventricular zone of r4 and r5, the FVM neurons in the ventral ventricular zone of r5. After leaving the ventricular zone after E10.5, FVM neurons migrate first dorsolaterally within r5 and then towards the pial surface to form the the superior salivatory nucleus. FBM neurons migrate caudally along the ventricular surface to r6 (or r5 in avian species) and then dorsolaterally and radially to form the facial nucleus. Both nuclei are in a superficial position, close to the pial surface (Goffinet, 1984a; Garel et al., 2000; Jacob and Guthrie, 2000; Guthrie, 2007; Wanner et al., 2013).

The organization of the FBM neurons in the facial nucleus is altered in *reeler* mutants (Goffinet, 1984a; Terashima et al., 1993; Rossel et al., 2005). Based on morphological analysis and labeling with the motor neuron marker *Isl1 (Islet-1)*, the initial steps of migration appear not to be affected in *reeler* mutants, since no difference in the morphology, clustering or positioning of the presumptive FBM neurons was observed between wildtype and *reeler* mutants at E11.5 or E12.5 (Goffinet, 1984a; Rossel et al., 2005). By E14.5, when the migration of FBM neurons is largely complete, the large majority of FBM neurons reach their final superficial position in *reeler* mutants, but FBM neurons are more scattered than in wildtype brains (Goffinet, 1984a). At this stage, the facial nucleus is organized into a medial and a lateral lobe. Analysis of markers that label either the lateral lobe, Etv1 (Ets variant gene 1 also known as Er81) or medial lobe, *Lhx4* (Lim homeobox protein 4) demonstrated that the medial lobe is reduced in size while neurons in the lateral lobe seem to be more scattered in *reeler* mutants as compared to wildtype (Rossel et al., 2005; **Figures 2H,I**). By E17.5, the wildtype facial nucleus is divided into several different anatomical subsets in the wildtype brain, this segregation is less obvious in the *reeler* mutants (Goffinet, 1984a). Whether Reelin affects the late migration steps of FBM neurons or other aspects of their maturation has not been assessed (**Figures 2H,I**). Despite the apparent disorganization of the facial motor nucleus in *reeler* mutants, the topographic representation of the muscle targets appears to be preserved in the nucleus as shown by HRP-based retrograde tracing from the muscles innervated by the facial nerve (Terashima et al., 1993).

In contrast to the FBM neurons, the FVM neurons do not reach their final superficial position in the *reeler* mutants. Analysis of the hindbrain motor neuron markers *Isl1*, *Ret* (RET proto-oncogene) and *Phox2B* (paired like homeobox 2b) shows ectopic neuronal clusters in the lateral hindbrain that are either positioned close to the ventricular zone or in an intermediate position (between ventricular zone and pial surface; Rossel et al., 2005; **Figure 2G**). DiI labeling of cell bodies and projections of FVM neurons at E11.5 and E12.5 demonstrates that the FVM neurons are born at the correct time point and reach a lateral position. However, in wildtype embryos, the FVM neurons reach the pial surface by E12.5, while they remain deeper in the hindbrain tissue in the *reeler* mutants (Rossel et al., 2005; **Figure 2G**). These data suggest that FVM neurons undergo normal lateral migration in the absence of Reelin signaling, but are not able to relocate towards the pial surface. Radial glia fibers appear to be normal in the hindbrain of *reeler* mice (Rossel et al., 2005), suggesting that Reelin signaling affects the migration of these neurons directly. Whole-mount analysis of *Isl1* expressing motor neurons in the hindbrain of *scrambler* mutants, which are null mutants for *Dab1*, shows that the *scrambler* phenotype is comparable to the one in *reeler* mutants. In contrast, the *ApoER2/VLDLR* double knockout mice have no apparent phenotype (Rossel et al., 2005). Further analysis will be necessary to assess the cause for this discrepancy. In particular it should be investigated whether the canonical Reelin receptors are expressed in migrating FBM and FVM neurons.

Cochlear Efferent Nucleus

The neurons of the cochlear efferent nucleus (CEN) are generated from the motor neuron progenitor domain in r4. After leaving the progenitor area between E10.5 and E12.5, the differentiated neurons are initially intermingled with the facial brachial motor neurons. Subsequently, the two populations segregate and the neurons of the CEN take a dorsolateral migratory route within r4 where they settle close to the pial surface of the lateral hindbrain by E14.5 (Bruce et al., 1997; Rossel et al., 2005; **Figure 2F**). Analysis of the position of CEN neurons in E12.5 wildtype and *reeler* mutant brains with retrograde labeling, and the markers *GATA3* (Gata binding protein 3) and *Tbx20* (T-box 20) shows that similar to the FVM neurons, CEN

neurons do not reach their final superficial position. Instead, ectopically clustered cells are observed close to the ventricular zone in a lateral position where they should normally initiate their migration towards the pial surface. As described above for the FVM neurons, CEN neurons appear to be unable to reach their final position in *scrambler* mutants, but appear not to be affected in *ApoER2/VLDLR* double mutants (Rossel et al., 2005).

Ambiguus Nucleus

The branchimotor neurons of nucleus IX are clustered in the nucleus ambiguus in r6 of the hindbrain and innervate the esophagus, larynx, pharynx and palate. These neurons are important regulators of swallowing and speech. Little is known about their development in the rodent model, but analysis of human embryonic and fetal brains suggests that their migration path is similar to the one observed for trigeminal or FVM neurons in rodents (Brown, 1990; **Figure 2G**). Retrograde tracing with a lacZ-expressing adenoviral vector has been used to label the branchimotor neurons that project to the esophagus. While these neurons are tightly clustered in a lateral position close to the pial surface in the wildtype hindbrain, they are located deeper within the lateral hindbrain tissue in the *reeler* mutant mice, suggesting that their final migration step towards the pial surface could be altered in the absence of Reelin signaling (Fujimoto et al., 1998). The disorganization of the ambiguous nucleus (and of the trigeminal and facial nucleus) was also described in *shaking rat Kawasaki*, a rat strain that harbors an autosomal recessive mutation in the *Reelin* gene (Setsu et al., 2001; Kikkawa et al., 2003).

In summary, the migration of branchial motor neurons involves an initial lateral migration step followed by a change in direction of migration and radial migration towards the pial surface. The phenotypes observed in the absence of Reelin signaling suggest that Reelin is involved in the latter step of migration and/or in the change in direction of migration. Reelin appears to influence the migration of these neurons directly, since the radial glia scaffold has been described as being normal in the hindbrain of *reeler* mutants.

SPINAL CORD

The spinal cord is divided from rostral to caudal into cervical, thoracic, lumbar, sacral and coccygeal levels (**Figure 3A**). Ventral spinal cord neurons are arranged into columns that are established according to their final projection targets. For example, somatic motor neurons (SMNs) that project to the limbs are organized into the lateral motor column (LMC) at brachial (lower cervical) and lumbar levels of the spinal cord. Preganglionic neurons are clustered into the preganglionic column at thoracic (intermediolateral nucleus) or sacral levels (intermediolateral sacral nucleus). SMNs, V0–V3 interneurons, and preganglionic neurons originate from progenitors in the ventricular zone in the ventral spinal cord. Subsequently, they migrate to distinct dorsoventral positions: SMNs and V3 interneurons are located ventrally, preganglionic neurons are positioned at intermediate dorsoventral levels of the spinal cord and V0–V2 neurons are distributed in positions between SMNs and preganglionic neurons (Lee and Pfaff, 2001; Gaella, 2004; Dalla Torre di Sanguinetto et al., 2008).

Expression Pattern of Reelin and Its Downstream Pathway Components in the Spinal Cord

Reelin expression begins in the cervical region of the murine spinal cord at E9.5, when it appears to be restricted to subsets of differentiated neurons in the ventral and intermediate spinal cord (Ikeda and Terashima, 1997). By E11.5, Reelin mRNA and protein are expressed dorsally and medially to the motor column throughout the spinal cord (Ikeda and Terashima, 1997; Schiffmann et al., 1997; Yip et al., 2004a; Palmesino et al., 2010; Lee and Song, 2013). Between E11.5 and E12.5, Reelin protein is expressed in ventral and intermediate regions of the spinal cord but is excluded from SMNs and preganglionic neurons (Yip et al., 2000, 2004a; Phelps et al., 2002; Palmesino et al., 2010; Lee and Song, 2013; **Figures 3B,C**, **4B,C**). A similar pattern is observed in chick at E4.5 and E6.5 (Palmesino et al., 2010). Since Reelin protein is deposited around the cell bodies of V1 and V2 interneurons at this stage, it has been suggested that they are the source of Reelin (Yip et al., 2004b). Reelin expression is maintained in the ventral and intermediate spinal cord at subsequent stages of embryonic development. Starting between E13.5 and E14.5, Reelin can be also detected in a band of cells in the dorsal superficial horn (Phelps et al., 2002; Kubasak et al., 2004; Villeda et al., 2006) and in the ventricular zone at intermediate dorsoventral levels (Ikeda and Terashima, 1997; Phelps et al., 2002; Hochstim et al., 2008). The onset of expression in the ventricular zone appears to coincide with the onset of gliogenesis (Hochstim et al., 2008). In the prenatal and adult spinal cord, *Reelin* mRNA is expressed in the intermediate gray, the superficial dorsal horn and in a subset of white matter astrocytes in the lateral funiculus (Ikeda and Terashima, 1997; Phelps et al., 2002; Kubasak et al., 2004; Hochstim et al., 2008). The Reelin expression pattern is largely conserved between rat and mouse. In chick spinal cord, Reelin is already expressed by E4 and the Reelin expression pattern is comparable to the patterns observed in rodents (Bernier et al., 2000; Kubasak et al., 2004; Palmesino et al., 2010).

From E10.5 onwards, Dab1 mRNA and protein is expressed in the lateral intermediate and ventral region in the spinal cord, where it colocalizes with different neuronal populations, depending on the rostrocaudal level (Phelps et al., 2002; Yip et al., 2004a; Palmesino et al., 2010; Lee and Song, 2013). At E12.5, Dab1 protein expression in the somatic motor column is weak at upper cervical levels, but strong at brachial, thoracic and lumbar levels (Palmesino et al., 2010; Lee and Song, 2013). Within the LMC, Dab1 is highly expressed in the lateral LMC subpopulation characterized by the expression of Foxp1 (forkhead box protein 1) and Lhx1 (LIM homeobox 1), while Dab1 is weakly expressed in the dorsomedial LMC subpopulation expressing Foxp1 and Isl1 (Palmesino et al., 2010; **Figure 4B**). In the chick, Dab1 also colocalizes with Lhx1-positive

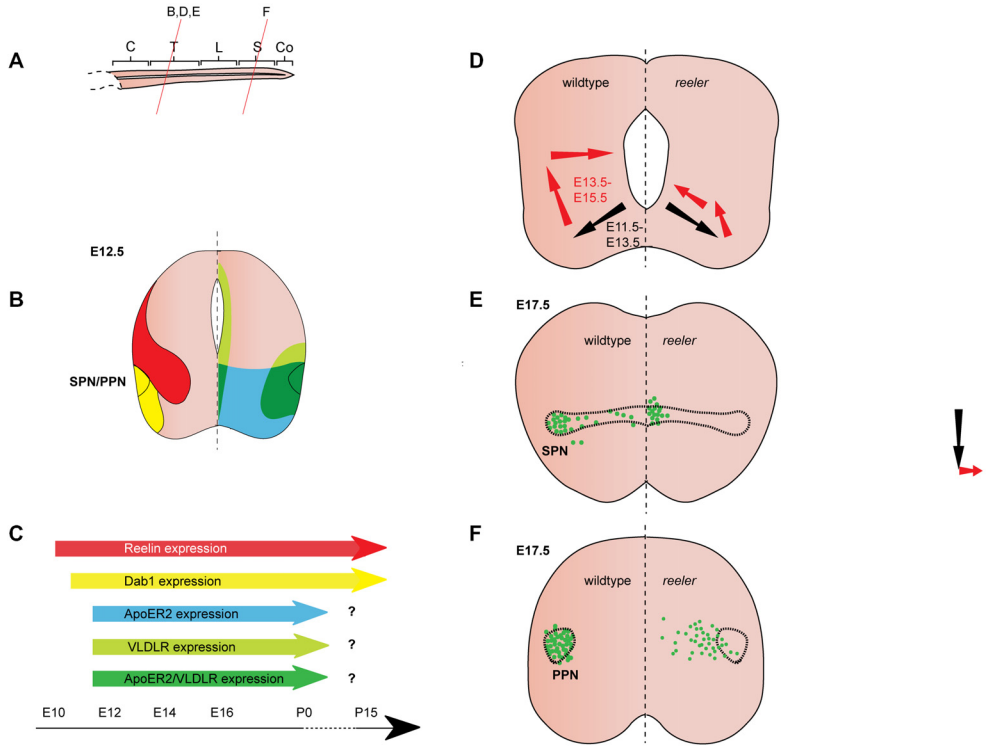

FIGURE 3 | Reelin signaling in the migration of autonomic preganglionic neurons. (A) Sagittal view of embryonic spinal cord (C, cervical; T, thoracic; L, lumbar; S, sacral; Co, coccygeal). Red lines indicate level of coronal section in **(D–F)**. **(B)** Expression patterns of Reelin (red), Dab1 (yellow), ApoER2 (green) and VLDLR (green) at the indicated time point. Reelin and Dab1 expression are only presented in the left half of the brain; ApoER2 and VLDLR expression are only presented in the right half of the brain. **(C)** Expression of Reelin, Dab1, ApoER2 and VLDLR over time. **(D)** Schematic of migratory paths of preganglionic autonomic neurons in wildtype mice (left half of the brain) and *reeler* mutants (right half of the brain). Black arrows indicate radial migration; red arrows indicate tangential migration. Radial migration is unaffected in *reeler* mutants, but in the second step of migration the majority of preganglionic autonomic neurons migrate to an ectopic position in the medial regions of the spinal cord. **(E)** SPN distribution at E17.5 in wildtype (left half of the brain) and *reeler* (right half of the brain) mice. Sympathetic preganglionic neurons (SPNs) are ectopically located close to the central canal in *reeler* mice. **(F)** Parasympathetic preganglionic neuron (PPN) distribution at E17.5 in wildtype mice (left half of the brain) and *reeler* mutants (right half of the brain). PPN are disorganized and distributed along the mediolateral axis of the intermediate spinal cord in *reeler* mutants.

SMNs at E4.5 and E6.5 (Palmesino et al., 2010). Dab1 protein colocalizes with migrating sympathetic preganglionic neurons (SPNs) in the intermediolateral nucleus at thoracic levels and with migrating parasympathetic preganglionic neurons (PPNs) at sacral levels at E12.5 (Phelps et al., 2002; Yip et al., 2004a; **Figure 3B**). SPNs, PPNs and lateral LMC neurons continue to express *Dab1* throughout development and well into adulthood (Abadesco et al., 2014). *ApoER2* is expressed throughout the ventral region of the spinal cord at thoracic levels at E12.5, while *VLDLR* expression is restricted to the lateral intermediate region (Yip et al., 2004a; **Figure 3B**). At the lumbar level, *ApoER2* is expressed in the ventricular zone and in LMC neurons in mouse at E11.5 (Palmesino et al., 2010; **Figure 4B**). In the chick spinal cord, *ApoER2* is only expressed in the ventricular zone at E4.5 and E6. In the mouse, VLDLR protein expression at E11.5 is higher in the lateral LMC neurons than in their medial counterparts. By E12.5, VLDLR is uniformly expressed in the LMC. VLDLR expression is also observed in chick embryos at E4 and E6.5 (Palmesino et al., 2010).

Function of Reelin signaling in the Ventral Spinal Cord

Throughout the spinal cord, Reelin and Dab1 expression appear to be mutually exclusive. Neurons of the LMC, SPNs, and PPNs are Dab1-positive and express either one or both Reelin receptors. This suggests that these Dab1-expressing neurons require Reelin signaling for their correct positioning, while populations expressing Reelin but not Dab1, such as the V1 and V2 interneurons, do not require Reelin signaling for their migration (Phelps et al., 2002; Yip et al., 2004b). In the following sections, we discuss the role of Reelin signaling in the migration of SPNs, PPNs, and neurons in the LMC.

Sympathetic Preganglionic Neurons (SPNs)

SPNs are acetylcholine-releasing neurons located in the intermediolateral column of the thoracic spinal cord. They project to pre- and paravertebral ganglia where they innervate

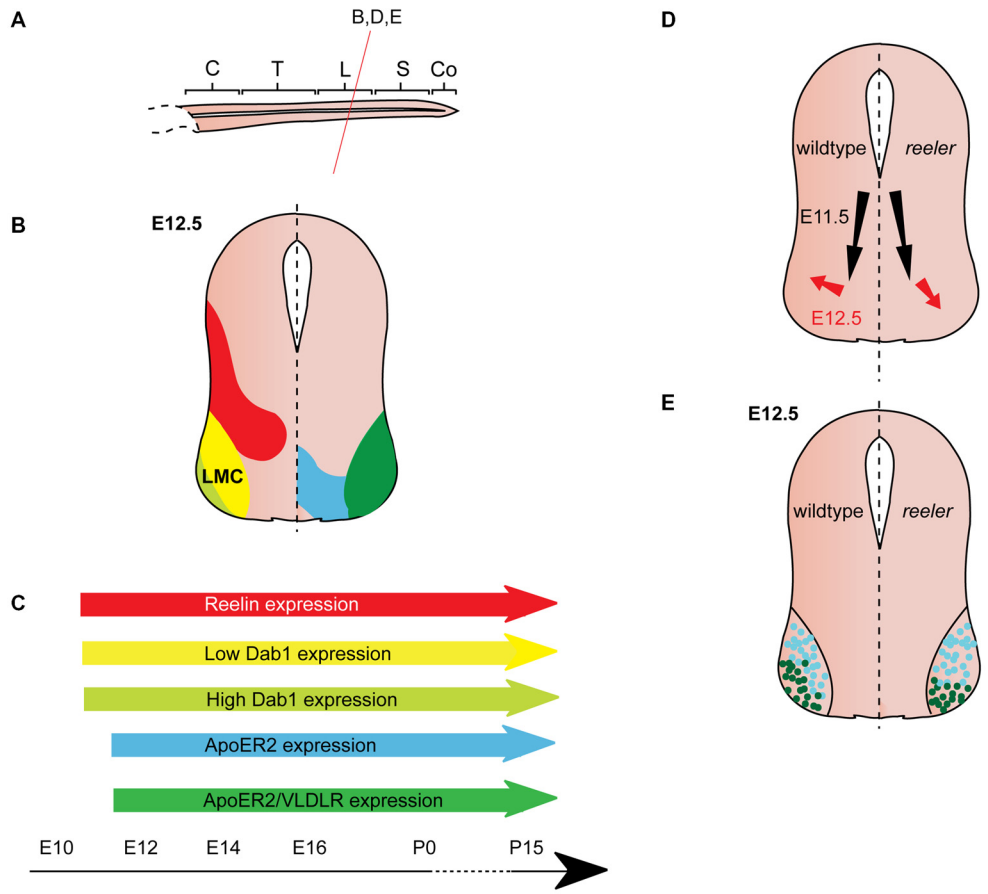

FIGURE 4 | Reelin signaling in motor neuron migration. (A) Sagittal view of embryonic spinal cord (C, cervical; T, thoracic; L, lumbar; S, sacral; Co, coccygeal). Red line indicates level of coronal section in **(D,E)**. **(B)** Expression patterns of Reelin (red), Dab1 (low levels: yellow, high levels: light green), ApoER2 (blue) and ApoER2/VLDLR (green) at the indicated time point. Low-level Dab 1 expression is restricted to Foxp1+, Isl1+ neurons in the medial part of the lateral motor column (LMC), high-level Dab1 expression is found in Foxp1+, Lhx1+ neurons in the lateral LMC. Reelin and Dab1 expression are only presented in the left half of the brain; ApoER2 and VLDLR expression are only presented in the right half of the brain. **(C)** Expression of Reelin, Dab1, ApoER2 and VLDLR over time. **(D)** Schematic of migratory paths of somatic motor neurons (SMNs) in wildtype mice (left half of the brain) and *reeler* mutants (right half of the brain). Black arrows indicate ventral radial migration of SMNs from the neuroepithelium to the LMC; red arrows indicate a short tangential migratory step, which enables a subset of SMNs to migrate dorsolaterally to form the lateral LMC. Radial migration is unaffected in *reeler* mice, but the dorsolateral migration is altered. **(E)**. As compared to their wildtype counterparts, lateral LMC neurons (Foxp1+, Lhx1+; dark green dots) settle in more ventromedial positions in the *reeler* spinal cord. Turquoise dots represent medial LMC neurons (Foxp1+, Isl1+).

postganglionic autonomic motor neurons, which in turn innervate visceral organs and neuroendocrine systems (Yip et al., 2000; Gaella, 2004). Beginning at E11.5 in the mouse, SPNs migrate radially from the ventral neuroepithelium towards the ventrolateral regions of the forming mantle layer. Together with SMNs they establish a rudimentary motor column. In the second step of their migration, SPNs separate from the SMNs by migrating dorsally in the lateral spinal cord. Once they reach the intermediate dorsoventral levels of the spinal cord they form the intermediolateral column. A small subset of SPNs undergoes further migration towards medial regions and settles close to the central canal. By E15.5, SPN migration is essentially complete (Phelps et al., 1991, 2002; Yip et al., 2000, 2003; **Figure 3D**). In *reeler* mice, a large number of SPNs are localized close to the central canal, while very few neurons are observed in the intermediolateral column (Yip et al., 2000; Phelps et al., 2002; **Figure 3E**). In addition, some SPNs are located outside of the spinal cord in *reeler* mice (Yip et al., 2003). *Dab1* knockout and *ApoER2/VLDLR* double knockout mice have a *reeler* like phenotype. *VLDLR* or *ApoER2* single knockout mice have no apparent phenotype, suggesting that the receptors can compensate for each other's function (Yip et al., 2004b). Monitoring the position of migrating SPNs at half-day intervals between E11.5 and E15.5 in wildtype and *reeler* mice, Yip et al. (2003) showed that the initial radial migration takes place normally in the mutant mice. At E12.5, when SPNs initiate their dorsolateral migration, SPNs in the wildtype orient themselves parallel to the dorsoventral axis, while SPNs in *reeler* mutants show a medial or a dorsomedial orientation. Consistent with this abnormal orientation, SPNs in E13 *reeler* mutant mice

migrate in a dorsomedial direction (parallel to radial glia fibers) towards the central canal, instead of migrating dorsolaterally to form the intermediolateral column (Yip et al., 2003; **Figure 3D**).

In the chick, the final position and migration of SPNs differ from the one described in mouse: after moving to the ventrolateral spinal cord, SPNs migrate close to the ventral midline to reach a more dorsal position. Once they arrive at the intermediate spinal cord they settle in the column of Terni next to the central canal. During this dorsally directed migration, the neurons appear to avoid Reelin rich areas in the lateral spinal cord. Misexpression of Reelin in the migratory path of dorsally-migrating SPNs leads to the stalling of these neurons ventral to the Reelin expressing cells (Yip et al., 2011). These data suggest that Reelin acts as a repellent for dorsally migrating SPN neurons in the chick.

There is also evidence for Reelin as a repellent in murine SPNs. To test whether ectopic expression of Reelin can rescue the defective SPN migration in *reeler* mutants, Yip et al. (2009) analyzed *reeler* mutants, in which Reelin is misexpressed under the control of the *Nestin* promoter (referred to as *reeler-NeReelin* mice; Magdaleno et al., 2002; Yip et al., 2009). In these mice, Reelin is strongly expressed in the neuroepithelium at the midline of the spinal cord starting at E9.5, but it is still absent from the areas where Reelin is normally expressed in the wildtype. The ectopic expression of Reelin does not affect the initial migration of SPNs to the ventrolateral spinal cord in the *reeler-NeReelin* mice, but SPNs still undergo abnormal dorsally-directed migration. This is evident in a lateral-to-medial shift in the location of SPNs. The SPNs in the *reeler-NeReelin* mice are distributed between their normal lateral position and the central canal up to E16.5. After E18.5 neurogenesis ceases in the spinal cord and with the disappearing neuroepithelium the midline expression of Reelin vanishes. SPNs in E18.5 and postnatal *reeler-NeReelin* mice are localized in two clusters, one in the intermediolateral column and one close to the central canal. Thus, the phenotype in the *reeler-NeReelin* mice is milder than in the *reeler* mice (Yip et al., 2009; **Figure 3E**), suggesting that the ectopic expression of Reelin in the neuroepithelium results in a partial rescue of the *reeler* phenotype. The fact that a subset of SPNs only accumulates close to the central canal after Reelin expression ceases in the midline, has been interpreted as an indication for a repellent function of Reelin in the migration of SPNs. A repellent function of Reelin would be consistent with the observation that ventrolaterally located SPNs are separated from V1 and V2 interneuron clusters (a likely source of Reelin protein) in E12.5 wildtype mice, while SPNs and interneurons are intermingled in *reeler* mice (Yip et al., 2004b). In addition, several other hypotheses have been posited regarding the function of Reelin in migration of murine SPNs. Reelin signaling might sensitize migrating SPNs to attractive cues in the intermediolateral column (Yip et al., 2003). Alternatively, Reelin signaling could affect SPN migration by facilitating the correct orientation of the neuronal cell bodies, since secreted Reelin can be detected laterally and directly adjacent to migrating SPNs (Kubasak et al., 2004). Such a role for Reelin signaling would be consistent with one of the known functions of Reelin in cortical migration (Jossin and Cooper, 2011). Radial glia fibers are not grossly altered in the spinal cord of *reeler* mice (Yip et al., 2003; Lee and Song, 2013) but Reelin might act by modulating the attachment of SPNs to radial glia fibers (Kubasak et al., 2004).

As in the cerebral cortex, Reelin signaling in the spinal cord requires the phosphorylation of Dab1 via Src/Fyn tyrosine kinases (Howell et al., 1997; Hiesberger et al., 1999; Arnaud et al., 2003; Yip et al., 2007). A *reeler*-like phenotype has been reported in the SPNs of *Src*/*Fyn* double knockout mice and in mice that are homozygous for a null allele of *Dab1* ($Dab1^{lacZ/lacZ}$) or that express a Dab1 protein in which the tyrosine phosphorylation sites are abolished ($Dab1^{5F/5F}$ mice; Howell et al., 2000; Yip et al., 2007; Abadesco et al., 2014). Src and Fyn might be able to compensate for each other's function, as the single mutants do not fully mimic the *reeler* phenotype (Yip et al., 2007). Phosphorylated Dab1 can recruit the adaptor molecules Crk, CrkL and CrkII and this interaction has been shown to regulate cell adhesion properties of migrating cortical neurons via the Crk/CrkL-C3G-Rap1 pathway (Ballif et al., 2004; Franco et al., 2011). To investigate whether this pathway acts downstream of Dab1 in the spinal cord, Yip et al. (2007) analyzed the development of SPNs in *Crkl* and *C3G* mutants. In *CrkL* knockout mice, some SPNs were still located in the intermediolateral column, but the majority of SPNs were clustered close to the central canal or were interspersed between the central canal and the intermediolateral column. The phenotype in *CrkL* knockout mutant mice appears to be milder than the one observed in *reeler* mutants (**Figure 3E**), thus Crk might be able to compensate for the loss of CrkL. In mice homozygous for a hypomorphic allele of C3G ($C3G^{gt/gt}$) SPNs are scattered between the intermediolateral column and the central canal. This phenotype is less severe than in the *reeler* mutants, suggesting that there are other downstream pathways or that a small amount of C3G protein in the hypomorphs is sufficient for residual downstream signaling (Yip et al., 2012).

The actin binding protein Cofilin1, which is an actin depolymerizing protein, may be another factor acting downstream of Reelin. Activation of Reelin signaling results in the LIM kinase-dependent phosphorylation of Cofilin1 (p-Cofilin1) and the attenuation of the actin-depolymerizing activity of Cofilin1. Thus, the phosphorylation of Cofilin1 contributes to the stabilization of the actin cytoskeleton (Chai et al., 2009). p-Cofilin1 is strongly expressed in the intermediolateral column of wild-type mice at E13.5 (Krüger et al., 2010). In contrast, almost no p-Cofilin1 expression is detected in the SPNs of *reeler* or *Dab1* knockout mice at this time point. Low-level p-Cofilin1 labeling is observed in *ApoER2* knockout mice while *VLDLR* knockout mice show weak but more distributed p-Cofilin1 expression compared to the wildtype. Moreover, the level of p-Cofilin1 increases when spinal cord tissue of *reeler* mutant mice is treated with recombinant Reelin protein (Krüger et al., 2010). This evidence suggests that Reelin plays a role in the stabilization of the cytoskeleton in migrating SPNs via p-Cofilin1, possibly causing their migratory arrest.

Parasympathetic Preganglionic Neurons

Parasympathetic preganglionic neurons (PPNs) at the sacral levels of the spinal cord are located in the intermediolateral sacral nucleus. These neurons are cholinergic and project over long distances to synapse on their postganglionic targets, which in turn innervate non-voluntary muscles and neuroendocrine glands. PPNs migrate in a similar fashion to SPNs: first radially towards a ventrolateral position and then tangentially (dorsally) to form the intermediolateral sacral nucleus. PPNs are laterally clustered, but unlike the SPNs, are completely absent from medial regions (Phelps et al., 2002). In *reeler or Dab1*$^{lacZ/lacZ}$ mice, PPNs are disorganized and dispersed along the mediolateral axis of the intermediate region of the sacral spinal cord (**Figure 3F**). Expression patterns of Reelin and Dab1 are similar at thoracic and sacral levels (**Figure 3B**) and most of the mechanisms proposed for SPN migration have been extended to PPNs (Phelps et al., 2002; Kubasak et al., 2004; Abadesco et al., 2014).

Somatic Motor Neurons

SMNs are located in the motor column of the ventrolateral spinal cord and are found at all rostrocaudal levels of the spinal cord. Their projections exit through the ventral root of the spinal cord, innervate skeletal muscle, and regulate voluntary movement. SMNs are born in the ventral neuroepithelium from where they migrate along radial glia fibers towards the ventrolateral mantle layer along with preganglionic neurons (Phelps et al., 1991). After their radial migration has ended, they maintain more or less the same position, and undergo only a small tangential migration step to arrange themselves in their final position (Palmesino et al., 2010).

SMN migration was generally believed to be normal in the absence of Reelin signaling (Phelps et al., 2002) until a study reported that Reelin is required for correct positioning of LMC neurons at lumbar-sacral levels, where SPNs and PPNs are absent (Palmesino et al., 2010; **Figure 4A**). Delineation of a medial subpopulation (Foxp1-positive, Isl1-positive, low-level Dab1 expression) from a lateral subpopulation (Foxp1-positive, Lhx1-positive, high-level Dab1 expression) reveals subtle defects in the positioning of these LMC neuronal subsets in *Dab1* knockout mice and *reeler* mice (Palmesino et al., 2010; **Figures 4B,C**). The initial radial migration of LMC neurons is normal in the absence of Reelin signaling. However, the tangential migration of these neurons is altered: the high-level Dab1-expressing, lateral LMC subpopulation is shifted to a more medioventral position in the mutants (**Figures 4D,E**). These findings are further corroborated by the analysis of *lacZ*-expressing SMNs (corresponding to the Dab1-expressing lateral LMC neurons) at lumbar levels of the adult spinal cord of *Dab1*$^{LacZ/LacZ}$ mice showing that the position of lateral LMC neurons is shifted medially and ventrally (Abadesco et al., 2014). Despite the disorganization of the LMC in the absence of *Dab1*, projections of medial and lateral LMC follow their normal trajectories in the ventral and dorsal limb nerve, respectively (Palmesino et al., 2010). In contrast, dendrites of lateral LMC neurons that project towards the lateral funiculus appear to be reduced and disorganized (Abadesco et al., 2014).

When Dab1 is misexpressed in LMC neurons in chick lumbar spinal cord at E6, the lateral subpopulation, which already expresses a high level of Dab1, is unaltered in position, but the medial subpopulation, which normally expresses low levels of Dab1, is shifted to a more lateral position (Palmesino et al., 2010). These data suggest that Reelin signaling promotes LMC neuronal migration directly, or enables LMC neurons to sense cues required for their lateral migration. As the medial subpopulation of LMC neurons expresses low levels of Dab1, the authors hypothesize that Dab1 is quickly degraded after phosphorylation downstream of Reelin signaling and hence results in their migratory arrest. Lateral LMC neurons migrate more laterally due to their high level of Dab1 expression.

How are the different expression levels of Dab1 in the two LMC subpopulations regulated? Analysis of transgenic and knockout mice showed that Foxp1 positively controls the expression of Dab1 in motor neurons: misexpression of Foxp1 in all motor neurons leads to abnormally high expression of Dab1 in motor neurons at upper cervical, where Foxp2 and Dab1 are normally absent or expressed at very low levels (Palmesino et al., 2010). Concordantly, in *FoxP1* knockout mice, Dab1 expression is reduced in motor neurons at lower cervical levels, where Foxp2 and Dab1 are expressed at high levels in the wildtype. Despite these results, it is unlikely that Foxp1 determines the different levels of Dab1 expression in medial and lateral LMC neurons, since it is expressed in both populations. Isl1 and Lhx1 determine the fate of medial and lateral LMC neurons, respectively, and are thus more likely regulators of Dab1 expression levels in the two LMC subpopulations. Indeed, Isl1 misexpression downregulates Dab1 expression, while Lhx1 misexpression upregulates Dab1 expression. Consistent with these results, conditional inactivation of *Lhx1* in the LMC neurons leads to decreased Dab1 levels in lateral LMC and a medial clustering of these neurons (Palmesino et al., 2010).

Finally, at brachial and thoracic levels of the spinal cord in *reeler* mutants, ectopic Foxp1-positive, Isl1-positive LMC neurons and medial motor column neurons are found outside the spinal cord (Lee and Song, 2013). Since Reelin is localized to radial glia end feet, the authors propose that Reelin might inhibit motor neurons from migrating out of the spinal cord, while allowing axonal efferents to exit the spinal cord and reach target areas. Hence Reelin may be involved in regulating two independent aspects of motor neuron migration: positioning lateral LMC neurons and hindering motor neurons from migrating out of the spinal cord.

CONCLUSION

In conclusion, the common role of Reelin in the migration of neurons in the ventral brain stem and spinal cord seems to be restricted to the late or final step of neuronal migration. Interestingly, Reelin regulates both tangential (midbrain and spinal cord) and radial migration (hindbrain). How Reelin influences the behavior of these migrating neurons is still not completely understood and several possible roles

have been suggested: Reelin might act as an attractant, repellent, or permissive factor. Since Reelin appears to influence only the second or third step of migration and leads to changes in tangential as well as radial migration routes, it is tempting to speculate that the main function of Reelin might be in changing the orientation of migrating neurons at the time when they have to alter the direction of their migratory route. This change in orientation could possibly be mediated by a change in cell adhesion properties of migrating neurons through the Crk/CrkL-C3G-Rap1 pathway, or by the stabilization of the cytoskeleton through the Lim Kinase-Cofilin1 pathway. Reelin signaling affects the final neuronal positioning in these nuclei but does not appear to affect their fate or their projections to target areas. Whether the aberrant position of these neurons alters their afferent connections or their functional output has not been examined.

Further studies will be necessary to elaborate the precise function of Reelin signaling in the migration of neurons in the ventral brain stem and spinal cord and to uncover whether additional ventral structures, including neuronal clusters in the forebrain, are affected in the absence of Reelin signaling. Moreover, at least some of the brainstem and spinal cord nuclei that respond to Reelin during development maintain expression of Dab1 and the Reelin receptors into adulthood, raising the question whether Reelin signaling is important for the maturation, maintenance, or function of these neurons.

AUTHOR CONTRIBUTIONS

ARV and SB contributed to the overall concept and the writing of the review.

ACKNOWLEDGMENT

This work was supported by the Maria von Linden-Program and BONFOR (both University of Bonn) and the Deutsche Forschungsgemeinschaft (BL 767/2-1, BL 767/3-1). ARV is a recipient of a German Academic Exchange Service doctoral fellowship.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 12 January 2017
doi: 10.3389/fncel.2016.00308

Reelin-Haploinsufficiency Disrupts the Developmental Trajectory of the E/I Balance in the Prefrontal Cortex

*Lamine Bouamrane, Andrew F. Scheyer, Olivier Lassalle, Jillian Iafrati, Aurore Thomazeau and Pascale Chavis**

INMED, Aix-Marseille University, INSERM, Marseille, France

The reelin gene is a strong candidate in the etiology of several psychiatric disorders such as schizophrenia, major depression, bipolar disorders, and autism spectrum disorders. Most of these diseases are accompanied by cognitive and executive-function deficits associated with prefrontal dysfunctions. Mammalian prefrontal cortex (PFC) development is characterized by a protracted postnatal maturation constituting a period of enhanced vulnerability to psychiatric insults. The identification of the molecular components underlying this prolonged postnatal development is necessary to understand the synaptic properties of defective circuits participating in these psychiatric disorders. We have recently shown that reelin plays a key role in the maturation of glutamatergic functions in the postnatal PFC, but no data are available regarding the GABAergic circuits. Here, we undertook a cross-sectional analysis of GABAergic function in deep layer pyramidal neurons of the medial PFC of wild-type and haploinsufficient heterozygous reeler mice. Using electrophysiological approaches, we showed that decreased reelin levels impair the maturation of GABAergic synaptic transmission without affecting the inhibitory nature of GABA. This phenotype consequently impacted the developmental sequence of the synaptic excitation/inhibition (E/I) balance. These data indicate that reelin is necessary for the correct maturation and refinement of GABAergic synaptic circuits in the postnatal PFC and therefore provide a mechanism for altered E/I balance of prefrontal circuits associated with psychiatric disorders.

Keywords: prefrontal cortex, GABA, reelin, postnatal maturation, synaptic transmission, E/I balance

OPEN ACCESS

Edited by:
Adalberto Merighi, University of Turin, Italy

Reviewed by:
Alberto Granato, Università Cattolica del Sacro Cuore, Italy
Maria Concetta Geloso, Università Cattolica del Sacro Cuore, Italy

****Correspondence:***
Pascale Chavis
pascale.chavis@inserm.fr

Received: *24 November 2016*
Accepted: *26 December 2016*
Published: *12 January 2017*

Citation:
Bouamrane L, Scheyer AF, Lassalle O, Iafrati J, Thomazeau A and Chavis P (2017) Reelin-Haploinsufficiency Disrupts the Developmental Trajectory of the E/I Balance in the Prefrontal Cortex. Front. Cell. Neurosci. 10:308. doi: 10.3389/fncel.2016.00308

INTRODUCTION

Reelin is a signaling glycoprotein (Bock and May, 2016) serving multiple functions in the brain throughout life which has also emerged as a psychiatric risk factor in a wide spectrum of psychiatric disorders (Folsom and Fatemi, 2013). Secreted by Cajal-Retzius cells in the marginal zone of the cerebral cortex and hippocampus and by pioneer granule cells of the cerebellum during embryonic development, reelin plays an essential role in neuronal migration, positioning and layer formation (Sekine et al., 2014). In addition to being a developmental molecule, reelin is an important contributor to postnatal and adult central nervous system (CNS) physiology. In postnatal forebrain, once migration and layering are completed, reelin production is shifted to subpopulations of GABAergic interneurons distributed throughout cellular layers of the hippocampus and neocortex

(Alcantara et al., 1998; Pesold et al., 1998; Campo et al., 2009). In the maturing and adult CNS, reelin modulates several aspects of excitatory synaptic function and morpho-functional plasticity. Reelin plays an important role in dendritic maturation and spine development (Niu et al., 2008; Chameau et al., 2009), hippocampal long-term potentiation, synaptic transmission, and cognitive ability (Weeber et al., 2002; Beffert et al., 2005; Pujadas et al., 2010; Rogers et al., 2011). Additionally, previous data from our laboratory have shown that reelin is necessary for the maturation of NMDA receptors (Sinagra et al., 2005; Groc et al., 2007; Campo et al., 2009). More recently, we have shown that spine density, excitatory synaptic transmission and plasticity of prefrontal pyramidal neurons as well as cognitive traits are altered during the postnatal maturation of the reelin-haploinsufficient heterozygous reeler mice (HRM) prefrontal cortex (PFC) (Iafrati et al., 2014; Iafrati et al., 2016). We showed that reelin is necessary for the correct structural and functional maturation of deep layer excitatory synapses of the prelimbic area of the PFC (PrPFC) and that reelin happloinsufficiency delineates prefrontal endophenotypes thus identifying reelin as a risk gene for PFC maturational cognitive deficits (Iafrati et al., 2014; Iafrati et al., 2016). Despite these advancements, and apart from studies reporting alterations in GABAergic markers and reduced number of purkinje cells in HRM (Hadj-Sahraoui et al., 1996; Biamonte et al., 2009; Nullmeier et al., 2011) as well as the correlation between firing properties and neurochemical identity of reelin-expressing interneurons (Pohlkamp et al., 2014), the role of reelin in the maturation and plasticity of GABAergic connectivity has not been investigated.

A significant contribution of reelin to the etiology of psychiatric and neurodevelopmental disorders has been proposed based on evidences of the pleiotropic roles of reelin in adult and developing brain together with patients' data showing alteration in reelin levels (Folsom and Fatemi, 2013). Patients suffering from psychiatric disorders such as schizophrenia, bipolar disorder, major depression, and autism spectrum disorders (ASDs) exhibit an approximate reelin downregulation of 50% in several brain structures, most notably the hippocampus and PFC (Impagnatiello et al., 1998; Guidotti et al., 2000; Folsom and Fatemi, 2013). In schizophrenic patients, reduced reelin levels were accompanied by the decrease of other interneurons markers (Impagnatiello et al., 1998; Guidotti et al., 2000; Fatemi et al., 2005). As such, the identification of the mechanisms by which reelin contributes to GABAergic circuit dysfunctions in these diseases is of considerable interest.

The PFC is an associative brain region that supports complex cognitive functions. In rodents, the prelimbic area is one of the regions of the medial PFC which likely mediates cognitive functions similarly to the primate dorsolateral PFC (Kesner and Churchwell, 2011). One distinctive feature of the PFC is its protracted maturation through early adulthood (Gogtay et al., 2004) characterized by connectivity refinement in parallel to maturation of cognitive abilities (van Eden et al., 1990; Luna et al., 2001). This extended period of maturation constitutes a sensitive period of increased vulnerability to injuries leading to development of neurophychiatric disorders (Lewis, 1997; McEwen and Morrison, 2013; Iafrati et al., 2016; Labouesse et al., 2016). Several studies suggest that alterations of postnatal PFC maturation may contribute to the development of psychiatric diseases including depression, addiction, ASD and schizophrenia (Lewis, 1997; Raedler et al., 1998; Iafrati et al., 2016). Neuronal deficits associated to these disorders could include reduced elaboration of inhibitory connectivity leading to altered excitation–inhibition (E/I) balance in the PFC (Insel, 2010). Indeed, a general reduction of the GABAergic system has been described in the PFC of schizophrenic (Volk et al., 2000; Guidotti et al., 2005; Torrey et al., 2005; Gonzalez-Burgos and Lewis, 2008) autistic (Fatemi et al., 2002; Fatemi et al., 2010; Oblak et al., 2011; Fatemi et al., 2014) and depressive patients (Sanacora et al., 1999, 2004; Hasler et al., 2007; Bhagwagar et al., 2008; Karolewicz et al., 2010). Down-regulation of the GABAergic system is also reported in several animal models of psychiatric diseases (Beninger et al., 2010; Cellot and Cherubini, 2014). However, it is not known whether prefrontal GABAergic function and connectivity is affected by reelin haploinsufficiency.

In the present study, we analyzed the polarity of GABAergic signaling, maturation of GABAergic synaptic transmission and the E/I balance in deep layer PrPFC pyramidal neurons of wild-type mice and HRM throughout the first 3 months of postnatal life. We provide evidence that reelin is crucial for correct maturation of GABAergic synaptic functions and E/I balance in the postnatal PFC.

MATERIALS AND METHODS

Animals

The HRM (B6C3Fe a/a-Relnrl/J strain) breeding pairs were purchased from Jackson Laboratory. Offsprings were genotyped by PCR as previously described (Iafrati et al., 2014). Males and females were used in electrophysiological and morphological studies and no significant sex-dependent differences were observed. All mice were weaned at 21 days and then caged socially in same-sex groups. Mice were housed in standard 12 h light–dark cycle and supplied food pellets and water *ad libitum*. Animals were treated in strict compliance with the criteria of the European Communities Council Directive (agreement number 2015121715284829-V4).

Electrophysiology

Coronal slices containing the prelimbic area of the medial prefrontal cortex (PrPFC) were prepared as previously described (Lafourcade et al., 2007). Briefly, mice were anesthetized with isoflurane and 300 μm-thick coronal slices were prepared in a sucrose-based solutiuon at 4°C using an Integraslice vibratome (Campden Instruments). Slices were stored for 30 min at 32°C in artificial cerebrospinal fluid (ACSF) containing 130 mM NaCl, 2.5 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 23 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 11 mM glucose, equilibrated with 95% O_2/5% CO_2. Slices were then stored at room temperature until recording. All experiments were conducted at 30–32°C in ACSF. Whole-cell and cell-attached patch-clamp recordings were made in PrPFC layer 5/6, collected using an Axopatch-1D amplifier (Axon Instruments) and acquired

with Clampex 10.2 acquisition Software via a Digidata 1440A (Axon Instruments). Pyramidal neurons in PrPFC layer 5/6 were visualized using an infrared illuminated upright microscope (Olympus BX51WI).

Spontaneous Spiking Activity

Spontaneous spiking activity was recorded in cell-attached configuration with a patch pipette filled with ACSF. A gigaOhm seal was obtained in current clamp configuration before recording spike-activity in $I = 0$ mode. Data were filtered at 2 kHz and digitized at 10 kHz. Spontaneous spike activity was analyzed in Clampfit 10.5 (Molecular Devices) threshold detection with a trigger threshold of >2x SD of baseline noise. Mean spike activity was calculated as an average of spikes per minute over a 10-min baseline period. For drug-effects, mean spike activity was calculated as an average of spikes per minute over a 10-min period following at least 5 min of bath perfusion.

Spontaneous and Evoked GABA-IPSCs, Spontaneous AMPA-EPSCs, and Intrinsic Properties

To record $GABA_A$-mediated PSCs, NBQX (10 μM) and DL-APV (100 μM) were added to the ACSF to block glutamatergic synaptic transmission. Spontaneous $GABA_A$ receptor-mediated inhibitory post-synaptic currents sIPSCs and evoked IPSCs (eIPSCs) were recorded at −70 mV using the following intracellular solution which contained (mM): Cesium-Cl (125), KCl (20), EGTA (1), HEPES (10), Na_2ATP (2), NaGTP (0.3), and cAMP (0.2) (pH 7.3 and 290 mOsm). In these conditions, chloride reversal potential was around 0 mV. To record spontaneous AMPA-EPSCs (AMPA-sEPSCs) picrotoxin (100 μM; Sigma) was added to the ACSF to block $GABA_A$ synaptic transmission. AMPA-sEPSCs were recorded at −70 mV using internal solution containing (mM): K-Gluconate (145), NaCl (5), $MgCl_2$ (1), EGTA (1), $CaCl_2$ (0.3), Hepes (10), Na_2ATP (2), NaGTP (0.3), and 0.2 cAMP (0.2) (pH 7.3 and 290 mOsm) (Iafrati et al., 2014). To perform current-voltage curves and test neuronal excitability a series of hyperpolarizing and depolarizing current steps were applied immediately after breaking in the cell.

Whole-cell recording electrodes had resistances of 4–6 MOhms. Access resistance was continuously monitored (<25 MOhms) and recordings were rejected if there was a >20% change during the course of the experiment. Spontaneous and evoked currents were filtered at 2 kHz and digitized at 10 kHz.

Paired-pulse ratio (PPR) was measured from IPSCs evoked by a stimulating glass electrode filled with ACSF placed in layer 2/3. Time intervals between stimulations were 30, 50, 100, 150, 200, 300, and 400 ms.

Spontaneous post-synaptic currents amplitude and inter-interval time were detected and analyzed with Axograph X using a double exponential template: $f(t) = \exp(-t/\text{rise}) + \exp(-t/\text{decay})$. For GABA-sIPSCs, rise = 0.2 ms and decay = 10 ms, and for AMPA-sEPSCs, rise = 0.5 ms and decay = 3 ms. The threshold of amplitude detection was set at 7 pA.

Total charge was calculated by summing the charge transfer of all individual events (sEPSCs or sIPSCs) detected over a 6 min acquisition period for each neuron.

Statistical Analysis

All values are given as mean ± SEM and statistical significance was set at $P < 0.05$. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Two sample comparisons were made with the non-parametric Mann–Whitney test and multiple comparisons were made using a one-way analysis of variance (ANOVA) followed, if significant, by Tukey's test.

RESULTS

In order to evaluate the effects of an extracellular neuronal environment impoverished in reelin on the polarity of GABA action, maturation of GABAergic synaptic transmission and the E/I balance of the PrPFC, we compared wild-type and reelin-haploinsufficient HRM during the first 3 months of postnatal life: pre-weaning (Pw, P14–20), juvenile (Juv, P22–28), adolescent (Ado, P30–45), and adulthood (Adu, P50–90). These developmental epochs match our recent report studying the effect of reelin-haploinsufficiency on the maturation of deep layer PrPFC excitatory synapses (Iafrati et al., 2016). Here, we also focused on layer 5/6 pyramidal neurons, one of the main output cells of the PrPFC microcircuit. Layer 5/6 pyramidal neurons were identified as previously described (Thomazeau et al., 2014; Iafrati et al., 2016; Martin et al., 2016) based on their morphology and/or their intrinsic properties (Supplementary Figure S1).

Polarity of GABA Action Is Not Affected by Reelin Haploinsufficiency during the P14–20 Pre-weaning Period

One of the first events during postnatal brain maturation is the switch in GABA polarity from depolarization/excitation in immature neurons to hyperpolarization/inhibition in adult neurons (reviewed in (Ben-Ari et al., 2012). Alteration in the timing of this polarity switch has been consensually reported under pathological conditions and in mouse models of ASD and intellectual disability (He et al., 2014; Tyzio et al., 2014; Deidda et al., 2015). Although GABA is depolarizing in immature cortical neurons, it has also been reported to inhibit network activity of the neonatal cortex *in vivo* (Kirmse et al., 2015). In light of these findings, we felt that a prerequisite to studying the maturation of GABAergic synaptic transmission was to examine the polarity of GABA signaling before weaning between P14 and P18 and whether it was altered at this maturational stage in pathological conditions, e.g., in HRM (**Figure 1**).

We tested the direction of GABAergic actions by observing the impact of the $GABA_A$ receptor antagonist picrotoxin (PTX) and the $GABA_A$ receptor positive-allosteric modulator isoguvacine on cell-attached recorded spontaneous spiking activity of layer 5/6 pyramidal neurons (Khazipov et al., 2004). First, we examined the average baseline spontaneous spiking frequency which was found to be similar in P14–18 HRM and wild-type littermates ($P = 0.9101$, Mann–Whitney U-test; **Figure 1A**), suggesting that reelin haploinsufficiency does not affect basal network activity. In P14–18 wild-type mice, application of PTX increased the

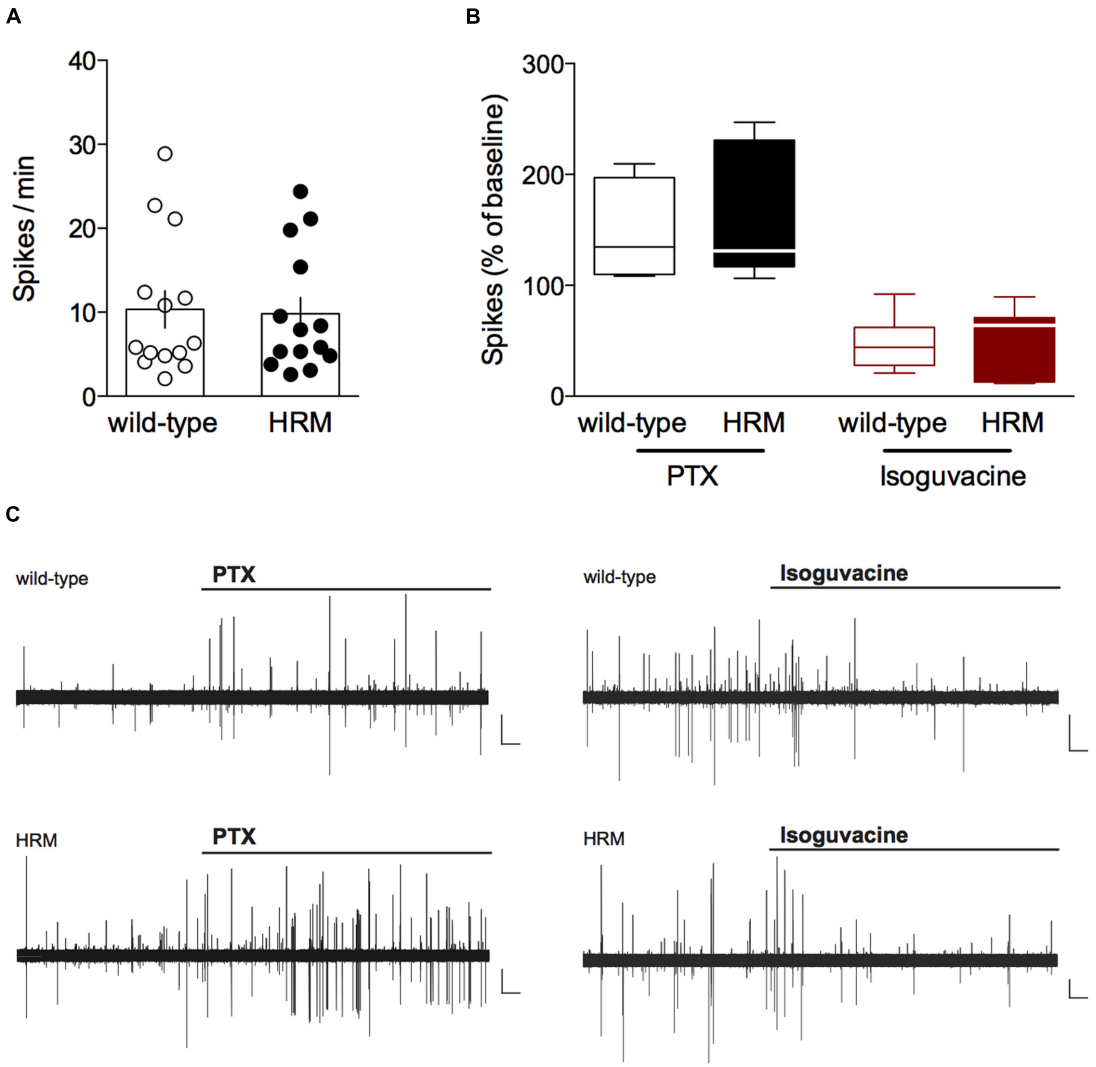

FIGURE 1 | Inhibitory action of GABA in P14–18 wild-type and reelin-haploinsufficient mice. (A) Average baseline spontaneous spiking activity (number of spikes per min) from cell-attached recorded visually identified deep layer pyramidal neurons from P14-P18 wild-type mice and HRM. Spike frequency was 10.3 ± 2.2 spikes/min (n = 14 cells/10 mice) in wild-type mice and 9.8 ± 2.0 spikes/min (n = 14 cells/8 mice) in HRM. Data points represent baseline spontaneous spiking frequency from individual cells. Error bars represent SEM. **(B)** Effect of the $GABA_A$ antagonist picrotoxin (PTX, 20 μM, black) or the $GABA_A$ agonist isoguvacine (7 μM, red) on the spontaneous spiking activity of P14–18 wild-type mice (open symbols) and HRM (filled symbols). Box plot showing the interquartile range with whiskers at minimum and maximum data points of the effect of PTX (wild-type: n = 7 neurons/5 mice; HRM: n = 7 neurons/4 mice) or isoguvacine (wild-type: n = 7 neurons/5 mice; HRM: n = 7 neurons/4 mice) expressed as the percentage of baseline spontaneous spiking activity. Horizontal lines represent the median. Note the inhibitory action of PTX and excitatory effect of isoguvacine in both genotypes. **(C)** Representative traces of the excitatory action of PTX and inhibitory effect of isoguvacine on the spontaneous spiking activity recorded in cell attached configuration in layer 5/6 pyramidal neuron from P16 wild-type and HRM. Calibration: 100 pA, 1 s.

spontaneous spiking activity to 146.5 ± 15.6% whereas it was reduced to 46.8 ± 9.1% following application of isoguvacine (**Figures 1B,C**). These results demonstrate that in wild-type mice GABA exerts a classical inhibitory action from P14. PTX and isoguvacine produced the same effects on baseline spontaneous activity recorded in P14–18 HRM (increase to 155.5 ± 22.0% and reduction to 53.4 ± 11.3% respectively; **Figures 1B,C**).

Together, these data show that from the pre-weaning P14–18 period GABA exhibits an inhibitory action on layer 5/6 pyramidal neurons in the PrPFC, and that reelin haploinsufficiency does not impact local network activity nor the inhibitory action of GABA during the pre-weaning period.

Reelin Haploinsufficiency Disrupts the Maturation of GABAergic Synaptic Transmission in Layer 5/6 PrPFC

Spontaneous $GABA_A$-mediated inhibitory post-synaptic currents (sIPSCs) were recorded in layer 5/6 PrPFC pyramidal

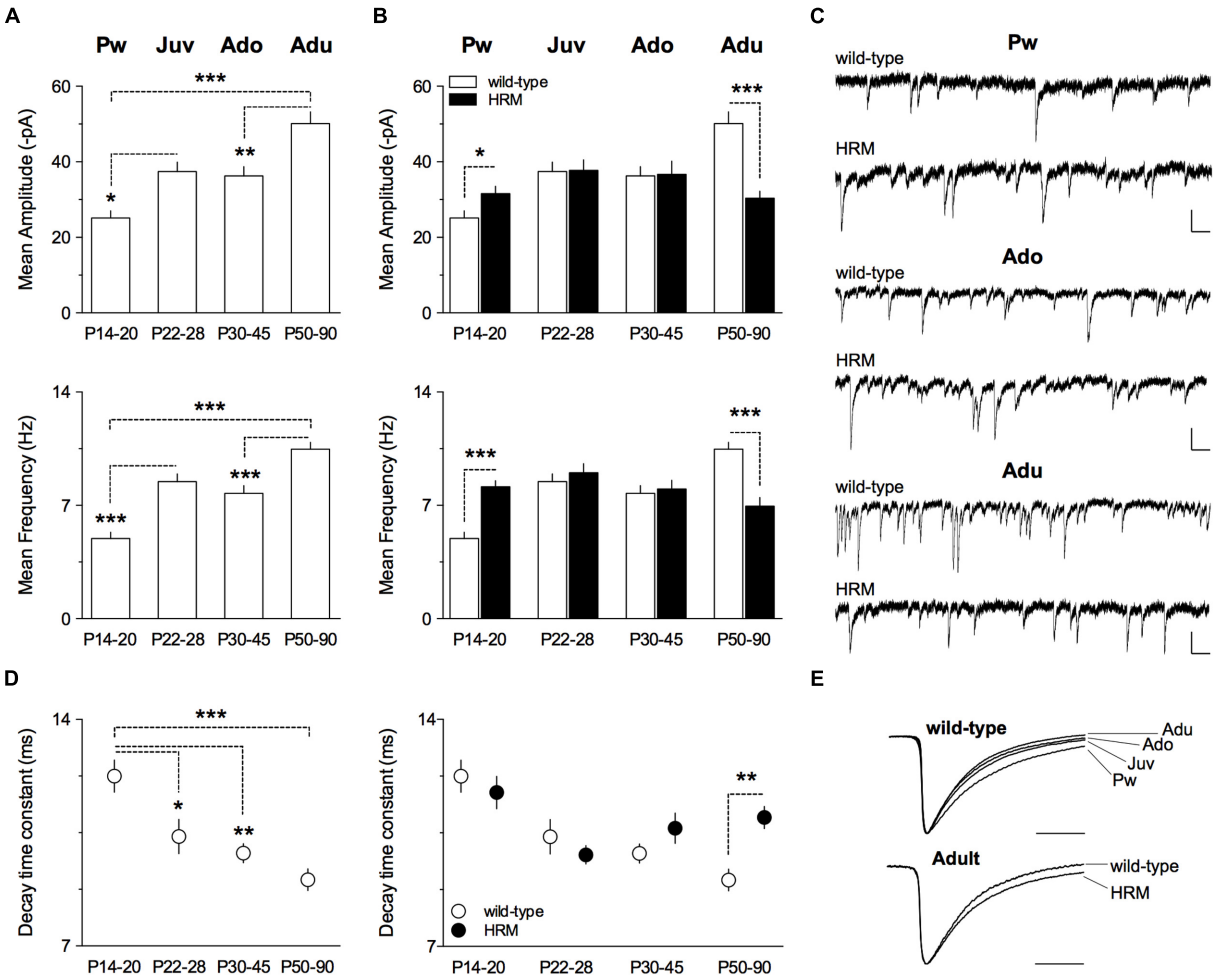

FIGURE 2 | Maturational profile of GABAergic spontaneous activity in wild-type mice and HRM. (A) Mean amplitude and frequency of spontaneous GABA-IPSCs in wild-type mice from pre-weaning to adult stage. Values for mean amplitude were: 25.1 ± 1.9 pA ($n = 10$ neurons/5 mice) at P14–20, 37.4 ± 2.5 pA ($n = 13$ neurons/9 mice) at P22–28, 36.3 ± 2.5 pA ($n = 12$ neurons/6 mice) at P30–45 and 50.1 ± 3.1 pA ($n = 16$ neurons/7 mice) at P50–90. Values for mean frequency were: 5.0 ± 0.4 Hz ($n = 10$ neurons/5 mice) at P14–20, 8.5 ± 0.5 Hz ($n = 13$ neurons/9 mice) at P22–28, 7.8 ± 0.5 Hz (12 neurons/6 mice) at P30–45 and 10.5 ± 0.4 Hz ($n = 16$ neurons/7 mice) at P50–90. **(B)** Mean amplitude of GABA-sIPSCs is augmented in P14–20 HRM compared to aged-matched wild-type (31.6 ± 1.9 pA, $n = 12$ cells/6 mice HRM) and reduced in adult HRM compared to adult wild-type (30.4 ± 1.9 pA, $n = 17$ cells/7 mice HRM). At P22–28 and P30–45, mean amplitude was similar between both genotypes (37.8 ± 2.8 pA, $n = 15$ neurons/8 mice HRM Juv; 36.7 ± 3.5 pA, $n = 16$ neurons/7 mice HRM Ado). $F_{(7,103)} = 7.421$, $P < 0.0001$, ANOVA. Mean frequency of GABA-sIPSCs is augmented in P14–20 HRM compared to aged-matched wild-type (8.1 ± 0.4 Hz, $n = 12$ cells/6 mice HRM) and reduced in adult HRM compared to adult wild-type (6.9 ± 0.5 Hz, $n = 17$ cells/7 mice HRM). In juvenile and adolescent, mean frequency was similar between both genotypes (9.0 ± 0.6 Hz, $n = 15$ neurons/8 mice HRM Juv; 8.0 ± 0.5 Hz, $n = 16$ neurons/7 mice HRM Ado). $F_{(7,103)} = 9.499$, $P < 0.0001$, ANOVA. **(C)** Representative traces of GABA-sIPSCs recorded at −70 mV from both genotypes at indicated developmental stages. Calibration: 50 pA, 200 ms. **(D)** Left: decay time constant of GABA-sIPSCs during maturation of wild-type mice. Values were: 12.3 ± 0.5 ms ($n = 10$ neurons/5 mice) at P14–20, 10.4 ± 0.5 ms ($n = 13$ neurons/9 mice) at P22–28, 9.9 ± 0.3 ms ($n = 12$ neurons/6 mice) at P30–45 and 9.0 ± 0.3 ms ($n = 16$ neurons/7 mice) at P50–90. Right: decay time constant is slower in adult HRM compared to aged-matched wild-type (11.0 ± 0.3 ms, $n = 17$ cells/7 mice HRM) and was not different between both genotypes from pre-weaning to adolescent period. $F_{(7,103)} = 6.212$, $P < 0.0001$, ANOVA. **(E)** Representative normalized traces illustrating decay acceleration of sIPSCs during maturation in wild-type (top) and slower decay in adult HRM compared to age-matched wild-type. Calibration: 10 ms. **(A,B,D)** Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA.

neurons in whole-cell configuration (**Figure 2**). In wild-type mice, both mean amplitude and frequency increased between P14 and P90 ($F_{(3,47)} = 14.39$, $P < 0.0001$ and $F_{(3,47)} = 24.53$, $P < 0.0001$ respectively, ANOVA; **Figure 2A**). In contrast, both parameters remained similar throughout maturation in HRM ($F_{(3,56)} = 1.943$, $P = 0.1331$, ANOVA mean amplitude and $F_{(3,56)} = 3.515$, $P = 0.3188$, ANOVA mean frequency; **Figure 2B**). When compared between genotypes, mean amplitude and frequency were higher in pre-weaning HRM, similar during the juvenile and the adolescent periods and reduced in adult HRM (**Figures 2B,C**). These results indicate that synaptic activity at inhibitory synapses increases with maturation in wild-type layer 5/6 PrPFC whereas it does not change during maturation of HRM.

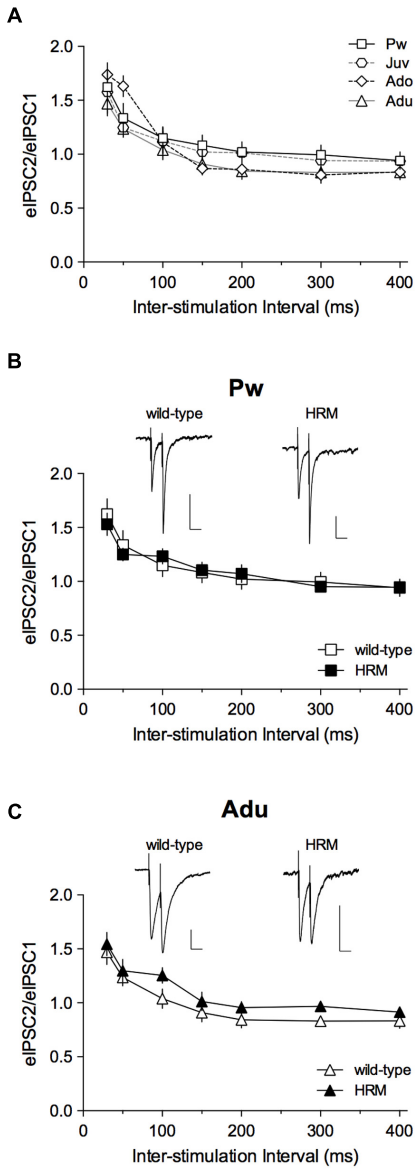

FIGURE 3 | Maturational profile of short-term plasticity. (A) Paired-pulse ratios (PPRs) of evoked GABA-IPSCs in wild-type mice indicate no difference from pre-weaning to adulthood. Two-way ANOVA (age × inter-stimulation interval) revealed a significant effect of inter-stimulation interval ($F_{(6,186)} = 61.80$, $P < 0.001$), a non-significant effect of age ($F_{(3,31)} = 0.6857$, $P = 0.5676$) and a non-significant interaction between factors ($F_{(18,186)} = 1.423$, $P = 0.1248$). Pw: $n = 11$ cells/5 mice; Juv: $n = 7$ cells/4 mice; Ado: $n = 8$ cells/4 mice; Adu: $n = 9$ neurons/4 mice. **(B,C)** PPRs of eIPSCs show no difference between wild-type and HRM in the pre-weaning **(B)** and adult **(C)** periods. At both ages, two-way ANOVA (genotype × inter-stimulation interval) revealed a significant effect of inter-stimulation interval (**B**: $F_{(6,96)} = 25.40$, $P < 0.001$; **C**: $F_{(6,96)} = 32.28$, $P < 0.001$), a non-significant effect of genotype (**B**: $F_{(1,16)} = 5.6 \times 10^{-3}$, $P = 0.9411$; **C**: $F_{(1,16)} = 1.461$, $P = 0.2444$) and a non-significant interaction between factors (**B**: $F_{(6,96)} = 0.6067$, $P = 0.7244$; **C**: $F_{(6,96)} = 0.2614$, $P = 0.9534$). HRM: $n = 7$ neurons/4 mice Pw **(B)** and $n = 9$ neurons/6 mice Adu **(C)**. Representative recordings of 50 ms interval evoked GABA-IPSCs in pre-weaning wild-type and HRM (**B**; calibration: 100 pA, 50 ms) and in adult wild-type and HRM (**C**; calibration: 50 pA, 50 ms). Data are expressed as mean ± SEM.

During brain maturation, the subunit composition of $GABA_A$ receptors undergoes changes from predominantly containing α2 to α1 subunit, thus contributing to faster kinetics observed with age (Dunning et al., 1999; Davis et al., 2000; Eyre et al., 2012; Ehrlich et al., 2013). We next examined whether GABA-sIPSCs kinetics from layer 5/6 PrPFC pyramidal neurons displayed maturation-dependent changes. In wild-type, the decay time constant exhibited a large decrease form the pre-weaning period to adulthood ($F_{(3,47)} = 9.763$, $P < 0.0001$, ANOVA; **Figures 2D,E**) showing that GABA-sIPSCs became faster with age. In contrast, the decay time constant remained similar between pre-weaning, adolescent and adult HRM (**Figure 2D**). Of note, the decay time constant was higher in adult HRM compared to age-matched wild-type (**Figures 2D,E**), showing that in HRM GABA-sIPSCs remained in an immature stage.

Maturation of Short-Term GABAergic Plasticity in Layer 5/6 PrPFC

To test whether this altered pattern in pre-weaning and adult HRM resulted in modification of short-term plasticity, we analyzed the PPR of evoked IPSCs (eIPSCs; **Figure 3**) during maturation in wild-type mice (**Figure 3A**) and then compared PPR between both genotypes during the pre-weaning (**Figure 3B**) and adult periods (**Figure 3C**). This form of plasticity, which depends on release probability was identical in wild-type at all developmental epochs and all intervals tested (**Figure 3A**) and between both genotypes at P14–20 and P50–90 at all intervals tested (**Figures 3B,C**). These results show that decreased reelin levels do not affect short-term plasticity of layer 5/6 pyramidal neurons GABAergic synapses during the first 3 months of PrPFC maturation.

Reelin Haploinsufficiency Impairs the Developmental Trajectory of the E/I Balance

Alterations in the ratio of excitatory (glutamatergic)/ inhibitory (GABAergic) neurotransmission in the PFC have been proposed to play a role in psychiatric disorders of schizophrenic and ASD patients (Bicks et al., 2015). An altered E/I balance has also been reported in mouse models of several psychiatric disorders (Gandal et al., 2012; Gkogkas et al., 2013; Lee et al., 2015).

Therefore, we next examined the maturation of the E/I balance (**Figure 4**) and whether it was modified by reelin haploinsufficiency (**Figure 5**). First, we examined the total charge transfer from whole-cell recorded spontaneous AMPA-mediated EPSCs (sEPSCs; **Figure 4A**) and sIPSCs (**Figure 4B**), a parameter which accounts for both frequency and amplitude of spontaneous synaptic events. The total charge transfer of sEPSCs was augmented from pre-weaning to adolescence in wild-type ($F_{(3,30)} = 4.235$, $P = 0.0131$, ANOVA; **Figure 4A**). It was neither significantly different in HRM ($F_{(3,48)} = 1.845$, $P = 0.1517$, ANOVA; **Figure 4A**) nor between the two genotypes within each developmental period ($F_{(7,78)} = 2.551$, $P = 0.203$, ANOVA; **Figure 4A**). These results are in accordance with the maturational profile of AMPA-sEPSCs mean amplitude (Iafrati et al., 2016) and frequency in both wild-type mice and HRM (Supplementary

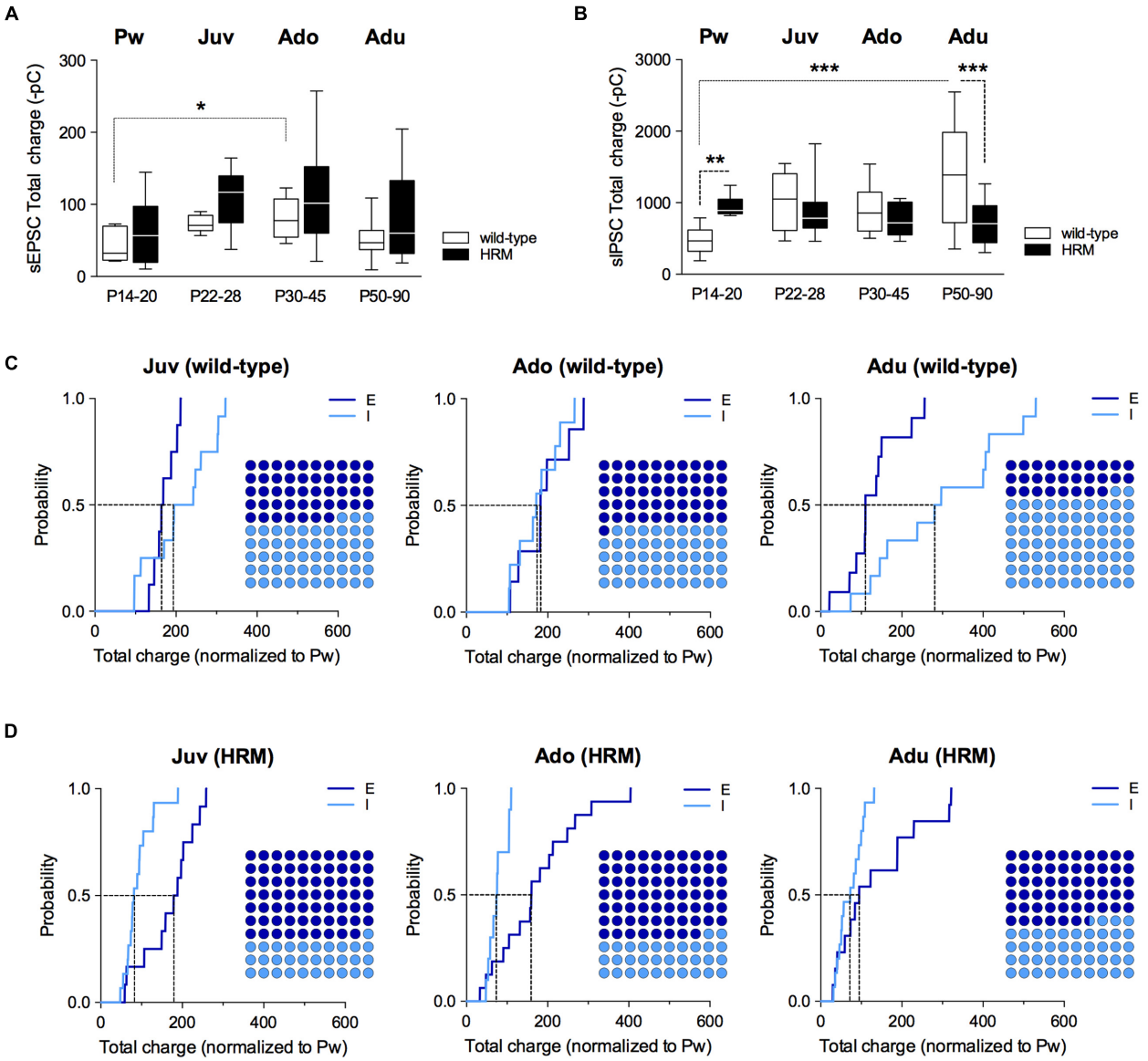

FIGURE 4 | Maturational profile of the E/I balance in wild-type and reelin-haploinsufficient mice. (A) Box plot showing the interquartile range with whiskers at minimum and maximum data points of the AMPA-sEPSCs total charge measured over a 6 min period in wild-type mice ($n = 8$ neurons/4 mice P14–20, $n = 8$ neurons/6 mice P22–28, $n = 7$ neurons/4 mice P30–45 and $n = 11$ neurons/9 mice P50–90) and HRM ($n = 11$ neurons/6 mice P14–20, $n = 12$ neurons/7 mice P22–28, $n = 16$ neurons/7 mice P30–45 and $n = 13$ neurons/6 mice P50–90). Horizontal lines represent the median sEPSCs total charge. **(B)** Same as A for total charge of GABA-sIPSCs measured over a 6 min period in wild-type mice (Pw: $n = 9$ cells/5 mice, Juv: $n = 12$ cells/8 mice, Ado: $n = 10$ cells/5 mice and Adu: $n = 12$ cells/5 mice) and in HRM (Pw: $n = 9$ cells/5 mice, Juv: $n = 15$ cells/8 mice, Ado: $n = 10$ cells/5 mice and Adu: $n = 15$ cells/7 mice). Error bars represent SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Mann–Whitney t-test. **(C)** Cumulative frequency distributions of sEPSC (E) and sIPSC (I) total charge transfer obtained for each wild-type neurons within juvenile, adolescent and adult epochs normalized to the mean value of total charge transfer calculated for wild-type P14–20 neurons. E: $n = 8$ Juv, 7 Ado and 11 Adu; I: $n = 12$ Juv, 10 Ado and 12 Adu. Insets: dot plots showing the proportion of E versus I during maturation extrapolated at $P = 0.5$ from the corresponding cumulative distributions. **(D)** Same as C but for HRM. Total charge transfer of each HRM neuron was normalized to the mean value of total charge transfer obtained for pre-weaning HRM. E: $n = 12$ Juv, 16 Ado and 13 Adu; I: $n = 15$ Juv, 10 Ado and 15 Adu. n represents the number of neurons.

Figure S2). The total charge transfer of sIPSCs increased in wild-type adult compared to pre-weaning ($F_{(3,39)} = 6.913$, $P = 0.0008$, ANOVA; **Figure 4B**) as expected from the maturational profile of wild-type sIPSCs mean amplitude and frequency (**Figure 2A**). The sIPSCs total charge transfer was higher in P14–20 HRM and reduced in HRM at P50–90 compared to age-matched wild-type mice whereas it was identical between both genotypes at juvenile and adolescent stages ($F_{(7,84)} = 5.836$, $P < 0.0001$, ANOVA; **Figure 4B**).

Subsequently, we computed the relative changes in excitation and inhibition using normalized total charge transfer from sEPSCs and sIPSCs (**Figures 4C,D** and **5**; Gkogkas et al.,

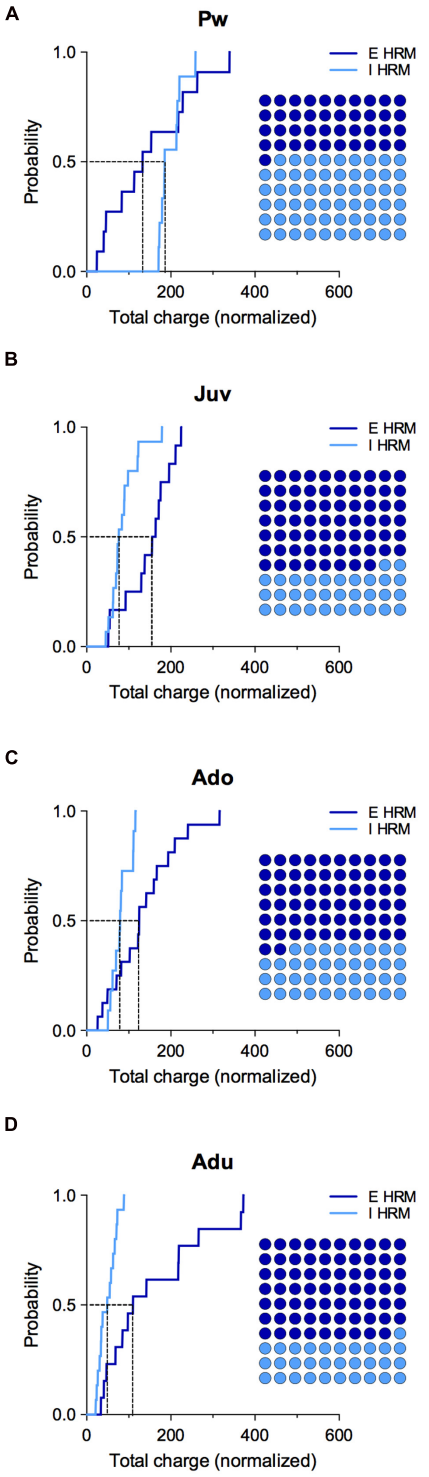

FIGURE 5 | Effect of reelin haploinsufficiency on the maturation of E/I balance. (A–D) Cumulative frequency distributions of E and I total charge transfer for HRM neurons normalized to the wild-type age-matched mean value are shown at all maturational epoch. Insets: dot plots showing the maturation of the proportion of E versus I extrapolated at $P = 0.5$ from the corresponding cumulative distributions. Pw: E = 11 and I = 9 neurons, Juv: E = 12 and I = 15 neurons, Ado: E = 16 and I = 10 neurons, Adu: E = 13 and I = 15 neurons.

2013). We first analyzed the maturation of E/I balance within each genotype (**Figures 4C,D**). In wild-type, we found that the normalized total charge transfer from sEPSCs and sIPSCs was similar between P22 and P45 showing that the E/I balance did not vary during juvenile and adolescent stages (**Figure 4C**, Juv and Ado), and that E and I were represented in equal proportion (**Figure 4C**, Dot plots). However, at P50–90 the normalized sIPSCs total charge transfer was significantly increased compared to juvenile and adolescent as shown by the right-shift in the sIPSC cumulative distribution (**Figure 4C**, Adu), indicating that the E/I balance dramatically shifted to an increased proportion of I at adulthood. In HRM, the E/I balance remained identical from juvenile to adolescent stage and was characterized by a higher proportion of E as shown by a right-shift in the normalized sEPSC total charge transfer cumulative distributions compared to age-matched sIPSC cumulative distributions (**Figure 4D**, Juv and Ado). In adult HRM, the normalized sEPSC total charge transfer cumulative distribution shifted to the left compared to juvenile and adolescent, resulting in an equal proportion of E versus I that was comparable to the E/I balance observed in juvenile and adolescent wild-type (**Figure 4D**, Adu). These data show that in wild-type PrPFC the postnatal maturation of the E/I balance is characterized by a shift toward increased I during adulthood. In contrast, in HRM the E/I balance did not dramatically change during the same developmental epochs and remained in an immature stage characterized by a larger proportion of E versus I.

To evaluate whether reelin-haploinsufficiency altered the maturation of the E/I balance, we compared the E/I balance between both genotypes at all developmental epochs (**Figure 5**). During the pre-weaning period, the normalized total charge transfer in HRM relative to wild-type mice was larger for sIPSCs as indicated by the right-shift of I cumulative distribution (**Figure 5A**), indicating an increased proportion of I in the E/I balance of pre-weaning HRM compared to age-matched wild-type. At the juvenile stage, the E/I balance in HRM switched to the opposite direction and displayed a reduced proportion of I compared to wild-type as shown by the left shift in the cumulative distribution of total sIPSC charge transfer relative to wild-type mice (**Figure 5B**). The decrease in the proportion of I persisted throughout adolescence and adulthood (**Figures 5C,D**). Therefore, these results show that the maturational sequence of the E/I balance of the PrPFC is disrupted by reelin-haploinsufficiency.

DISCUSSION

This study describes for the first time analysis of the impact of reelin haploinsufficiency on multiple GABAergic parameters during postnatal maturation (2 weeks to 3 months) of deep layer PrPFC pyramidal neurons. Namely, we investigated alterations in the polarity of GABA action, postnatal maturation of GABAergic synaptic inputs and the developmental sequence of the E/I balance in both wild-type and HRM mice.

In accordance with studies performed in other developing brain structures (Ben-Ari et al., 2007; Kirmse et al., 2015), we found that after 2 weeks of postnatal development GABA exhibits an inhibitory action in PrPFC deep layers. It remains to be determined whether the time course of the developmental excitatory-inhibitory GABA sequence in the PrPFC is identical to other brain structures.

We provide the first evidence that GABAergic synaptic transmission undergoes significant changes during PrPFC postnatal development. In wild-type mice, we show a maturation of the function of GABAergic synapses on pyramidal layer 5/6 PFC neurons with an increase of transmission efficacy with age which reached maturity at ∼2–3 months of age. Similar results have been described in primate dorsolateral PFC (Gonzalez-Burgos et al., 2015). Specifically, we found that both the amplitude and frequency of $GABA_A$-mediated sIPSCs increased between P14 and P90 whereas the PPR did not change, suggesting a post-synaptic locus of developmental alteration. These changes could result from the functional maturation of the expression of $GABA_A$ receptor subunits (Fritschy and Panzanelli, 2014) and/or from an increase in the number of post-synaptic GABAergic sites as in the rat frontal cortex, where a transient increase in the expression of gephyrin, the post-synaptic scaffolding protein that anchors $GABA_A$ receptors has been reported around P21–25 (Pinto et al., 2013). Similar to changes reported in other developing brain areas (Hollrigel and Soltesz, 1997; Dunning et al., 1999; Ehrlich et al., 2013), we found changes in spontaneous $GABA_A$-mediated IPSC kinetics with slow IPSCs in early period followed by a sharp reduction of the decay time constant from juvenile to adulthood. It remains to be determined whether the maturation of IPSC kinetics in pyramidal layer 5/6 PFC neurons results from changes in expression of GABAa receptors subunits (Hollrigel and Soltesz, 1997; Dunning et al., 1999; Ehrlich et al., 2013) or other mechanisms (Draguhn and Heinemann, 1996).

In contrast, in HRM spontaneous GABAergic synaptic transmission remained stable during the same developmental period and displayed an immature phenotype similar to juvenile and adolescent wild-type. Whether GABAergic synaptic inputs on layer 5/6 pyramidal neurons reach their maturity before P14 or after P90 in HRM requires further investigation. The differences in the time course of maturation of GABAergic synaptic transmission in HRM and wild-type littermates resulted in an increased transmission efficacy in P14–20 HRM and a reduction at adult stages compared to age-matched wild-type mice. The latter has been similarly reported in CA1 pyramidal neurons of adult HRM (Qiu et al., 2006).

The E-I balance has been shown to shift during early development in the sensory cortex (Dorrn et al., 2010) and to be a trigger for the onset of critical periods in the developing cortex (Hensch and Fagiolini, 2005). Biochemical measurements support the finding that the E/I balance reaches maturity later in the frontal cortex compared to visual and somatosensory cortices (Pinto et al., 2013). Of particular interest, alterations of the E/I balance have been found in animal models of psychiatric disorders (Gatto and Broadie, 2010) and it was shown that direct alteration of the E/I balance within the PFC has a strong effect on social motivation in mice (Yizhar et al., 2011). Thus, we found it crucial to examine the sequence of the E/I balance during periods of development. We observed that in PrPFC the E/I balance reaches maturity during adulthood and is characterized by a shift toward increased inhibition. In contrast, in HRM the E/I balance did not shift and remained in an immature stage. We also showed that reelin-haploinsufficiency blocked the maturational shift of the E/I balance which occurs during adulthood in wild-type PrPFC. Our findings indicate that the developmental trajectory of the E/I balance is disrupted in HRM, which could prove deleterious for the proper initiation of intense periods of plasticity in the PFC. In turn, this aberrant development may increase vulnerability to PFC-related disorders.

The network of layer 5/6 pyramidal neurons consists of local connections with principal neurons and different types of GABAergic neurons present in the different layers in addition to long-range thalamic imputs. The effect of reelin-haploinsufficiency on local interconnectivity as well as long-range thalamocortical connectivity is unknown. Aberrant thalamo-cortical circuitry has been reported in homozygous reeler mice (Li et al., 2005) as well as modification in GABAergic markers in HRM (Nullmeier et al., 2011). Thus our findings could result from a direct mechanism such as changes in GABAergic local connectivity, may be secondary to a general disruption in cortical development, or result from homeostatic mechanisms involving reduced thalamic input dependent excitation which in turn could cause reduction in GABAergic terminals. Apical dendritic activity of layer 5 somatosensory pyramidal cells is highly sensitive to inhibition mediated by interneurons, presumably Martinotti cells, present in deep cortical layers (Murayama et al., 2009). Of note, some deep cortical Martinotti cells express reelin (Pesold et al., 1999) and constitute one of the main sources of secreted reelin in neocortical superficial layers (Ramos-Moreno and Clasca, 2014). Thus, GABAergic impairment and disruption of the E/I balance observed in HRM could have deleterious effects on the function of cortical circuits such as the dendritic filtering of inputs and encoding of stimuli and ultimately in the processing of information and behavioral adaptation (Barr et al., 2008; Teixeira et al., 2011; Labouesse et al., 2016).

Together, our data show that reelin is necessary for the fine-tuning of GABAergic connectivity and of the physiological E/I balance in the maturing PrPFC. Furthermore, these data indicate that a disrupted developmental trajectory of prefrontal GABAergic microcircuitry leads to an altered E/I balance. Combined with previous findings illustrating disrupted E/I balance in psychiatric disorders, it therefore follows that this aberrant maturation may ultimately manifest as behavioral deficits. While further research dissecting if reelin-haloinsufficiency affects a specific class of PFC interneurons is crucial for concluding the exact nature and mechanisms underlying such consequences, these data lay the groundwork for novel investigations into the mechanistic underpinnings of complex psychiatric diseases which manifest during development.

ETHICS STATEMENT

All experiments were performed according to INSERM ethic rules. This study and protocols were approved by the ethic committee of Marseille under the reference n°2015121715284829-V1n°#3279.

AUTHOR CONTRIBUTIONS

LB performed electrophysiology related to **Figures 2** to **5**, conducted the data analysis and contributed to the design of the experiments and to the writing of the manuscript. AS performed experiments related to **Figure 1**, conducted the data analysis and experimental design and contributed to the writing of the manuscript. OL, JI, and AT performed electrophysiology experiments. PC designed the experiments, conducted data analysis, supervised the entire project and wrote the manuscript.

FUNDING

Work in Dr. P. Chavis laboratory was supported by INSERM and Fondation Jérôme Lejeune (R13913AA). LB and JI were supported by the French Ministère de la Recherche (MENRT), AFS was supported by the NIDA-INSERM Drug Abuse Research Fellowship.

ACKNOWLEDGMENT

The authors thank L. Herrera and O. Montheil from the genotyping facility headed by Dr. Pallesi-Pocachard.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2016.00308/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

MINI REVIEW
published: 31 March 2016
doi: 10.3389/fncel.2016.00084

RELN Mutations in Autism Spectrum Disorder

*Dawn B. Lammert and Brian W. Howell**

Department of Neuroscience and Physiology, SUNY Upstate Medical School, Syracuse, NY, USA

RELN encodes a large, secreted glycoprotein integral to proper neuronal positioning during development and regulation of synaptic function postnatally. Rare, homozygous, null mutations lead to lissencephaly with cerebellar hypoplasia (LCH), accompanied by developmental delay and epilepsy. Until recently, little was known about the frequency or consequences of heterozygous mutations. Several lines of evidence from multiple studies now implicate heterozygous mutations in *RELN* in autism spectrum disorders (ASD). *RELN* maps to the AUTS1 locus on 7q22, and at this time over 40 distinct mutations have been identified that would alter the protein sequence, four of which are *de novo*. The *RELN* mutations that are most clearly consequential are those that are predicted to inactivate the signaling function of the encoded protein and those that fall in a highly conserved RXR motif found at the core of the 16 Reelin subrepeats. Despite the growing evidence of *RELN* dysfunction in ASD, it appears that these mutations in isolation are insufficient and that secondary genetic or environmental factors are likely required for a diagnosis.

Keywords: *RELN*, Dab1, autism, autism spectrum disorder, brain development, cerebellum, neocortex

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers, The State Univesity of New Jersey, USA

Reviewed by:
Ivan Iossifov, Cold Spring Harbor Laboratory, USA
Carlos Gustavo Perez-Garcia, The Salk Institute, USA

****Correspondence:***
Brian W. Howell
howellb@upstate.edu

Received: *29 December 2015*
Accepted: *18 March 2016*
Published: *31 March 2016*

Citation:
Lammert DB and Howell BW (2016) RELN Mutations in Autism Spectrum Disorder. Front. Cell. Neurosci. 10:84. doi: 10.3389/fncel.2016.00084

Autism spectrum disorder (ASD) currently affects as many as 1 in 45 children in the United States (Zablotsky et al., 2015). ASD incorporates Asperger, autism, and pervasive developmental disorder-not otherwise specified (PDD-NOS), and is characterized by social, behavioral, and language deficits. A small percentage (<20%) of ASD known as "syndromic autism" is attributable to monogenetic diseases, the two most common being fragile X syndrome and tuberous sclerosis (Miles, 2011; Persico and Napolioni, 2013). Other monogenetic disorders that have a high frequency of ASD but are less prevalent in the general population include Prader-Willi/Angelman, 15q microduplication, Rett, Smith-Lemli-Opitz, and Timothy syndromes.

The remaining 80% of ASD cases are considered "non-syndromic autism" and are the focus of high throughput sequencing efforts. A better understanding of how candidate genes contribute to ASD at the molecular level is key to understanding how so many variants converge on a common phenotype. *RELN*, encoding a large secreted glycoprotein, expressed in the brain and critical for proper brain development and synapse function, is consistently cited as a candidate gene for ASD (Persico and Napolioni, 2013).

In 2001 the International Molecular Genetic Study of Autism Consortium (IMGSAC) described a region on chromosome 7q as the peak region of linkage and first autism susceptibility locus (AUTS1; IMGSAC, 1998). Subsequent linkage studies supported this finding (IMGSAC, 2001a,b; Lamb et al., 2005). Given the role of *RELN* in neurodevelopment and its location at chromosome 7q22, *RELN* quickly emerged as a candidate gene for autism and numerous studies (>15) have investigated the occurrence of ASD risk-associated single nucleotide polymorphism (SNPs) in *RELN*

(DeSilva et al., 1997; Persico et al., 2001; Krebs et al., 2002; Zhang et al., 2002). These and other studies had mixed results, possibly due to varying study designs, ethnic populations, and mathematical interpretations. A recent meta-analysis considered three known SNPs in *RELN*, and concluded that one rs362691 was significantly associated with an increased risk of ASD (Wang et al., 2014a).

While SNP analysis supports heterozygous mutations in *RELN*, it cannot explain how they contribute to ASD. Many genes have been proposed as candidates for ASD on the basis of sequencing analysis, but like *RELN*, their pathological mechanism remains speculative. Instead of perseverating on a particular individual SNP, researchers are now considering candidate genes on a much broader scale. The sum of coding and non-coding variants from genome-wide screens coupled with network analyses, gene and protein expression, and epigenetic modifications provide evidence that helps understand functionally how a gene contributes to ASD (Neale and Sham, 2004). From these types of analyses emerges an approach for deciphering the role of *RELN* in ASD at the molecular level, beyond association.

RELN expression is both spatially and temporally consistent with ASD, which is thought to originate as a neurodevelopmental disorder that persists into postnatal life. Homozygous loss of *RELN* leads to severe neuronal dysplasia in several brain regions including the neocortex, hippocampus, and cerebellum. Patients homozygous null for *RELN* suffer from lissencephaly with cerebellar hypoplasia (LCH), a profoundly developmentally debilitating disease (Hong et al., 2000; Chang et al., 2007). Patients with LCH also suffer from epilepsy, but no autistic behavior has been reported in the patients or their parents.

RELN is first expressed by Cajal Retzius (CR) cells, and other less well defined marginal zone neurons, that act as pioneer neurons by regulating the positioning of projection neurons into discrete layers in the neocortex (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995; Ikeda and Terashima, 1997; Meyer et al., 2004). CR eventually degenerate and a population of GABA-ergic interneurons expresses *RELN* postnatally (Pesold et al., 1998). In the developing cerebellum, *RELN* is first expressed by cells of the rhombic lip that migrate to populate the external granule layer and regulate the position of Purkinje neurons (D'Arcangelo et al., 1995; Miyata et al., 1997). Postnatally, cerebellar granule cell neurons (GCNs) now positioned in the internal granule layer continue to secrete Reelin, although its postnatal role is not clear (Sinagra et al., 2008). The brain size and architecture are relatively normal in the heterozygous reeler mouse (HRM); however, male HRM, thought to model ASD, have decreased numbers of Purkinje cells (Hadj-Sahraoui et al., 1996; Biamonte et al., 2009).

Traditionally, the cerebellum is considered responsible for fine-tuning movement, but its role in cognitive and emotional functions is now appreciated (Buckner, 2013). Acute adult injury results in the classical cerebellar signs early, such as asynergy, followed by subtle, often overlooked cognitive and communication impairments. In contrast, damage to the cerebellum during development leads to cognitive and communication defects. Interestingly, these early injuries have also been associated with the occurrence of ASD, which highlights a role for the cerebellum in its etiology (Becker and Stoodley, 2013; Wang et al., 2014b).

One of the most consistent anatomic findings in ASD is a decrease in cerebellar Purkinje cells and decreased volume of the vermis (Fatemi et al., 2012; D'Mello et al., 2015; Hampson and Blatt, 2015). In the most recent stereologic study, Skefos et al. (2014) found that Purkinje cells were decreased in ASD individuals. Compared to previous studies, this group included patients with cognitive delay and epilepsy, showing that this defect is widespread across ASD. The role of Purkinje cells and their ability to drive ASD behaviors as seen in tuberous sclerosis, has been recently demonstrated in a mouse model (Tsai et al., 2012a). They used a conditional knock-out of TSC1 in Purkinje cells to show that dysfunction in these cells was sufficient to decrease interest in novel mouse social interaction, increase grooming, and increase ultrasonic vocalizations—behaviors consistent with other ASD mouse models (Silverman et al., 2010).

Not only is *RELN* expression consistent with ASD, but the Reelin signaling pathways intersect prominent ASD protein networks. To understand how so many disparate genes can converge on a similar phenotype, grouping candidate genes into networks has helped to uncover cellular processes that might be driving ASD. Network analysis continually implicates synaptic function and dysregulated protein translation, particularly at the synapse (Gilman et al., 2011; Sanders et al., 2012; Ebert and Greenberg, 2013; De Rubeis et al., 2014).

Canonical Reelin signaling is initiated by Reelin binding its receptors very-low-density-lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2; LRP8; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999). Disabled-1 (Dab1) is recruited to the receptors, which then activates Src family kinases and leads to reciprocal activation through tyrosine phosphorylation of Dab1 (Howell et al., 1997, 1999; Rice et al., 1998; Arnaud et al., 2003; Bock and Herz, 2003). This signaling initiates a number of signaling cascades, which have been extensively reviewed (Tissir and Goffinet, 2003; D'Arcangelo, 2014; Sekine et al., 2014). Dab1 and VLDLR have been suggested to be associated with ASD risk, but overall there is little evidence directly implicating Dab1, VLDLR, ApoER2, SRC, or FYN in ASD (Fatemi et al., 2005; Iwata et al., 2012; Li et al., 2013; Shen et al., 2015).

Particularly relevant to ASD network analyses are recent studies of the integral role of Reelin as a modulator of the postnatal synapse and the ability of Reelin to enhance LTP in the hippocampus (Weeber et al., 2002; Beffert et al., 2005; Chen et al., 2005). Secretion of Reelin, however, is constitutive and independent of synaptic activity (Lacor et al., 2000). Canonical Reelin-ApoER2/VLDLR-Dab1 signaling leads to phosphorylation of the NMDA receptor (NMDAR), increased calcium flux with glutamate stimulation, as well as altered intermembrane mobility of NR2B and NR2A subunit-containing

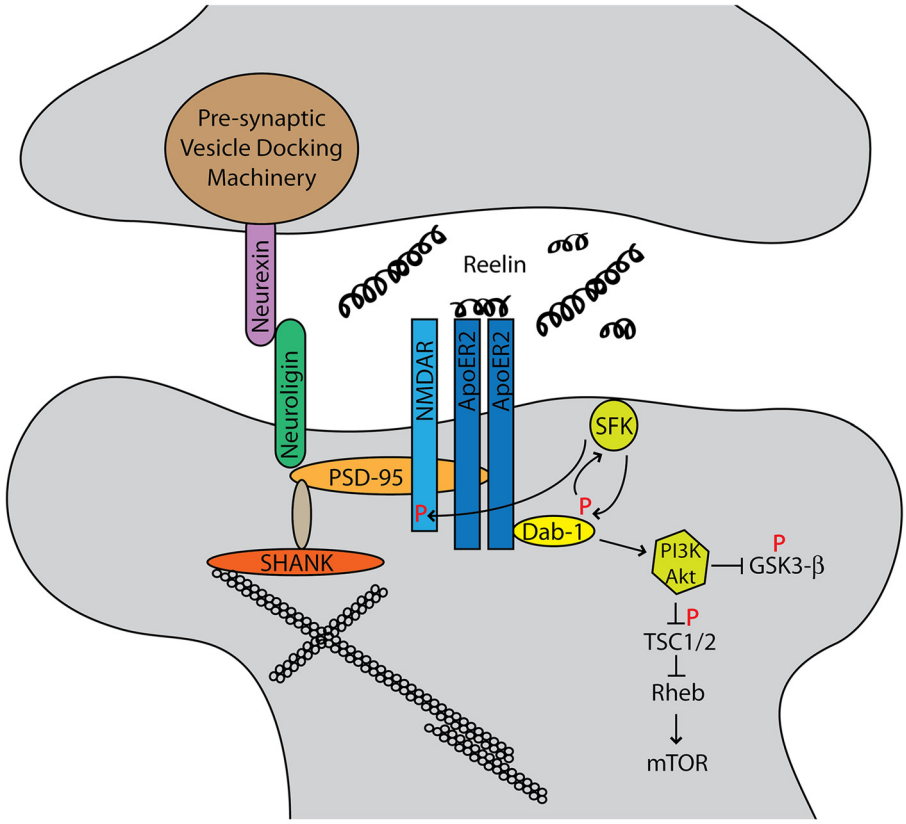

FIGURE 1 | Autism spectrum disorders (ASD) candidate genes cluster into functional networks, and the two most prominent are synapse structure/function and protein translational control. The Reelin-signaling pathway intersects both of these networks. Reelin binds its receptors ApoER2 and very-low-density-lipoprotein receptor (VLDLR). The adapter protein Dab1 binds the cytoplasmic NPXY motif of the receptors and is phosphorylated by Src family kinases. This reciprocally activates Src, which leads to phosphorylation of the NMDA receptor (NMDAR) as well as downstream AKT/PI3K signaling that intersects the mTOR pathway.

receptors (Beffert et al., 2005; Chen et al., 2005; Groc et al., 2007; Campo et al., 2009; Ventruti et al., 2011). Reelin is also capable of modulating presynaptic neurotransmitter release by regulating the VAMP7 and SNAP-25 interaction (Bal et al., 2013).

Structurally, ApoER2, NMDAR, PTEN, and PSD-95 form a complex at the post-synaptic density in a Reelin-dependent manner (Ventruti et al., 2011; **Figure 1**). Neurexins and neuroligins, pre- and post-synaptic cell adhesion molecules respectively, organize the synapse, and each has been implicated in ASD (Dean and Dresbach, 2006). Neuroligins interact with PSD-95, which in turn is anchored to the cytoskeleton through SHANK proteins (Ebert and Greenberg, 2013). SHANK3 mutations often lead to Phelan-McDermid syndrome, which frequently presents with ASD (Grabrucker et al., 2011). PSD-95 expression is also regulated by fragile X mental retardation protein (FMRP), the protein implicated in fragile X syndrome (Tsai et al., 2012b). Hypermethylation of a trinucleotide expansion leads to decreased expression of FMRP and subsequent augmented synaptic mRNA translation.

Reelin also directly intersects protein translation control, the second major candidate gene network and hallmark of Fragile X and tuberous sclerosis syndromes. Tuberous sclerosis is caused by mutations in either TSC1 or TSC2 genes, which leads to hyperactivation of mTORC1 and subsequent increases in protein translation (Crino, 2011). Canonical Reelin signaling activates Akt, which phosphorylates TSC1/2 and leads to dendrite growth and branching (Jossin and Goffinet, 2007). Recently, Reelin and Dab1 protein expression were shown to be increased in TSC2 conditional knock-out mice as well as human cortical tubers (Moon et al., 2015). Here, activation of mTOR signaling may impair Cul5-mediated Dab1 degradation. Although Reelin signaling through mTOR is still incompletely understood, it is clear that it plays a significant role.

Further evidence for *RELN* involvement in ASD is the observation of decreased expression of *RELN* transcript and encoded protein in ASD patients. Decreased Reelin was detected in the cerebellum of ASD subjects as compared to controls (Fatemi et al., 2001, 2005) and in the superior frontal cortex (Fatemi et al., 2005). *RELN* mRNA in these areas was

decreased, as was the *dab1* transcript. *VLDLR* mRNA levels were increased.

Part of the elusiveness of ASD etiology is the likelihood of gene-environment interactions. Maternal stressors during gestation have been shown to alter *RELN* expression through promoter methylation (Giovanoli et al., 2014). MeCP2, the gene implicated in Rett and MeCP2 duplication syndromes, which share features of ASD, shows increased binding to the *RELN* promoter in human ASD cerebella (Zhubi et al., 2014). This binding corresponds with decreased *RELN* mRNA expression, consistent with the aforementioned reduced *RELN* expression in ASD tissue samples.

With the advent of more efficient and affordable sequencing technologies, whole-exome sequencing (WES) has become a new, popular approach for identifying candidate genes. WES identifies probable disease-contributing mutations that disrupt protein function. The average rate of mutation for the human genome is 1.2×10^{-8} per nucleotide. Over the entire genome, Kong et al. (2012) detected 63.2 *de novo* mutations per trio studied, but only 2% of the human genome is actually coding sequence (Kong et al., 2012). Therefore, in agreement with these findings, each exome has approximately only a single *de novo* protein changing allele (Gratten et al., 2013). Focusing then on only detected *de novo* events is a way to streamline candidate gene discovery.

Initial expectations were that individuals with yet unsolved complex disorders would have increased indels, CNVs, and frameshift, nonsense, and missense mutations compared to controls. While findings support that nonsense mutations may be more frequent in ASD than controls, the general finding is that there is not a dramatic overall increase in *de novo* mutation rates in ASD (Neale et al., 2012; Sanders et al., 2012; Samocha et al., 2014). Furthermore, *de novo* mutations do not make up a large enough proportion of cases to explain the elusive genetics of ASD, and likely represent less than 5% of the overall ASD risk (Neale et al., 2012).

Currently more than two *de novo* mutations in a gene support its candidacy, although this threshold will increase with increasing patients to control for multiple testing. *RELN* currently has four unique documented *de novo* ASD-associated mutations, three of which are likely pathological (Neale et al., 2012; De Rubeis et al., 2014; Iossifov et al., 2014; Yuen et al., 2015). Furthermore, *RELN* was 1 of 22 genes with a false discovery rate of < 0.05 in a study of nearly 4000 ASD patients (De Rubeis et al., 2014). *De novo* mutations, while directly explaining very few cases, are likely to contribute, at least in part, to disease in the proband in whom they were discovered. Given the repeated implication of particular gene signaling networks in ASD, understanding how a single *de novo* mutation influences this system at the molecular level will help explain a much larger number of ASD cases (Gratten et al., 2013).

Large and small scale WES studies of ASD individuals consistently identify missense and nonsense mutations in *RELN*, leading researchers to emphasize its importance in ASD (De Rubeis et al., 2014). There are currently over 40 unique *RELN* variants identified in ASD probands that are absent in controls (Bonora et al., 2003; Neale et al., 2012; Koshimizu et al., 2013; De Rubeis et al., 2014; Iossifov et al., 2014; Yuen et al., 2015; Zhang et al., 2015; **Figure 2**). These mutations have not been functionally characterized; however, strong predictions regarding their consequences can be deduced based on Reelin structure and function.

Reelin, a large 410 kDa protein, comprises eight Reelin repeat domains (D'Arcangelo et al., 1995; Nogi et al., 2006; Panteri et al., 2006; Yasui et al., 2007). Each Reelin repeat domain is composed of two subrepeat domains (A and B) linked by an EGF-like domain that share highly conserved sequences and are structurally similar. Reelin binds its receptors ApoER2 and VLDLR through two lysine residues on subrepeat 6A (Yasui et al., 2010).

The first prediction from structure-function analysis is that any nonsense mutation that truncates Reelin before the receptor-binding domain will be loss-of-function. In this instance, the transcript would either be degraded by nonsense-mediated decay or it would produce a protein product unable to initiate canonical signaling. Two mutations with this characteristic have been identified—a *de novo* mutation Q417X and a frameshift mutation that disrupts Reelin after E221 (De Rubeis et al., 2014). Both of these mutations occur before the receptor binding residues K2359 and K2466 (mouse equivalents K2360, K2467; Yasui et al., 2010). Whether these mutations could also contribute to a possible gain-of-function, perhaps through a non-canonical Reelin pathway is unclear, since the receptor-ligand domain is unknown (Lee et al., 2014).

Alternatively, one may predict that mutations may interfere with conserved domains, altering Reelin function in a way that contributes to the ASD phenotype. Aligning the subrepeat sequences of Reelin (Clustal Omega) and plotting the mutations identified in ASD genetic studies, we have found that Reelin is enriched in mutations that lie within an RXR consensus sequence that occurs once in each subrepeat (Bonora et al., 2003; De Rubeis et al., 2014; Iossifov et al., 2014; **Figure 2**). Of the identified variants, seven unique mutations fall within the RXR consensus sequence—a much larger percentage than would be expected by chance (R1742W, R1742Q, R2290C, R2290H, R2292C, R2639H, R2833S). R2290C, a mutation falling within the RXR consensus sequence, was discovered as a *de novo* variant originating on the paternal chromosome (Iossifov et al., 2014). This RXR consensus region is highly conserved across evolution, suggesting a particular functional relevance for this region that is linked to ASD pathogenesis.

Each subrepeat is composed of an 11-stranded beta-jelly roll fold, and the RXR consensus sequence is found at the beginning of the 10th beta sheet (Nogi et al., 2006). Arginine is important structurally for hydrogen bonding with the protein backbone (Borders et al., 1994). Disruption at this position could compromise protein folding, exposing the hydrophilic pore and enabling novel interactions. Alternatively, considering that Reelin may serve as an extracellular matrix (ECM) molecule, these closely spaced

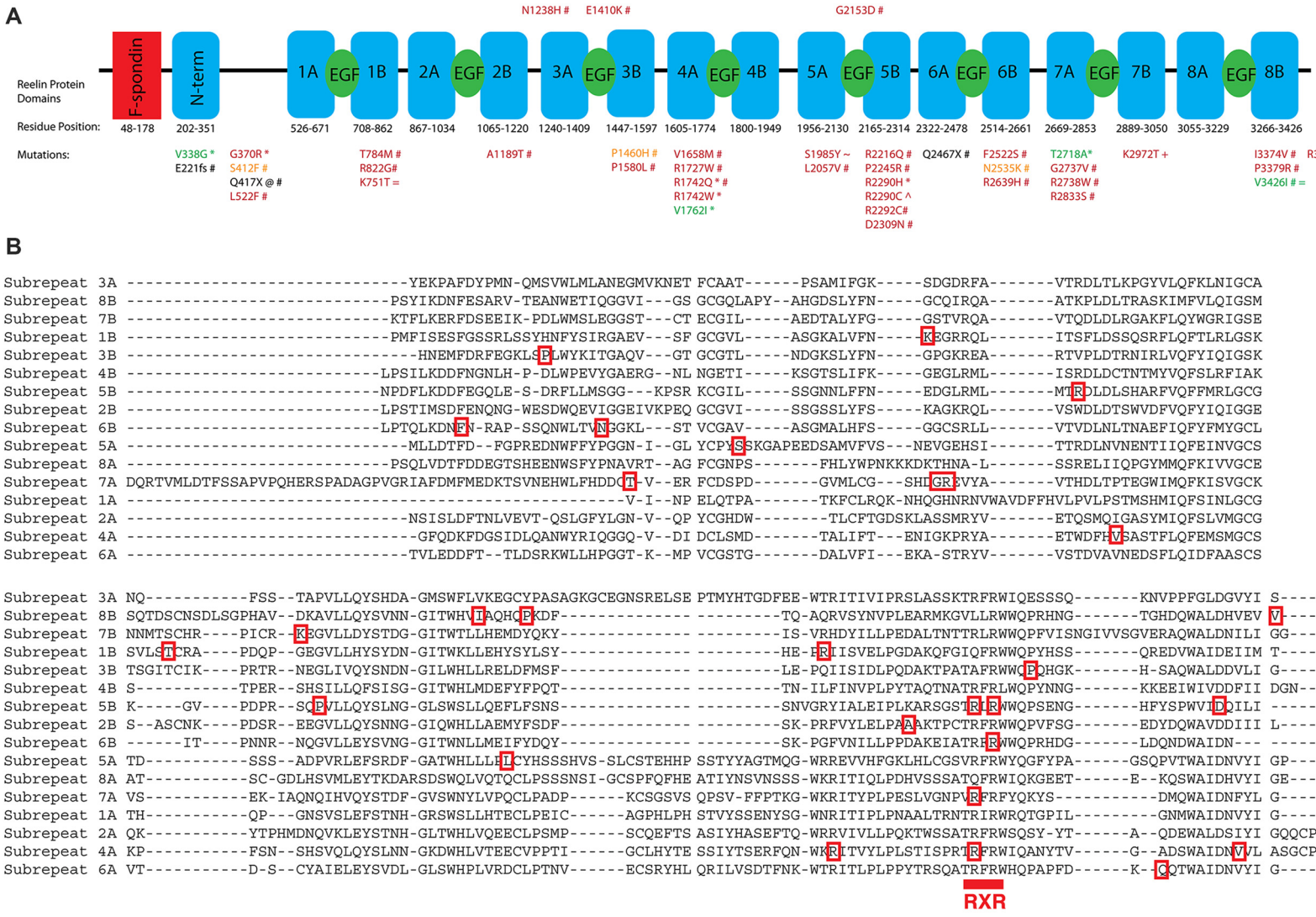

FIGURE 2 | The structure of Reelin is diagrammed with conserved domain boundaries mapped (NCBI Conserved Domain Database). (A) Missense, nonsense, and frameshift mutations that are absent in controls and thus more likely to contribute to ASD are indicated (*Bonora et al., 2003; @Neale et al., 2012; +Koshimizu et al., 2013; #De Rubeis et al., 2014; ^Iossifov et al., 2014; ~Yuen et al., 2015, =Zhang et al., 2015). Specifically, mutations identified by Bonora et al. (2003) and De Rubeis et al. (2014) as occurring in isolated controls from case-control studies, even if they overlap with mutations identified in other studies, are not pictured. This process did not remove any RXR mutations identified in ASD probands; however, three RXR mutations were identified in controls: R1198H, R2104H, and R2292H. Missense mutations are colored based on PolyPhen2 predictions (green, benign; orange, possibly damaging; red, probably damaging). Nonsense and frameshift mutations are indicated in black. **(B)** Clustal Omega alignment of the sub-repeat domains is annotated with the corresponding missense mutations from **(A)**. The RXR consensus sequence is indicated below the aligned repeat sequence. Note: corrections to annotations for missense mutations from supplementary file S4 of De Rubeis et al. (2014) are as follows: R3439Q to R3441Q, G254V to G2737V, R156H to R2639H.

arginines and their neighboring tryptophan residues may be important for glycosaminoglycan binding (Panteri et al., 2006).

Clustering of mutations within this RXR consensus sequence argues against random mutations leading to complete loss-of-function. Presumably many mutations throughout the 3460 amino acids of Reelin could disrupt function. Therefore, a mutational hotspot might suggest a particular mode of loss-of- or gain-of-function, the details of which will need to be determined experimentally.

Animal models of *RELN* mutations may ultimately be necessary to parse out the link between *RELN* and ASD. Thus far, simple loss-of-function alleles have not provided overwhelming evidence that heterozygous *RELN* mutations in the mouse produce overt or consistent behavioral phenotypes reminiscent of ASD (Moy and Nadler, 2008). Similarly, the human genetics of *RELN* mutations suggests that a second hit, either environmental or genetic, may be necessary for ASD. Parents of patients with LCH are heterozygous for *RELN* loss-of-function alleles but do not have ASD (Hong et al., 2000; Chang et al., 2007). Approximately half of the ASD-associated mutations identified in *RELN*, including truncating and RXR mutations, are inherited from normal parents. In addition, following the same method of characterizing mutations in controls from ASD studies, here too there are examples of a nonsense mutation truncating Reelin before the receptor binding domain (Q849X) and RXR consensus mutations (R1198H, R2104H, and R2292H; Bonora et al., 2003; De Rubeis et al., 2014).

Since *RELN* is particularly susceptible to environment-driven epigenetic changes, one can hypothesize that perhaps a single mutation, which decreases its expression, combined with environmental down-regulation of Reelin production, could drive Reelin protein levels below a critical threshold in the brain. Or, perhaps another modifying gene allele in trans provides this added susceptibility. One likely contributing factor is sex. *RELN* mutations occur in approximately four times as many male as female probands. And indeed, testosterone and estrogen have differing effects on *RELN* expression and HRM phenotypes (Hadj-Sahraoui et al., 1996; Absil et al., 2003; Biamonte et al., 2009; Macri et al., 2010).

Adding to the excitement and promise of deciphering the role of *RELN* in ASD is evidence that Reelin supplementation or increased production could potentially reverse behavioral consequences of decreased Reelin signaling (Rogers et al., 2011, 2013; Hethorn et al., 2015). As mutations in *RELN* continue to be identified in genetic studies and the molecular mechanisms of these mutations are elucidated, we will better understand the role of Reelin in neuronal signaling, development, and ASD.

In the same way that science stresses transitioning from the bench to the bedside, we must also start to move gene candidates from the computer to the bench. *RELN* is now well positioned to make such a transition in ASD research. This approach will not be without its challenges, since it is predicted that for *RELN* and many of the other candidate genes, a single mutation may not be sufficient to cause overt ASD phenotypes, and gene-gene or gene-environment interactions will need to be considered.

AUTHOR CONTRIBUTIONS

DBL wrote the manuscript and BWH suggested the topics to be covered and edited its content.

FUNDING

This work was supported by R01 NS073662 (BWH) and F31 NS086731 (DBL). National Institute of Neurological Disorders and Stroke/National Institutes of Health.

ACKNOWLEDGMENTS

We would like to thank Eric Olson for discussions, Eric Larsen for explaining the processes for filtering and listing mutations on SFARI Gene-Autism Database, and Bonnie Lee Howell for editing the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 05 April 2016
doi: 10.3389/fncel.2016.00089

Epigenetic RELN Dysfunction in Schizophrenia and Related Neuropsychiatric Disorders

Alessandro Guidotti[1]*, *Dennis R. Grayson*[1] and *Hector J. Caruncho*[2]

[1] Department of Psychiatry, The Psychiatric Institute, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA,
[2] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

REELIN (RELN) is a large (420 kDa) glycoprotein that in adulthood is mostly synthesized in GABAergic neurons of corticolimbic structures. Upon secretion in the extracellular matrix (ECM), RELN binds to VLDL, APOE2, and α3β2 Integrin receptors located on dendritic shafts and spines of postsynaptic pyramidal neurons. Reduced levels of RELN expression in the adult brain induce cognitive impairment and dendritic spine density deficits. RELN supplementation recovers these deficits suggesting a trophic action for RELN in synaptic plasticity. We and others have shown that altered RELN expression in schizophrenia (SZ) and bipolar (BP) disorder patients is difficult to reconcile with classical Mendelian genetic disorders and it is instead plausible to associate these disorders with altered epigenetic homeostasis. Support for the contribution of altered epigenetic mechanisms in the down-regulation of RELN expression in corticolimbic structures of psychotic patients includes the concomitant increase of DNA-methyltransferases and the increased levels of the methyl donor S-adenosylmethionine (SAM). It is hypothesized that these conditions lead to RELN promoter hypermethylation and a reduction in RELN protein amounts in psychotic patients. The decreased synthesis and release of RELN from GABAergic corticolimbic neurons could serve as a model to elucidate the epigenetic pathophysiological mechanisms acting at pyramidal neuron dendrites that regulate synaptic plasticity and cognition in psychotic and non-psychotic subjects.

Keywords: RELN, synaptic plasticity, promoter methylation, schizophrenia, bipolar disorder, Dab1

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers, The State Univesity of New Jersey, USA

Reviewed by:
Marija Kundakovic, Fordham University, USA
Tania L. Roth, University of Delaware, USA

***Correspondence:**
Alessandro Guidotti
aguidotti@psych.uic.edu

Received: *22 January 2016*
Accepted: *21 March 2016*
Published: *05 April 2016*

Citation:
Guidotti A, Grayson DR and Caruncho HJ (2016) Epigenetic RELN Dysfunction in Schizophrenia and Related Neuropsychiatric Disorders Front. Cell. Neurosci. 10:89. doi: 10.3389/fncel.2016.00089

INTRODUCTION

REELIN (RELN) is an extracellular matrix (ECM) glycoprotein that controls neuronal cell migration and the lamination of the corticolimbic structures during embryonic development (D'Arcangelo et al., 1995). RELN also plays a role in controlling dendritic spines, and synapse structure and function in adulthood (Costa et al., 2001). Research in the last 20 years, has suggested that abnormal brain RELN expression is a feature that associates with major neuropsychiatric disorders including schizophrenia (SZ), bipolar (BP) disorder (Impagnatiello et al., 1998; Fatemi et al., 2000; Guidotti et al., 2000), autism (Fatemi, 2002), depression (Lussier et al., 2009, 2011, 2013), and Alzheimer's disease (Herz and Chen, 2006). While the role of RELN in dendritic spine structure, synapse plasticity, and cognitive function in adulthood has been extensively studied, considerably less research has focused on the mechanisms whereby RELN expression is altered in neuropsychiatric conditions.

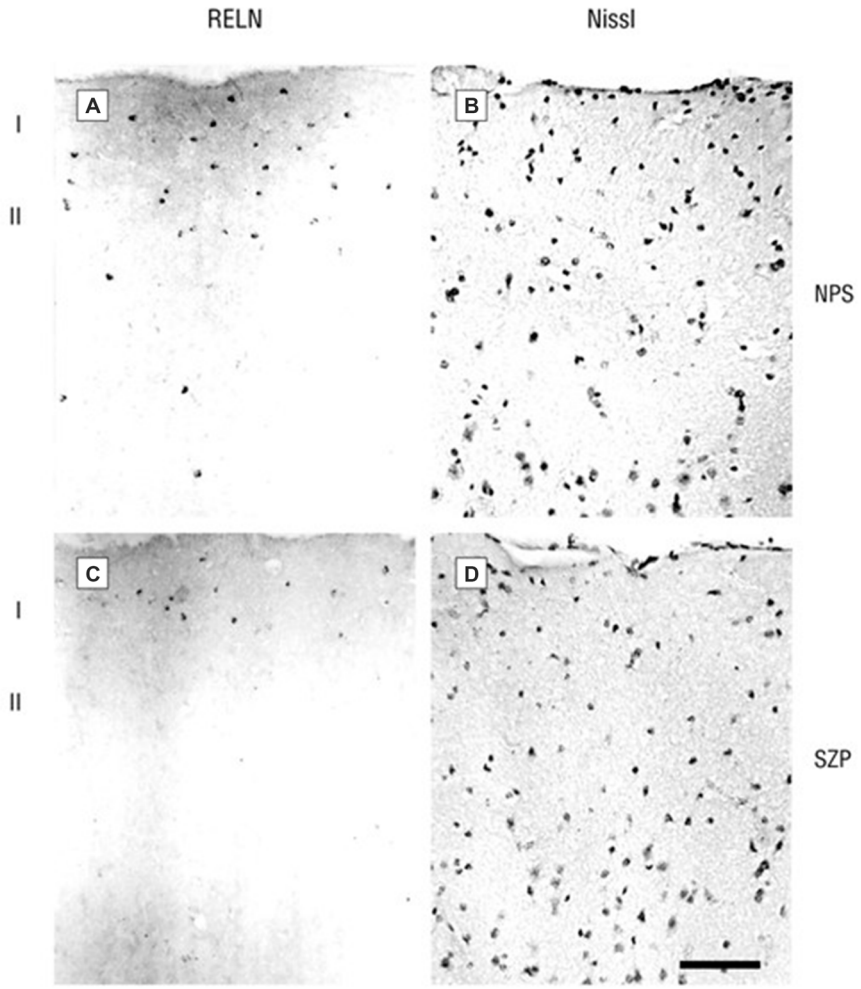

FIGURE 1 | Photomicrographs of 20 μm sections of prefrontal cortex (PFC) of a non-psychiatric subject (NPS) and of a schizophrenia patient (SZP) immunolabeled for RELN (A,C, left side) or Nissl-stained (B,D, right side). RELN positive neurons are mostly localized in layer 1. Note that the NPS has a higher density of RELN-positive cells and also a stronger extracellular diffuse RELN immunostaining halo. Reprinted with permission from Guidotti et al. (2000).

Here, we review evidence for a role of the epigenetic control of the expression of RELN in the regulation of neuronal plasticity and behavior in SZ and BP disorder patients compared with controls devoid of major psychiatric disorders.

RELN IN THE ADULT MAMMALIAN BRAIN

Neuronal Location

In the cortex and hippocampus of adult rodents and primates, RELN is predominantly synthesized and secreted by GABAergic interneurons (Alcántara et al., 1998; Impagnatiello et al., 1998; Pesold et al., 1998, 1999; Guidotti et al., 2000; Rodriguez et al., 2000; Kadriu et al., 2012). Immunohistochemistry coupled to *in situ* hybridization studies distinguishes at least two sets of GABAergic interneurons based on their ability to synthesize and secrete RELN. The first synthesizes and secretes RELN onto apical and basal dendrites of pyramidal neurons and includes GABAergic horizontal, double bouquet, multipolar and Martinotti neurons in layers 1 and 2 of the mammalian cortices (**Figure 1**). The second set of GABAergic neurons, which do not usually express RELN, include chandelier and basket interneurons that innervate the axon initial segment or somata of pyramidal neurons, respectively (Pesold et al., 1998, 1999). In contrast, in cerebellum, RELN is predominately synthesized by glutamatergic granule neurons, and is secreted by their parallel fiber axon terminals into the ECM surrounding the dendrites of GABAergic Purkinje cells (Pesold et al., 1998). Studies in primary cultures of rat cerebellar granule cells suggest that RELN is secreted in the extracellular medium in a manner that is blocked by the constitutive secretory pathway inhibitor brefeldin. Moreover, secretion of RELN is independent of neuronal activity (Lacor et al., 2000). These findings suggest the possibility that secretory pathway activators might be useful in facilitating RELN secretion when RELN expression is compromised.

Extracellular Location

Once released in the extracellular space, RELN binds to VLDL, APOE and α3β1 integrin receptors activating the signal transduction system in the effector cells including apical and basilar dendrites of pyramidal neurons in the neocortex or Purkinje cells in the cerebellum (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Dong et al., 2003; Strasser et al., 2004).

Using electron microscopic techniques, Costa et al. (2001) demonstrated the presence of RELN-like immunoreactivity decorating the dendritic shafts and spines of distal apical dendrites of pyramidal neurons in the frontal cortex. This area, as well as the hippocampal fissure, is characterized by strong diffuse RELN–immunoreactivity (Pesold et al., 1998). The colocalization of RELN with the α3 subunit of the integrin receptor at post-synaptic densities of adult rat and primate brains suggests that a RELN signaling mechanism involving integrin and VLDL and APOE2 receptors may be operative in modulating the strength of synaptic function (Rodriguez et al., 2000; Dong et al., 2003; Niu et al., 2004). It has been shown that RELN interacting with VLDL, APOE2, or integrin receptors results in activation of the Src-tyrosine kinase family Fyn-kinase, leading to tyrosine phosphorylation and recruitment of the cytoplasmic adaptor protein DAB1 (**Figure 2**; Jossin et al., 2003; Bock et al., 2004; Kuo et al., 2005). Studies suggest that DAB1 phosphorylation is a crucial step in the activation of RELN signal transduction pathways (Rice et al., 1998; Trommsdorff et al., 1999; Niu et al., 2004; Howell and Pillai, 2015). DAB1 is frequently expressed in proximity of synapses located on dendritic spines or shafts of cortical pyramidal neurons (Rodriguez et al., 2000). Hence, phosphorylated DAB1 may regulate cytoskeletal protein synthesis at dendrites by activating the translation of dendritic resident mRNAs (see **Figure 2**, from Costa et al., 2001).

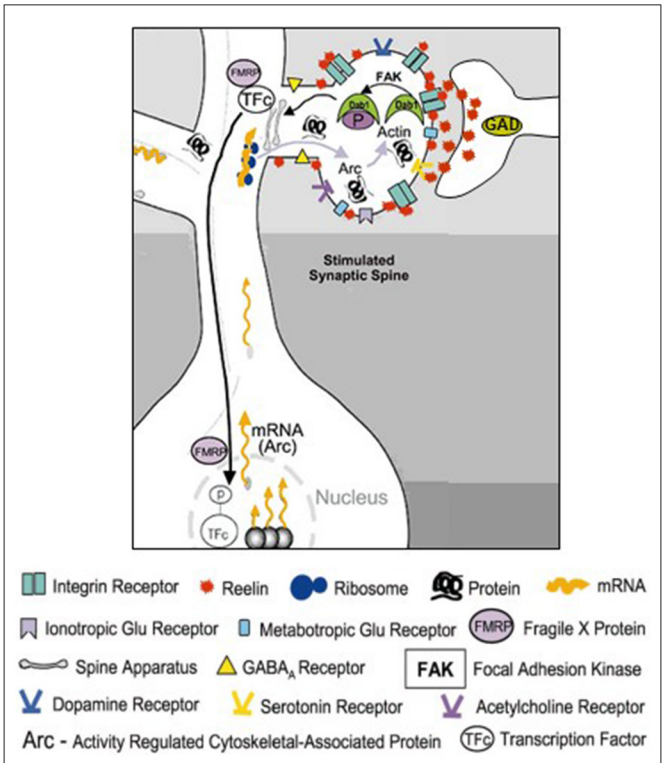

FIGURE 2 | Schematic representation of the RELN signaling pathways in dendritic spines. RELN released in the extracellular matrix (ECM) from the terminal of cortical GABAergic neuron binds to dendritic shafts and spines and modulates the transcriptional function of activated dendritic synapses. Modified from Costa et al. (2001). RELN binding to Integrin receptors located on dendritic spines mediates activation of focal adhesion kinase (FAK) that directly or indirectly, via activation of Fyn kinase, phosphorylates DAB1 adaptor protein resident in dendrites. Phosphorylated dendritic DAB1 may recruit or activate ribosomal structures and induce the synthesis of ARC (Activity regulated cytoskeletal-associated protein) or cytoskeletal proteins like β-actin.

RELN REGULATES SPINE DENSITY AND EXCITATORY SYNAPTIC FUNCTION

Animal models in which RELN expression is genetically reduced provide important information on the impact of RELN on synaptic plasticity and cognition. *Reeler* mice display altered LTP and deficits in active avoidance tasks (Goldowitz and Koch, 1986; Marrone et al., 2006). Young adult heterozygous *reeler* mice (HRM) which exhibit a 50% reduction in RELN content have significantly reduced dendritic spine densities and also show a deficit in LTP (Tueting et al., 1999, 2006; Liu et al., 2001; Levenson et al., 2008; Niu et al., 2008; Iafrati et al., 2014). Adult HRM also have a defective molecular composition of the synaptic structure (Ventruti et al., 2011), as well as deficits in excitatory postsynaptic responses to glutamate receptor agonists and reduced LTP (Levenson et al., 2006). Addition of recombinant RELN to hippocampal slices or direct injection of RELN into the cerebral ventricles enhances hippocampal LTP (Beffert et al., 2006; Pujadas et al., 2010; Rogers et al., 2011).

HRM also display deficits in cognitive function (Krueger et al., 2006; Stranahan et al., 2011), executive function (Brigman et al., 2006), fear conditioning learning (Ammassari-Teule et al., 2009), anxiety and motor impulsivity (Ognibene et al., 2007). Importantly, RELN supplementation recovers sensory motor gating, synaptic plasticity, and associative learning deficits in HRM (Rogers et al., 2013). In addition to the HRM data described above, Lussier et al. (2013) reported that hippocampal RELN deficiency elicited by chronic stress (repeated corticosterone treatment) can impair adult hippocampal neurogenesis and lead to the development of a depression-like phenotype. Co-treatment with antidepressant drugs prevents both RELN deficit and the development of the depression-like phenotype (Fenton et al., 2015).

Addition of recombinant RELN to cortical synaptosomes *in vitro* induces the expression of activity-regulated cytoskeleton-associated protein (Arc; Dong et al., 2003), and augments the density and clustering of proteins in postsynaptic membranes (i.e., neurotransmitter receptors), which provides further evidence of a functional

role for RELN in regulating the synaptic strength of glutamatergic inputs onto dendritic spines (Caruncho et al., 2004).

RELN IN THE BRAINS OF SCHIZOPHRENIA (SZ) AND BIPOLAR (BP) DISORDER PATIENTS

A number of molecular, anatomical (dendritic spine density), behavioral, and cognitive deficits associated with reduced RELN expression (mRNA and protein) are observed in subjects with SZ and BP disorder. In different post-mortem brain cohorts, we and others have demonstrated that RELN expression is reduced by approximately 50% in the prefrontal cortex (PFC), temporal cortex, hippocampus, and caudate nucleus of patients with SZ and BP disorder (Impagnatiello et al., 1998; Fatemi et al., 2000; Guidotti et al., 2000). In successive studies we found that the expression of RELN was paralleled by decreases in the levels of GAD67 but not DAB1 or GAD65. Slices from the same samples immunostained for RELN and counterstained for Nissl or NeuN to recognize neurons showed that RELN-positive neurons were significantly decreased by 30–50% in patients with SZ or BP disorder with psychosis but not in those with unipolar depression when compared to non-psychiatric subject (NPSs; **Figure 1**). Differences were absent for GAD65, and NeuN expression implying that RELN and GAD67 down-regulation is unrelated to neuronal damage (Guidotti et al., 2000). The RELN and GAD67 downregulation is also unrelated to postmortem interval, dose, duration, or presence of antipsychotic medication. Similar to HRM, RELN deficiency in the neocortex of SZ and BP disorder patients is associated with a decrease in GAD67, reduced prepulse inhibition to startle, and loss of dendritic spines, all features associated with SZ pathology (Tueting et al., 1999; Glantz and Lewis, 2001; Liu et al., 2001; Grayson and Guidotti, 2013).

Like the RELN deficiency in the cerebellar cortex of HRM, the RELN deficiency in cerebellar cortex of SZ and BP disorder patients is associated with a 20% decrease of GABAergic Purkinje neurons (Hadj-Sahraoui et al., 1996; Maloku et al., 2010). Collectively these data suggest that RELN plays a central role in inducing and maintaining the structure and function of GABAergic and glutamatergic neurons and their reciprocal interactions (Grayson and Guidotti, 2013).

Since SZ and BP disorder have a neurodevelopmental origin (Folsom and Fatemi, 2013) and RELN is a major player in brain development and maturation (D'Arcangelo et al., 1995), an important question raised by these studies is whether the altered epigenetic (promoter hypermethylation) regulation of RELN in brains of SZ and BP patients is initiated early in embryonic or perinatal life or develops later in life as the consequence of the GABAergic neuropathology related to the development of SZ morbidity. To address this question, the extent of methylation of the RELN promoter was measured in offspring born from mice stressed during pregnancy. These offspring, at adulthood, display SZ-like behavioral endophenotypes (increased locomotor activity, PPI, social recognition deficits), and a decrease of RELN, GAD67, and BDNF expression associated with an increase in methylation at their respective promoters. We also found that the amount of Methyl CpG Binding Protein 2 (MECP2) binding to the *RELN* promoter at birth was higher than that observed in the adult (Matrisciano et al., 2013). These data suggest that RELN promoter hypermethylation is likely initiated early in life, including during embryonic life, and is then maintained throughout adulthood.

IS AN ALTERED EPIGENETIC REGULATION OF GENE EXPRESSION THE MOLECULAR MECHANISM MEDIATING RELN EXPRESSION DOWN-REGULATION IN SZ AND BP DISORDER?

Mutations in the RELN gene are associated with a form of autosomal recessive lissencephaly with abnormal axonal connectivity, and cerebellar hypoplasia (Hong et al., 2000). Human subjects with RELN gene mutations exhibit marked ventricular dilation, mental retardation, and epilepsy and a marked decrease in muscle tone that appears of neurogenic origin (Hourihane et al., 1993). Heterozygous RELN mutations have been shown to cause autosomal-dominant lateral temporal epilepsy (Dazzo et al., 2015).

A highly conserved single nucleotide polymorphism (SNP) has been identified in the vicinity of the regulatory region of the RELN gene (Shifman et al., 2008; Wedenoja et al., 2010). This polymorphism is associated with an increased risk of psychotic symptoms. Although these studies highlight the importance of RELN gene variants as risk factors in the etiopathogenesis of psychiatric disorders, it is important to note that variants in the RELN gene are rare and cannot explain the high frequency of RELN expression downregulation observed in the general population of SZ, BP disorder and autism spectrum disorder patients (Zhang et al., 2002; Lintas and Persico, 2010; Grayson and Guidotti, 2013; Wang et al., 2014; Zhubi et al., 2014).

The epidemiological and clinical evidence that SZ and BP disorders do not follow the rules expected for a Mendelian-genetic disorder led to the proposal that environmental insults may influence RELN gene expression by altering epigenetic regulatory mechanisms and led to the hypothesis that epigenetic factors are operative in mediating changes in the expression of RELN and other SZ candidate genes in psychotic patients (Costa et al., 2003). To better understand the rules governing the epigenetic regulation of *RELN*, we cloned the human gene and experimentally examined its regulation in both neuroprogenitor NT2 cells (Chen et al., 2002; Mitchell et al., 2005) and mouse cortical neurons *in vitro* (Dong et al., 2003; Noh et al., 2005). Data from these studies support the concept that the *RELN* promoter is regulated epigenetically through changes in DNA methylation.

TABLE 1 | Summary showing studies of RELN methylation relevant to neurobiology[a].

Reference	Location[b]	Species	Tissue	Design	Method	Result
Abdolmaleky et al. (2005)	Promoter: below −700 bp	Human	Frontal Lobe	RELN methylation in SZ vs. Con were compared	Bisulfite seq, Methylation specific PCR	*RELN* promoter is hypermethylated in SZ
Aberg et al. (2014)	First Intron	Human	Whole Blood	RELN methylation in SZ vs. Con were compared	Methyl Binding Domain-profiling	*RELN* is hypermethylated in SZ
Blaze et al. (2013)	Promoter	Rats	Medial Prefrontal Cortex	Comparison of methylation status at the *Reln* promoter as a function of post-natal maltreatment or nurturing care	Methylation specific PCR	*Reln* methylation varies by condition, age and sex
Chen et al. (2002)	Promoter: below −527 bp	Human	NT2 Cells	*RELN* methylation in differentiated vs. control neuroprogenitor cells *in vitro* were examined	Bisulfite seq	*RELN* hypermethylation in NT2 cells that is demethylated on differentiation
Dong et al. (2007)	Promoter: −520 to −198 bp	Mouse	Frontal Cortex	Methionine (MET) induced hypermethylation of the *RELN* promoter was examined and the effects of VPA and MS-275 on this methylation *in vivo* were evaluated	Methylation specific PCR	MET induces *RELN* methylation, while VPA and MS-275 reverse this methylation
Dong et al. (2016)	Promoter: −220 to +70 bp	Mouse	Frontal Cortex	PRS mice were examined for changes in *Reln* promoter methylation or hydroymethylation at PND 75 following either vehicle (VEH), clozapine (CLZ) or haloperidol (HAL)	MeDIP, hMeDIP	At PND 75, PRS strongly induces RELN promoter hypermethylation and, to a lesser extent hydroxyl methylation, of the *Reln* promoter. CLZ, but not HAL, attenuates the PRS-induced hypermethylation
Grayson et al. (2005)	Promoter: below −527 bp	Human	BA 9 and 10	*RELN* methylation in SZ vs. Con were compared	Bisulfite seq	*RELN* is hypermethylated in SZ
Kobow et al. (2009)	Promoter: below −500 to +100 bp	Human	Hippocampus	Human temporal lobe epilepsy (TLE) biopsy specimens vs. autopsied control tissue were compared	Bisulfite seq	*RELN* promoter hypermethylation was observed in TLE biopsies
Kundakovic et al. (2009)	Promoter: below −250 bp	Human	NT2 Cells	The effect of the HDAC inhibitor, MS-275, on RELN promoter methylation was determined	MeDIP followed by qPCR	MS-275 induces *RELN* promoter demethylation
Levenson et al. (2006)	Promoter: between −1000 and −500 bp	Mouse	Hippocampal Slice Preparation	The response of neurons in slices to the effects of Protein Kinase C (PKC) activation by phorbol esters and or by inhibitors of DNA methylation	Methylation specific PCR	Reln promoter methylation is decreased by inhibitors of DNA methylation and activators of PKC
Lintas and Persico (2010)	Promoter: below −413 bp	Human	BA 41 and 42	*RELN* promoter methylation was examined in pre- and post-pubertal post-mortem brain from non-psychiatric subjects	Bisulfite seq	Post-pubertal *RELN* promoter is hypermethylated compared with pre-pubertal *RELN*

(Continued)

TABLE 1 | (Continued)

Reference	Location[b]	Species	Tissue	Design	Method	Result
Matrisciano et al. (2011)	Promoter: −423 to −252 bp	Mouse	Frontal Cortex	PRS mice were examined for methylation vs. non-stressed mice and the effect of LY379268 on this methylation	MeDIP	PRS mice showed *Reln* promoter methylation which was reduced by LY379268
Matrisciano et al. (2013)	Promoter: −432 to −252 bp	Mouse	Frontal Cortex	PRS mice were analyzed for changes in Reln promoter methylation and hydroxymethylation vs. Con	MeDIP and hMeDIP	PRS mice showed elevated Reln promoter methylation and hydroxymethylation at PND 60
Mill et al. (2008)	Promoter	Human	Frontal Cortex	*RELN* methylation in SZ vs. BD vs. Con were compared	Pyrosequencing	No change between groups
Mitchell et al. (2005)	Promoter: below −500 bp	Human	NT2 Cells	Determined the effects of HDAC and DNMT inhibitors on *RELN* promoter methylation	Bisulfite seq	TSA, VPA and AZA induces *RELN* hypomethylation
Noh et al. (2005)	Promoter: −340 to +140 bp	Mouse	Cortical Neurons	MET was used to manipulate *RELN* promoter methylation *in vitro*	Bisulfite seq	MET induced *RELN* promoter hypermethylation
Palacios-Garcia et al. (2015)	Promoter: −786 to −625 bp	Rats	Whole Cortex Cultured Neurons	PRS rats were analyzed for changes in *Reln* promoter methylation *in vivo* and *in vitro*	Methylation sensitive restriction enzyme PCR	*Reln* promoter methylation is increased in newborn PRS rats and in cultured neurons *in vitro*
Qin et al. (2011)	Promoter	Rat	Hippocampus	The effects of maternal deprivation on *Reln* promoter methylation were examined	Methylation specific PCR	Maternal deprivation facilitated increased *Reln* promoter methylation
Sui and Li (2010)	Promoter: −700 to −400 bp	Rat	Hippocampus	Promoter methylation was analyzed in rats with perinatal hypothyroidism at PND 1 through 60	Methylation specific PCR	Hypothyroid rats show elevated *Reln* promoter methylation at PND 1, 5 and 15 relative to Con
Sui et al. (2012)	Promoter	Rat	Medial Prefrontal Cortex	Promoter methylation was analyzed following the induction of LTP as compared with Con	Methylation specific PCR	High frequency stimulations induce DNA demethylation at the *Reln* promoter vs. Con
Tremolizzo et al. (2002)	Promoter: −340 to +160 bp	Mouse	Frontal Cortex	The effects of VPA treatment on the MET-induced hypermethylation of the *RELN* promoter were evaluated	Bisulfite seq	Methionine induces *RELN* methylation, while VPA reverses this effect
Zhubi et al. (2014)	Promoter: −220 to +70 bp	Human	Cerebellum	Reln promoter methylation and hydroxymethylation were analyzed in autism spectrum disorder (ASD) vs. typically developed subjects (Con)	MeDIP and hMeDIP	While *RELN* promoter methylation levels are unchanged between ASD and Con, 5hmC content at the promoter is increased

[a]*The above studies do not include at least one report (Tochigi et al., 2008) that showed no detectable RELN promoter methylation in either SZ or control subjects by pyrosequencing. In addition, there are numerous studies showing that elevated RELN promoter methylation is associated with poor prognosis in various types of cancers. We apologize for any relevant studies that were inadvertently omitted from this list.* [b]*For precise locations of the RELN methylation see the associated reference. The locations provided are approximate and if no coordinates are indicated, then the information was not in the original report. For example, Promoter (without additional information) indicates that the authors specified the RELN promoter without giving additional coordinates. ASD, Autism spectrum disorder; Bisulfite seq, Bisulfite sequencing; CLZ, Clozapine; Con, Control; HAL, Haloperidol; hMeDIP, Hydroxymethyl DNA immunoprecipitation; LTP, Long-term potentiation; MeDIP, Methyl DNA immunoprecipitation; NT2 cells, Ntera2 cells; PCR, Polymerase chain reaction; PKC, Protein kinase C; PND, Post-natal day; PRS, Pre-natal restraint stress; TLE, Temporal lobe epilepsy; VEH, Vehicle; VPA, Valproic acid.*

Furthermore, we have reported that the down-regulation of RELN expression in GABAergic neurons of SZ and BP patients is associated with an overexpression of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3a (DNMT3a) in neocortical and striatal GABAergic neurons (Veldic et al., 2004, 2007; Ruzicka et al., 2007). DNMTs are a family of enzymes that catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the 5′ carbon of cytosine of many gene promoters (Grayson and Guidotti, 2013). Increased promoter methylation generally leads to decreased gene expression. Interestingly, the inhibitory action of DNMTs on RELN expression also likely occur through the formation of chromatin repressor complexes which include, in addition of DNMTs, also the methyl CpG binding domain proteins, SIN3A, and histone deacetylases (see Grayson and Guidotti, 2013 for review).

The hypothesis that an epigenetic pathology of the *Reln* promoter is operative in the transcriptional down-regulation of the corresponding gene in SZ or BP disorder patients is supported by the evidence that there is an increased level of SAM in the PFC of these patients (Guidotti et al., 2007), and that hypermethylation of the *RELN* promoter (Abdolmaleky et al., 2005; Grayson et al., 2005, 2006; Lintas and Persico, 2010) is associated with the down-regulation of the corresponding protein in the PFC of psychotic patients (Guidotti et al., 2000), although negative findings for RELN promoter hypermethylation have also been reported (Mill et al., 2008).

In other studies a decreased histone methylation at GABAergic gene promoters (Huang et al., 2007), and an increased histone deacetylase -1 expression and down regulation of GABAergic gene expression in PFC and hippocampus of SZ patients have been reported (Benes et al., 2007; Sharma et al., 2008). A summary of many of the methylation studies of *RELN* in neuropsychiatric patients, SZ-like epigenetic mouse models, and neuronal culture systems are summarized in **Table 1**. These data are consistent with the epigenetic GABAergic theory of major psychosis (Costa et al., 2003; Grayson and Guidotti, 2013) and suggest that *RELN* promoter methylation should be further studied to establish its temporal and casual association with the etiopathogenesis of SZ and BP disorder.

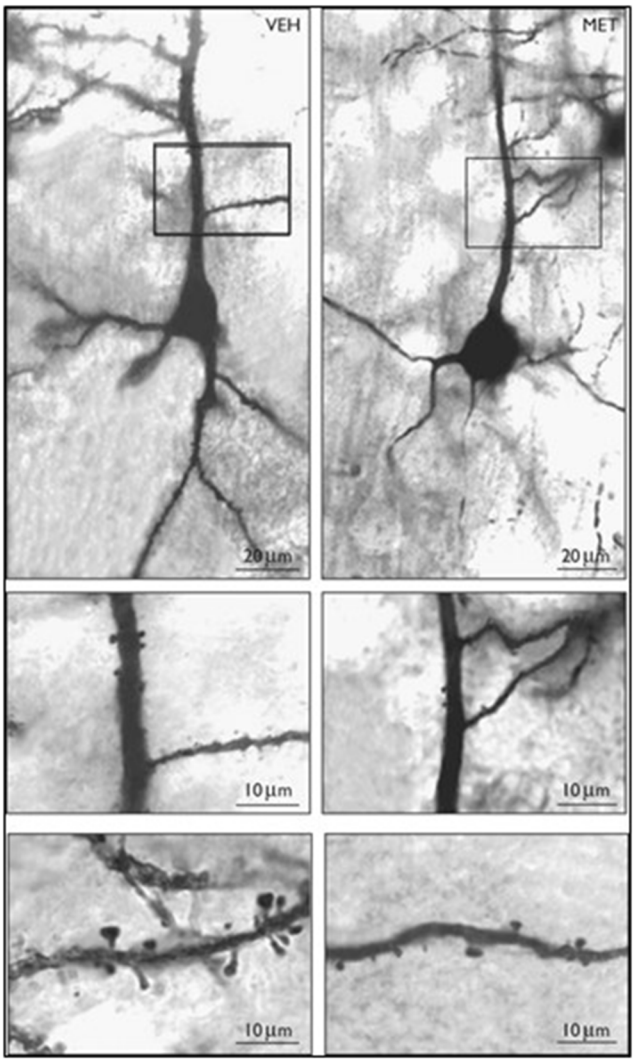

FIGURE 3 | Photomicrographs of Golgi stained layer III pyramidal neurons in vehicle (VEH) and methionine (MET) treated mouse frontal cortex. Top panel: 10× objective; Middle panel: 20×; Bottom panel: 100× Vehicle or L-methionine (5.2 mmol/kg/twice a day) was administered for 14 days. Reprinted with permission from Tueting et al. (2010).

RELN, SPINE DENSITY DOWN REGULATION AND COGNITIVE PERFORMANCE DEFICITS INDUCED BY L-METHIONINE TREATMENT

Support for the hypothesis that an increase of DNA methylation contributes to the down-regulation of RELN and other GABAergic or glutamatergic genes in psychotic patients is sustained by clinical studies conducted in the early 1970s (for review see Wyatt et al., 1971; Cohen et al., 1974; Grayson et al., 2009). In these studies L-methionine (MET, the precursor of SAM), administered in large doses (10–20 g/day) for 3–4 weeks to SZ patients was reported to exacerbate psychotic symptomatology (Cohen et al., 1974; Grayson et al., 2009). Patients were administered large doses of L-methionine either with or without a monoamine oxidase inhibitor in an attempt to reduce the levels of putative bioactive psychedelic compounds. Interestingly, many of the treated patients responded with a worsening of their symptoms (Cohen et al., 1974).

In both mouse FC and neuronal cultures, the administration of large doses of L-methionine increases SAM levels and facilitates the hypermethylation of GABAergic gene promoters, including *Reln*, and *GAD67* and the reduced expression of these genes (Tremolizzo et al., 2002, 2005; Mitchell et al., 2005; Noh et al., 2005; Chen et al., 2007). Similar to the

HRM, spine density is also decreased in the dendrites of mice treated with L-methionine (**Figure 3**, Tueting et al., 2010). Furthermore, L-methionine treated mice display SZ-like behavioral abnormalities (Tremolizzo et al., 2005). Collectively, these data suggest that the reduction of dendritic spines observed in brain of L-methionine-treated mice are likely due to MET-induced altered epigenetic mechanisms that lead to decreased expression of RELN (Tremolizzo et al., 2005; Tueting et al., 2010).

RELN PROMOTER METHYLATION IN NEURONS IS A DYNAMIC PROCESS THAT CAN BE TARGETED BY ENVIRONMENTAL FACTORS AND DRUGS

The dogma that in post-mitotic neurons DNA methylation patterns are established during development and remain stable thereafter (Razin and Shemer, 1995) has been challenged by convincing evidence that in post-mitotic neurons, methylation patterns of specific cytosine/guanine (CpG) dinucleotide-rich promoters, change rapidly. Thus, in neurons, promoter methylation provides a series of targets on which the environment, drugs, and/or toxins can modify transcription and affect neuronal phenotype profiles without altering the genotype (Szyf, 2009). To verify this hypothesis, we treated (Tremolizzo et al., 2002, 2005; Tueting et al., 2010) mice protractedly with L-methionine (as described above) and measured the ratio of 5 methyl cytosine (5mC) to unmethylated cytosine (C) of the murine RELN CpG-enriched promoter region from −340 to +160 bp (Tremolizzo et al., 2005) or the murine GAD_{67} CpG-enriched promoter region from −760 to −311 bp (Satta et al., 2008) by measuring the fraction of promoters immunoprecipitated by specific anti-5mC or anti-MeCP2 antibodies with competitive RT-PCR and internal standards (Dong et al., 2005). We found that (Dong et al., 2005; Tremolizzo et al., 2005) methionine induces an increase of brain RELN and GAD_{67} promoter methylation (Dong et al., 2005), and downregulation of RELN and GAD_{67} mRNA and cognate protein expression associated with decreased spine density (**Figure 3**), and SZ-like behavioral modifications (Tremolizzo et al., 2002, 2005; Tueting et al., 2010). The effects of methionine on the RELN promoter, RELN protein level, dendritic spine density, and SZ-like behavioral modifications are reversed by the administration of Valproic acid (VPA) and other HDAC inhibitors (Dong et al., 2005; Tremolizzo et al., 2005). These findings, together with data obtained in the HRM, suggest the working hypothesis that the down-regulation of spine density and SZ-like behavioral modifications in L-methionine treated mice may be, in part, due to decreased expression of RELN.

CONCLUDING REMARKS

SZ and BP are neurodevelopmental disorders with genetic risk load and behavioral and neurochemical SZ-like phenotypes triggered by exposure to prenatal or perinatal environmental insults: stress, toxins, infection, trauma. In mice exposed prenatally to restraint stress, we found increased DNMT levels that are associated with RELN promoter hypermethylation, RELN expression downregulation, SZ-like epigenetic behavioral modifications, and decreased dendritic spine density in adultood (Tremolizzo et al., 2005; Tueting et al., 2010; Dong et al., 2016). L-Methionine supplementation in rats induces epigenetic variations including RELN promoter hypermethylation in offspring (Weaver et al., 2005). Further, there is an epigenomic reprogramming of RELN and glucocorticoid receptors in hippocampal pyramidal neurons after methionine administration (Weaver et al., 2006). Our studies in cultured mouse cortical neurons (Noh et al., 2005) and human neuronal progenitors (Kundakovic et al., 2007, 2009) not only show that the hypermethylation of promoters induced by L-methionine is blocked by siRNA-mediated DNMT-KO or by reduction of DNMT activity with small molecule antagonists but also that this blockade induces the overexpression of RELN, GAD_{67}, or BDNF proteins (Kundakovic et al., 2007, 2009).

Collectively, these data challenge the classic concept that 5-methylcytosine patterns in DNA remain stable in post-mitotic neurons and strongly suggest that by increasing brain SAM content, L-methionine facilitates the promoter methylation mediated by DNMT1 or DNMT3a in the central nervous system (Grayson and Guidotti, 2013). Unlike the DNA sequence of a cell, which is stable and strongly conserved, epigenetic processes that impact DNA methylation and chromatin architecture are highly dynamic. That is, they can be tissue-specific, developmentally-regulated, and modified by a wide range of drugs and other environmental factors (Szyf, 2009; Ptak and Petronis, 2010; Grayson and Guidotti, 2013; Dong et al., 2016).

Studies using the L-methionine mouse model or offspring of restraint stressed mothers may be aimed at determining whether antipsychotics capable of reducing RELN promoter methylation (e.g., clozapine), enhance spine density, and relieve SZ-like epigenetic behaviors (Tremolizzo et al., 2005; Dong et al., 2016). These models should provide useful preclinical tools for screening small molecules for their capacity to reverse SZ candidate gene promoter methylation and the associated neuronal and behavioral deficits.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the ideas and editing of the manuscript.

FUNDING

AG is supported by the following NIH grants AA022538, R01 MH093348, R01 MH101043, DRG is supported by AA022538. HJC is supported by a SHRF Establishment Grant, and a NSERC Discovery grant.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 25 February 2016
doi: 10.3389/fncel.2016.00048

Reelin-Related Disturbances in Depression: Implications for Translational Studies

Hector J. Caruncho[1], Kyle Brymer[2], Raquel Romay-Tallón[3], Milann A. Mitchell[2], Tania Rivera-Baltanás[4], Justin Botterill[2], Jose M. Olivares[4] and Lisa E. Kalynchuk[3]*

[1] Neuroscience Cluster, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada, [2] Department of Psychology, University of Saskatchewan, Saskatoon, SK, Canada, [3] Department of Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [4] Department of Psychiatry, Alvaro Cunqueiro Hospital, Biomedical Research Institute of Vigo, Galicia, Spain

The finding that reelin expression is significantly decreased in mood and psychotic disorders, together with evidence that reelin can regulate key aspects of hippocampal plasticity in the adult brain, brought our research group and others to study the possible role of reelin in the pathogenesis of depression. This review describes recent progress on this topic using an animal model of depression that makes use of repeated corticosterone (CORT) injections. This methodology produces depression-like symptoms in both rats and mice that are reversed by antidepressant treatment. We have reported that CORT causes a decrease in the number of reelin-immunopositive cells in the dentate gyrus subgranular zone (SGZ), where adult hippocampal neurogenesis takes place; that down-regulation of the number of reelin-positive cells closely parallels the development of a depression-like phenotype during repeated CORT treatment; that reelin downregulation alters the co-expression of reelin with neuronal nitric oxide synthase (nNOS); that deficits in reelin might also create imbalances in glutamatergic and GABAergic circuits within the hippocampus and other limbic structures; and that co-treatment with antidepressant drugs prevents both reelin deficits and the development of a depression-like phenotype. We also observed alterations in the pattern of membrane protein clustering in peripheral lymphocytes in animals with low levels of reelin. Importantly, we found parallel changes in membrane protein clustering in depression patients, which differentiated two subpopulations of naïve depression patients that showed a different therapeutic response to antidepressant treatment. Here, we review these findings and develop the hypothesis that restoring reelin-related function could represent a novel approach for antidepressant therapies.

Keywords: reelin, depression, hippocampus, neurogenesis, neural plasticity, lymphocytes, membrane protein clustering

OPEN ACCESS

Edited by:
Adalberto Merighi, University of Turin, Italy

Reviewed by:
Alexander Dityatev, Magdeburg University, Germany
Alessandro Guidotti, University of Illinois at Chicago, USA

****Correspondence:***
Hector J. Caruncho
hector.caruncho@usask.ca

Received: *21 December 2015*
Accepted: *11 February 2016*
Published: *25 February 2016*

Citation:
Caruncho HJ, Brymer K, Romay-Tallón R, Mitchell MA, Rivera-Baltanás T, Botterill J, Olivares JM and Kalynchuk LE (2016) Reelin-Related Disturbances in Depression: Implications for Translational Studies. Front. Cell. Neurosci. 10:48. doi: 10.3389/fncel.2016.00048

BRIEF HISTORY OF REELIN IN THE NERVOUS SYSTEM

The reeler mouse has been widely studied since the 1950's as a model to understand neural development and developmental dysregulations (reviewed in Lambert de Rouvroit and Goffinet, 1998). However, when the reelin gene was cloned by D'Arcangelo et al. (1995), a new field focused on the neurobiology of reelin was firmly established. Early on, reelin was primarily thought

of as a developmental molecule highly expressed in cortical and hippocampal Cajal-Retzius cells and cerebellar granule cells (see Tissir and Goffinet, 2003), but very soon came the demonstration that reelin is preferentially expressed by GABAergic interneurons in the adult cortex and hippocampus of rodents (Alcantara et al., 1998; Pesold et al., 1998, 1999). This was subsequently shown in many other species, including non-human primates and humans (Martínez-Cerdeño et al., 2002; Rodriguez et al., 2002; Roberts et al., 2005; Ramos-Moreno et al., 2006). Although studies with reeler mice already indicated that reelin played an important role in regulating neural migration during brain development (see Lambert de Rouvroit and Goffinet, 1998), additional data demonstrated key roles for reelin in dendritic maturation and dendritic spine development (Niu et al., 2008; Chameau et al., 2009), in promoting synaptic plasticity in spine-impinging synapses, and in memory formation in the adult brain (Pesold et al., 1999; Rodriguez et al., 2000; Weeber et al., 2002; Beffert et al., 2005, 2006; Pujadas et al., 2010). More recently, it has become clear that reelin influences various aspects of hippocampal neurogenesis, including neural progenitor fate, neuronal migration, dendritic spine development and the integration of granule neurons into hippocampal circuitry. The absence of Dab1, a protein that is part of the reelin signal transduction pathway, limits dendritic development in dentate neuroprogenitor cells and causes those cells to migrate ectopically into the hilus (Teixeira et al., 2012). However, enhancing hippocampal reelin levels seems to normalize migration and increase the maturation rate of newborn granule neurons (Pujadas et al., 2010; Teixeira et al., 2011). Taken together, these results outline an important role for reelin in regulating hippocampal plasticity in the adult brain.

Although the vast majority of research on reelin has been conducted in the mammalian brain, reelin is also present in non-mammalian species. For example, reelin is highly expressed in the larval sea lamprey brain, particularly during the metamorphic stage of development (Pérez-Costas et al., 2002, 2004). Lampreys are primitive vertebrates with a laminar brain and no conventional radial-migration during development. Given that reelin is a key regulator of radial-migration in the rodent brain (Rakic and Caviness, 1995), the presence of reelin in larval sea lampreys suggests that in phylogenetic terms, reelin could have initially evolved as a molecule for regulating synaptic remodeling, and only later on became important for neural migration (Pérez-Costas et al., 2002).

The conceptualization of reelin as a pleiotropic extracellular matrix molecule with multiple roles in brain development and in adult brain plasticity captured the attention of Erminio Costa and Alessandro Guidotti, who hypothesized that reelin expression could be dysregulated in psychotic disorders (i.e., schizophrenia), and that baseline levels of reelin could be an important vulnerability factor in a two-hit neurodevelopmental hypothesis for the development of schizophrenia. Their initial studies revealed a downregulation of about 50% of brain reelin expression levels in both schizophrenia and bipolar disorder (Impagnatiello et al., 1998; Guidotti et al., 2000). These results were independently replicated by several research groups (Fatemi et al., 2000; Eastwood and Harrison, 2003; Knable et al., 2004; Torrey et al., 2005; Habl et al., 2012), opening the field for subsequent investigation of how reelin dysregulation might be operative in the pathogenesis and/or pathophysiology of multiple psychiatric disorders. Years later, Costa and Guidotti pioneered the study of epigenetic alterations as the possible cause of reelin downregulation in psychotic disorders (Veldic et al., 2004; recently reviewed in Grayson and Guidotti, 2013; Guidotti and Grayson, 2014).

It was Hossein Fatemi who first suggested that reelin may be downregulated in autism spectrum disorders (Fatemi et al., 2001b, 2002, 2005; Fatemi, 2002, 2005a,b). This led to the idea of a co-occurrence of reelin disturbances in autism and schizophrenia (Fatemi, 2010; Folsom and Fatemi, 2013). In addition, Fatemi's group also showed that a downregulation of reelin in the hippocampus occurred not only in schizophrenia and bipolar disorder, but also in patients with depression. In this case, they described a large but non-significant reduction of reelin-positive cells in the hippocampal CA4 region (the polymorphic region of the dentate gyrus; Fatemi et al., 2000).

The observation that hippocampal reelin is decreased in patients with depression led our research group to conduct a series of experiments to systematically examine whether reelin is altered in an animal model of depression. Beyond the patient data described above, it seemed to us that both chronic stress (an important risk factor for depression) and deficient reelin produce strikingly similar alterations in hippocampal plasticity and function. One example if this is the fact that both stress and deficient reelin can impair adult hippocampal neurogenesis and the proper maturation and integration of newborn neurons in the dentate gyrus (Pujadas et al., 2010; Lussier et al., 2013a), which have been repeatedly implicated in the pathogenesis of depression (as a recent review, see Schoenfeld and Cameron, 2015). The remainder of this review describes our studies of reelin-related events in the hippocampus and periphery of rats using a well characterized animal model of depression (Kalynchuk et al., 2004; Gregus et al., 2005; Johnson et al., 2006; Marks et al., 2009; Sterner and Kalynchuk, 2010), as well as our examination of peripheral biomarkers in depression patients (Rivera-Baltanás et al., 2012, 2014, 2015). We conclude by offering some hypotheses about how these ideas could be explored in a translational way in relation to modifying the reelin system to develop better diagnoses, prognoses, and therapeutics for patients with major depression.

REELIN EXPRESSION IN THE HIPPOCAMPUS: PUTATIVE ROLE IN DEPRESSION

We first assessed possible deficits in the number of reelin-immunopositive cells throughout the hippocampus in two chronic stress paradigms. Many current animal models of depression are based on chronic or repeated stress paradigms, because there is a well established relationship between exposure to traumatic or stressful life events and the onset of depressive symptoms in patients (Keller et al., 2007). In this case, we used a repeated corticosterone (CORT) injection paradigm, in which

rats receive a 40 mg/kg injection of CORT once per day for 21 consecutive days, and a repeated restraint stress paradigm, in which rats are placed in plastic restraint tubes for 6 h/day for 21 consecutive days. We chose these two stress paradigms because we and others had previously found that repeated CORT injections reliably increase depression-like behavior but repeated restraint stress does not (Gregus et al., 2005; Brummelte and Galea, 2010; Workmann et al., 2013; Kott et al., 2016). If alterations in hippocampal reelin are involved in depression, we would expect to see a loss of reelin-positive cells in rats subjected to the CORT injections but not restraint stress. Our results were consistent with this hypothesis, as we found a significant decrease (26%) in the number of reelin-positive cells specifically in the subgranular zone (SGZ) of the dentate gyrus after repeated CORT injections but not repeated restraint stress (Lussier et al., 2009). The fact that reelin-positive cells were lost in the SGZ was notable, as this is the location where adult hippocampal neurogenesis takes place and from where newborn neurons migrate into the granular cell layer and develop into mature granule cells, with dendrites extending through the dentate molecular layer and axons projecting toward CA3 pyramidal cells. This suggested to us that stress-induced alterations in reelin-expressing GABAergic interneurons located adjacent to the SGZ could influence the course of hippocampal neurogenesis. To investigate this idea, we compared the time course of changes in the number of reelin-positive cells in the SGZ, the maturation rate of newborn granule neurons, and the onset of a depression-like phenotype in rats subjected to 7, 14, or 21 days of CORT injections. We found that CORT-treated rats showed gradual increases in depression-like behavior over the course of the injections, which were paralleled by significant decreases in SGZ reelin expression (no changes at 7 days, 25% decrease at 14 days, and 26% decrease at 21 days) and significant decreases in the number of surviving immature dentate granule cells and the complexity of dendritic processes present in surviving immature granule cells (Lussier et al., 2013a). We interpreted these observations to indicate that reelin downregulation may delay the maturation of newborn granule cells and impair proper integration of these neurons into mature circuits, thereby disrupting hippocampal circuitry and enhancing depression-like behavior. This conclusion is consistent with other findings that reeler mice (with null reelin expression) have fewer mitotic cells in the dentate SGZ than wildtype mice (Sibbe et al., 2015) and that inactivation of the reelin pathway impairs hippocampal adult neurogenesis (Teixeira et al., 2012). It also adds a new component to the prominent hypothesis that adult hippocampal neurogenesis plays a causal role in major depression (Jacobs et al., 2000). This hypothesis was derived from data showing that patients with depression have reduced hippocampal volume (MacQueen et al., 2003; Campbell et al., 2004) and that chronic stress and antidepressant drugs can decrease and increase cell proliferation and survival respectively (Santarelli et al., 2003; Petrik et al., 2012). However, the putative causal role of neurogenesis in depression has been controversial because in animal models, both depression-like behavior and the behavioral actions of antidepressants can be dissociated from alterations in hippocampal neurogenesis (Surget et al., 2008; Bessa et al., 2009; David et al., 2009). Additionally, close examination of postmortem tissue from depressed patients has not revealed significant decreases in hippocampal stem cell proliferation (Lucassen et al., 2001; Reif et al., 2006). Our reelin data point to the idea that depressive symptoms could be associated with deficient neuronal maturation and integration rather than cell proliferation and survival *per se*. This idea has also been suggested by other research groups (Bessa et al., 2009; Mateus-Pinheiro et al., 2013).

Although many studies of reelin in animal models of depression have focused on the dentate SGZ and adult hippocampal neurogenesis, this is not the only region of the hippocampus where reelin is altered after a period of chronic stress. We have also reported that repeated CORT injections significantly decrease the number of reelin-positive cells in the CA1 stratum lacunosum-moleculare (by 21%), and dampen the co-expression of reelin and neuronal nitric oxide synthase (nNOS) in the molecular layer of the dentate gyrus (Lussier et al., 2009; Romay-Tallón et al., 2015). Early descriptions of reelin immunolabeling in the adult hippocampus identified a heavy "diffuse labeling" in both the distal molecular layer of the dentate gyrus and the CA1 stratum lacunosum-moleculare (Pesold et al., 1998), which was interpreted to indicate the presence of reelin secreted into the extracellular matrix that would regulate the strength of synaptic connections onto distal dendritic spines (Pesold et al., 1999; Rodriguez et al., 2000). Later on, it was shown that reelin signaling regulates glutamate receptor composition and activity particularly in the distal dendritic compartment (Chen et al., 2005; Sinagra et al., 2005; Qiu et al., 2006; Groc et al., 2007; Campo et al., 2009; Iafrati et al., 2014; Kupferman et al., 2014), and also that reelin could play a role in neurotransmitter release (Hellwig et al., 2011; Bal et al., 2013). The downregulation and neurochemical alterations of reelin-positive cells in the distal molecular layer and CA1 stratum-lacunosum-moleculare instigated by chronic stress could then result in changes in synaptic strength and/or glutamatergic receptors, and/or neurotransmitter release that will further affect hippocampal circuitry. This fits quite nicely with our report that repeated CORT injections significantly decrease expression of GAD65 and the $GABA_A$ α2 receptor subunit in the amygdala and hippocampus and increase expression of VGLUT2 within the hippocampus (Lussier et al., 2013b). These changes would create an imbalance in glutamatergic-GABAergic neurotransmission within the hippocampus, which could be another important pathophysiologic event in depression that is instigated by a deficit in reelin.

Heterozygous reeler mice have been used as a way to study the functional consequences of genetic deficits in reelin expression. These mice have about 50% of normal levels of reelin in bothe the brain and peripheral tissues. Previous work with these mice revealed mild neurochemical (Liu et al., 2001; Pappas et al., 2001; Ballmaier et al., 2002; Isosaka et al., 2006; Romay-Tallón et al., 2010; Nullmeier et al., 2011; Ventrutti et al., 2011; Varela et al., 2015), and behavioral (Tueting et al., 1999, 2006) alterations that did not appear to give rise to an overt pathological phenotype, but that could prime these animals to a high vulnerability to the deleterious effects of chronic stressors. We tested this idea

by investigating whether heterozygous reeler mice would be more susceptible to the depressogenic effects of repeated CORT injections than wildtype mice. Groups of heterozygous reeler mice and wildtype mice received daily injections of CORT (i.e., at 5 mg/kg, 10 mg/kg, or 20 mg/kg) over a 21-day period. We found that in the absence of CORT, heterozygous reeler mice do not show more depression-like behavior or deficits in the number or maturation rate of immature neurons compared to wildtype mice. However, the heterozygous reeler mice were more susceptible than wildtype mice to the damaging effects of CORT, as they showed dose dependent increases in depression-like behavior and decreases in neuronal survival and maturation (Lussier et al., 2011). Furthermore, analysis of the colocalization of reelin and nNOS in CORT-treated heterozygous reeler mice revealed a genotype × treatment interaction, with increased colocalization of both markers in the dentate SGZ in CORT treated heterozygous reeler mice. This indicates that chronic stress increases nNOS expression in reelin-positive cells when baseline levels of reelin are relatively low. This increase in nNOS expression could stimulate the release of nitric oxide, giving rise to an excitotoxic event through hyperactivation of NMDA glutamate receptors in these cells. Hypothetically, this excitotoxicity could have downstream consequences such as a loss of reelin release from GABAergic interneurons and the interruption of normal migration and maturation of newborn dentate granule cells (Romay-Tallón et al., 2015), as discussed above.

Overall, the evidence gathered to date clearly shows that reelin is important for adult hippocampal plasticity and that exposure to chronic stress or high levels of circulating glucocorticoids dampens reelin activity, with subsequent deficits in neuronal maturation and the development of depression-like behavior. **Figure 1** shows two possible roles for reelin in the pathogenesis of depression. It should be pointed out that the experiments that these ideas were developed from have all been conducted using male rodents, and there is some evidence that reelin may be differentially altered in male and female rodents under stress conditions (van den Buuse et al., 2012; Buret and van den Buuse, 2014). Nevertheless, the data described above beg the question of whether rescuing or enhancing hippocampal reelin could have antidepressant effects. We have recently demonstrated that administration of the tricylic antidepressant imipramine during the period of CORT injections prevents the downregulation of hippocampal reelin and rescues the behavioral phenotype (Fenton et al., 2015a). Other researchers have also shown that repeated citalopram administration counteracts the loss of hippocampal reelin after kainic-acid treatment (Jaako et al., 2011). Although these experiments do not provide definitive evidence that an enhancement of reelin is directly responsible for better behavioral outcomes, they are certainly consistent with that idea. There is a need for future studies to examine whether reelin enhancement is part of the mechanism underlying the therapeutic effects of antidepressant drugs. Furthermore, recent evidence has shown that intraventricular reelin infusions facilitate hippocampal-dependent cognition in a mouse model of Angelman syndrome (Hethorn et al., 2015). These authors did not report a specific mechanism by which reelin supplementation could facilitate memory in these mice. The identification of this mechanism is an important next step, which could also inform the development of novel antidepressant drugs or mechanisms of action for antidepressant drug actions (see discussion below).

PERIPHERAL REELIN IN RELATION TO PSYCHONEUROIMMUNOLOGY

Soon after the cloning of the reelin gene came the demonstration that reelin expression is not exclusive to the central nervous system, but that it is also expressed in other body regions both during developmental stages and adulthood. These regions were primarily identified as the yolk sac and blood vessels during developmental stages, and throughout life in the kidney, liver, and blood (Ikeda and Terashima, 1997; Smalheiser et al., 2000). Since then, reelin expression has also been shown in lymphatic tissues (Samama and Boehm, 2005; Lutter et al., 2012), platelets (Tseng et al., 2010), the enteric nervous system (Bottner et al., 2014), bone marrow (Chu et al., 2014), and some adult brain endothelial cells (Pérez-Costas et al., 2015). Although many studies have focused on the functional roles of reelin in brain development and the adult nervous system, there is scarce knowledge about the functional role reelin might play in the periphery. The picture so far illustrates reelin as a pleiotropic molecule with diverse functional roles both in the brain and periphery: as such, reelin is known to be released from liver and/or kidney cells into blood plasma (Smalheiser et al., 2000), where it plays a role in regulating erythropoiesis in the bone marrow (Chu et al., 2014), and in hemostasis (Tseng et al., 2010, 2014). Reelin also regulates lymphatic vessel formation (Lutter et al., 2012); and the remodeling of the vascular network in reelin-deficient mice (Lindhorst et al., 2012) together with the expression of reelin in yolk sac and developing blood vessels (Ikeda and Terashima, 1997), has led to the idea that reelin may be involved in blood vessel formation.

After our studies indicating that the addition of recombinant reelin to synaptosomes can increase protein expression (Dong et al., 2003), and that this increase was also accompanied by an augmentation of protein clustering on synaptosomal membranes (Caruncho et al., 2004), we wondered whether reelin could regulate membrane protein clustering along the cell membrane of peripheral blood cells (i.e., lymphocytes). Several observations informed this question, including the fact that reelin is highly expressed in blood plasma (Smalheiser et al., 2000), that plasma reelin is altered in mood and psychotic disorders (Fatemi et al., 2001a), that reelin induces clustering of its own receptors receptors and this is an important event for signaling (Strasser et al., 2004), and that lymphocytes contain reelin receptors whose expression is changed in psychiatric disorders (Suzuki et al., 2008). We hypothesized that a decrease in reelin levels or null reelin expression, as observed in heterozygous or homozygous reeler mice respectively, would alter the clustering of some specific proteins, such as the serotonin transporter, that tend to bunch into lipid rafts. We subsequently observed important alterations in the number and size of serotonin transporter clusters in both hererozygous and homozygous reeler mice, particularly in the latter where most lymphocytes showed

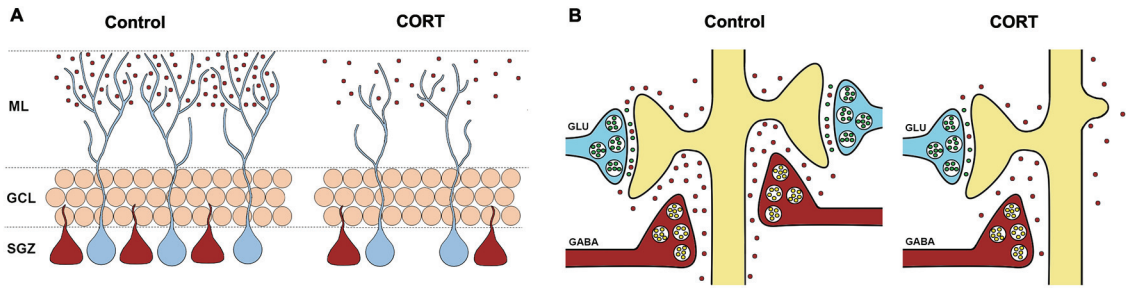

FIGURE 1 | Graphical depiction of two possible mechanisms by which reelin down-regulation may be involved in the pathophysiology of depression. **(A)** Reelin secreted by some hippocampal GABAergic interneurons in the dentate subgranular zone (SGZ) as well as in cells in the distal molecular layer (shown in red) is involved in hippocampal neurogenesis, particularly the rate and extent of dendritogenesis of newborn granule cells (shown in blue). Repeated CORT administration reduces the number of reelin+ cells in the dentate SGZ and the amount of extracellular reelin in the distal molecular layer, which delays the maturation (e.g., reduced dendritogenesis) of newborn neurons. These neurons may fail to properly integrate into existing hippocampal circuits. **(B)** Reelin secreted by GABAergic interneurons promotes and stabilizes synapses impinging onto dendritic spines. The downregulation and neurochemical alterations of reelin-positive cells in the distal molecular layer and CA1 stratum-lacunosum-moleculare (shown in red) instigated by chronic stress would decrease in the number of dendritic spines, resulting in a loss of glutamatergic synaptic strength and possibly a dampening of neurotransmitter release from glutamatergic terminals (shown in blue). This would further affect hippocampal circuitry.

a diffuse serotonin transporter immunostaining that made it difficult to identify individual clusters (Rivera-Baltanás et al., 2010). Not surprisingly, heterozygous and homozygous reeler mice have dysregulated secretion of cytokines by lymphocytes and macrophages (Green-Johnson et al., 1995).

It is now commonplace to conceptualize inflammatory events as key components in the pathophysiology of depression (see as reviews Raison et al., 2006; Maes et al., 2009; Miller et al., 2009; Leonard, 2010; Blume et al., 2011; Sperner-Unterweger et al., 2014; Young et al., 2014). In fact, levels of inflammatory cytokines are one of the best characterized biomarkers of depression (see as reviews Mössner et al., 2007; Howren et al., 2009; Li et al., 2011; Lichtblau et al., 2013; Valkanova et al., 2013). Taking into account the just mentioned alterations in immune cells in mice expressing low levels of reelin, as well as the disturbances in reelin immunoreactive cells in the dentate gyrus of both depressive patients (Fatemi et al., 2000) and rats showing depression-like behavior (Lussier et al., 2013a), we examined the pattern of lymphocyte membrane protein clustering in peripheral blood samples taken from rats treated with repeated CORT injections (i.e., showing a depressive-like phenotype) and from patients with depression both before treatment and after 8 weeks of antidepressant drug treatment. The pattern of protein clustering of the serotonin transporter and the serotonin 2A receptor on the cell membrane of lymphocytes from rats treated for 3 weeks with CORT showed a significant increase in cluster size for both markers in comparison with control rats, and also a significant positive correlation between larger membrane protein clusters and more depression-like behavior in the forced-swim test (Fenton et al., 2015b). Studies of lymphocytes from depression patients revealed an increase in the size of both serotonin transporter and serotonin 2A receptor protein clusters along the plasma membrane, similar to the alterations found in the CORT-treated rats. However, an important difference is that the analysis of the pattern of clustering of these markers also allowed us to differentiate two subpopulations of naïve depression patients that showed different therapeutic outcomes after antidepressant treatment (i.e., a subpopulation of naïve depression patients that had a poor response to treatment showed smaller clusters and a high percentage of clusters of 0.05–0.10 μm^2, whereas a subpopulation of naïve patients with better therapeutic outcomes showed larger clusters and a small percentage of clusters of 0.05–0.10 μm^2). These observations led us to propose that analyses of membrane protein clustering may be a new approach to identify novel biomarkers of depression, and perhaps of other psychiatric disorders as well (Rivera-Baltanás et al., 2012, 2014). Recently, we demonstrated that alterations in serotonin transporter clustering in lymphocytes in depression also correlate with remittance of anhedonia symptoms after antidepressant treatment (Rivera-Baltanás et al., 2015). This finding is of great interest when considering that anhedonia not only represents a cardinal symptom of depression, but it also is generally considered a symptom that shows a relatively poor response to conventional antidepressant treatment (Spijker et al., 2001; Pizzagalli, 2014). Therefore, being able to identify naïve depression patients that will show good or poor improvement in anhedonia symptoms may be useful in a clinical setting.

We are currently evaluating if different subpopulations of lymphocytes are differentially affected in terms of membrane protein clustering in depression, and also how these alterations may give rise to changes in the release of cytokines, which is of special interest for us when considering the depressogenic effects of cytokines in anhedonia (Anisman et al., 2002). The hypothesis driving this work is that alterations in reelin expression and membrane protein clustering in peripheral lymphocytes and monocytes may underlie some of the dysregulation in expression of pro-inflammatory and/or anti-inflammatory cytokines that may play a key role in the pathophysiology of major depression. **Figure 2** depicts a possible mechanism of how reelin dysregulation may relate to these events.

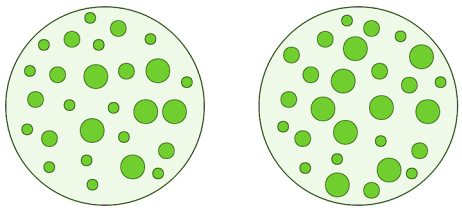

FIGURE 2 | Schematic representation of alterations in membrane protein clustering in depression. One of the roles of peripheral reelin may be to regulate membrane protein clustering in lymphocytes (see text for details). If peripheral reelin is decreased in depression, the pattern of protein clustering in peripheral immune cells could be altered. The figure shows our findings that patients with depression have generally larger protein clusters (shown in green) along the lymphocyte plasma membrane than non-depressed subjects (see Rivera-Baltanás et al., 2012, 2014). We propose that alterations in protein clustering in depression patients could stimulate cytokine secretion, as has been frequently reported in depression.

In relation to reelin expression in plasma and membrane protein clustering in depression, it is also of interest to consider that alterations in the pattern of clustering appear to affect proteins that tend to integrate within lipid rafts, such as the serotonin transporter (Magnani et al., 2004), and that shifting of Gα-proteins to and from lipid raft domains has been postulated as a possible mechanism involved in the chronic effects of antidepressants (Zhang and Rasenick, 2010; Czysz et al., 2015). This opens the possibility of studying combined alterations in membrane protein clustering and Gα-protein translocation to/from lipid rafts as an additional operative mechanism in the pathophysiology of depression that may be amenable to novel therapeutic intervention.

THE REELIN SYSTEM AS A TARGET FOR NOVEL ANTIDEPRESSANTS

The idea that reelin could be a part of the mechanism of antidepressant drug action or that it could have beneficial effects on its own was introduced in an earlier section of this review. This idea appears to be gaining momentum in the field: Several investigators have used exogenous reelin (or alternatively, rodent models of reelin overexpression) to investigate if high reelin levels can revert some of the neurochemical, anatomical, functional, and behavioral alterations in various animal models of human pathology. These studies have revealed that reelin supplementation can enhance synaptic plasticity, dendritic spine density, and cognitive ability in wild-type mice (Rogers et al., 2011), and that it can recover synaptic plasticity and learning deficits in heterozygous reeler mice (Rogers et al., 2013). The addition of exogenous reelin also prevents hippocampal dentate granule cell dispersion in experimental epilepsy (Müller et al., 2009), has a preventive effect on phencyclidine-induced behavioral deficits (Ishii et al., 2015), and recovers the neurochemical and behavioral phenotype in a mouse model for Angelman syndrome (Hethorn et al., 2015). Overexpression of reelin also prevents the development of behavioral alterations related to schizophrenia and bipolar disorder (Teixeira et al., 2011), and delays amyloid-beta fibril formation and rescues cognitive deficits in an animal model of Alzheimer's disease (Pujadas et al., 2014). Overall, these studies paint a tantalizing picture of the possible benefits reelin could have for a number of brain pathologies centered on hippocampal dysfunction. This is of course, also true for the case of major depression. The neurochemical and behavioral deficits associated with a loss of reelin in animal models of depression, together with the rescuing of behavioral phenotypes by addition or overexpression of reelin in models of several neuropsychiatric disorders, strongly suggest that tackling the reelin system (i.e., by the addition of recombinant reelin, by activating the reelin receptors VLDLR and/or ApoER2, or by neuroprotection of reelin-positive cells) could be a good strategy for the development of novel antidepressants.

The studies mentioned above have all used infusions of reelin into the brain. Reelin is a very large protein, and it has been unclear whether peripheral administration of reelin could influence functions within the brain. However, the recent finding of reelin immunoreactivity product within caveolar vesicles in endothelial cells in brain regions showing a high level of extracellular reelin labeling suggests that reelin peptides might indeed cross the blood-brain-barrier (Pérez-Costas et al., 2015). This opens the possibility that peripherally administered reelin could influence brain function. It also suggests that alterations in reelin could be important in relation to vascular and/or brain-blood-barrier disturbances in major depression, as these factors appear to be operative in the pathophysiology of depression (Najjar et al., 2013; Taylor et al., 2013).

The pattern of alterations in membrane protein clustering in lymphocytes, together with the multiple observations of disturbances in proinflammatory cytokines in major depression, as discussed in the previous section of this report, also raises the possibility of developing novel therapeutic strategies based on interventions that act peripherally in the immune system. In fact, an interesting recent report has shown that lymphocytes from chronically-stressed mice confer antidepressant-like effects when transferred to naïve mice (Brachman et al., 2015). In translating this remarkable observation to the human condition, one could imagine the possibility of extracting peripheral lymphocytes from patients with treatment-resistant depression and developing conditions to treat them *in vitro* (i.e., conditions that could result in alterations in membrane protein clustering that we have found to relate to a good therapeutic outcome) before re-implanting them in the patients, with the hope that there would be an effective antidepressant outcome from this intervention.

In conclusion, the alterations in reelin expression in both the CNS and periphery in depression (and in animal models of depression), the analysis of the functional roles of reelin (and dysfunctions in depression), and the observation of how reelin can rescue behavioral phenotypes in different paradigms, strongly suggest that systematically tackling the reelin system may be a good strategy for developing novel antidepressants.

However, additional studies at multiple levels will be necessary to further this field and translate it to the clinic.

AUTHOR CONTRIBUTIONS

All authors have contributed to the acquisition of data, designing of the work, drafting and reviewing the manuscript, and gave the final approval to the version to be published. HJC and LEK have been involved in all the studies on which this manuscript is based, while KB, RR-T, MAM, and JB have been primarily involved in the hippocampal studies; and TR-B and JMO have been involved in the membrane protein clustering studies in depression patients.

FUNDING

This work was funded by start-up funds from the College of Medicine and the College of Pharmacy and Nutrition at the University of Saskatchewan, a Saskatchewan Health Research Foundation Establishment Grant, and an NSERC-Discovery Grants to HJC; and by an NSERC- Discovery Grant to LEK.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the depression patients and control subjects who participated in the studies on biomarkers of diagnosis and treatment responsivity.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 18 October 2016
doi: 10.3389/fncel.2016.00229

Reelin and Neuropsychiatric Disorders

*Kazuhiro Ishii†, Ken-ichiro Kubo and Kazunori Nakajima**

Department of Anatomy, Keio University School of Medicine, Tokyo, Japan

Proper neuronal migration and laminar formation during corticogenesis is essential for normal brain function. Disruption of these developmental processes is thought to be involved in the pathogenesis of some neuropsychiatric conditions. Especially, Reelin, a glycoprotein mainly secreted by the Cajal-Retzius cells and a subpopulation of GABAergic interneurons, has been shown to play a critical role, both during embryonic and postnatal periods. Indeed, animal studies have clearly revealed that Reelin is an essential molecule for proper migration of cortical neurons and finally regulates the cell positioning in the cortex during embryonic and early postnatal stages; by contrast, Reelin signaling is closely involved in synaptic function in adulthood. In humans, genetic studies have shown that the *reelin* gene (*RELN*) is associated with a number of psychiatric diseases, including Schizophrenia (SZ), bipolar disorder (BP) and autistic spectrum disorder. Indeed, *Reln* haploinsufficiency has been shown to cause cognitive impairment in rodents, suggesting the expression level of the Reelin protein is closely related to the higher brain functions. However, the molecular abnormalities in the Reelin pathway involved in the pathogenesis of psychiatric disorders are not yet fully understood. In this article, we review the current progress in the understanding of the Reelin functions that could be related to the pathogenesis of psychiatric disorders. Furthermore, we discuss the basis for selecting Reelin and molecules in its downstream signaling pathway as potential therapeutic targets for psychiatric illnesses.

Keywords: reelin, psychiatric disorder, schizophrenia, animal model, *reeler*

OPEN ACCESS

Edited by:
Gabriella D'Arcangelo, Rutgers University, USA

Reviewed by:
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****Correspondence:***
Kazunori Nakajima
kazunori@keio.jp

†Present address:
Kazuhiro Ishii, Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Received: *11 April 2016*
Accepted: *22 September 2016*
Published: *18 October 2016*

Citation:
Ishii K, Kubo K-i and Nakajima K (2016) Reelin and Neuropsychiatric Disorders. Front. Cell. Neurosci. 10:229. doi: 10.3389/fncel.2016.00229

INTRODUCTION

Falconer (1951) first described the mutant *reeler* mouse, which is characterized by reeling gait caused by dysregulation of motor coordination and ataxia. More than four decades later, the gene responsible for the *reeler* phenotype was identified and the protein encoded by the gene was named Reelin (Bar et al., 1995; D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995). Until date, much work has been carried out towards understanding the functions of Reelin during cortical development, because the Reelin-deficient mutant mouse, *reeler*, shows largely inverted cortical layers (Caviness and Sidman, 1973; Tissir and Goffinet, 2003; Honda et al., 2011; Sekine et al., 2014).

Reelin is a glycoprotein secreted mainly from the Cajal-Retzius cells in the developing cerebral cortex and hippocampus, and acts as a key regulator of various aspects of laminar formation, including neuronal migration, cell aggregation and dendrite formation (Nakajima et al., 1997; Kubo et al., 2010; Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2014; Kohno et al., 2015). Many downstream molecules and several pathways involved in Reelin signaling during

development have been elucidated from animal studies (Howell et al., 1997; Hiesberger et al., 1999; Ballif et al., 2004; Honda et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2012; **Figure 1**).

In the postnatal period, the distribution and expression patterns of Reelin are dramatically changed as compared to those during the embryonic period (Alcántara et al., 1998). This suggests that the roles of Reelin in the postnatal brain might also be changed. Intriguingly, a number of lines of evidence indicate that Reelin signaling modulates synaptic function in the adult brain (Herz and Chen, 2006; **Figure 2**).

In humans, genetic studies have reported that the *RELN* locus is associated with neuropsychiatric disorders, such as Schizophrenia (SZ), bipolar disorder (BP), autism spectrum disorder (ASD) and Alzheimer's disease (AD; Ovadia and Shifman, 2011; Wang et al., 2014; Bufill et al., 2015; Li et al., 2015). Indeed, homozygous (*rl/rl*) and heterozygous *reeler* (*rl/-*) mice haploinsufficient for Reelin show cognitive and behavioral abnormalities (Tueting et al., 1999; Qiu et al., 2006a), supporting the notion that *RELN* haploinsufficiency may lead to higher brain dysfunctions relevant to neuropsychiatric disorders in humans. A number of studies have revealed that Reelin also plays a pivotal role in regulating synaptic functions, including N-methyl-d-aspartate (NMDA) receptor signaling (Beffert et al., 2005; Qiu et al., 2006b), and these findings may give a clue to uncover the impairments in the Reelin signaling pathway underlying the development of psychiatric disorders.

In this article, we first provide an overview of the current progress in Reelin research at the molecular, cellular and tissue levels. Second, we review human genetic studies of

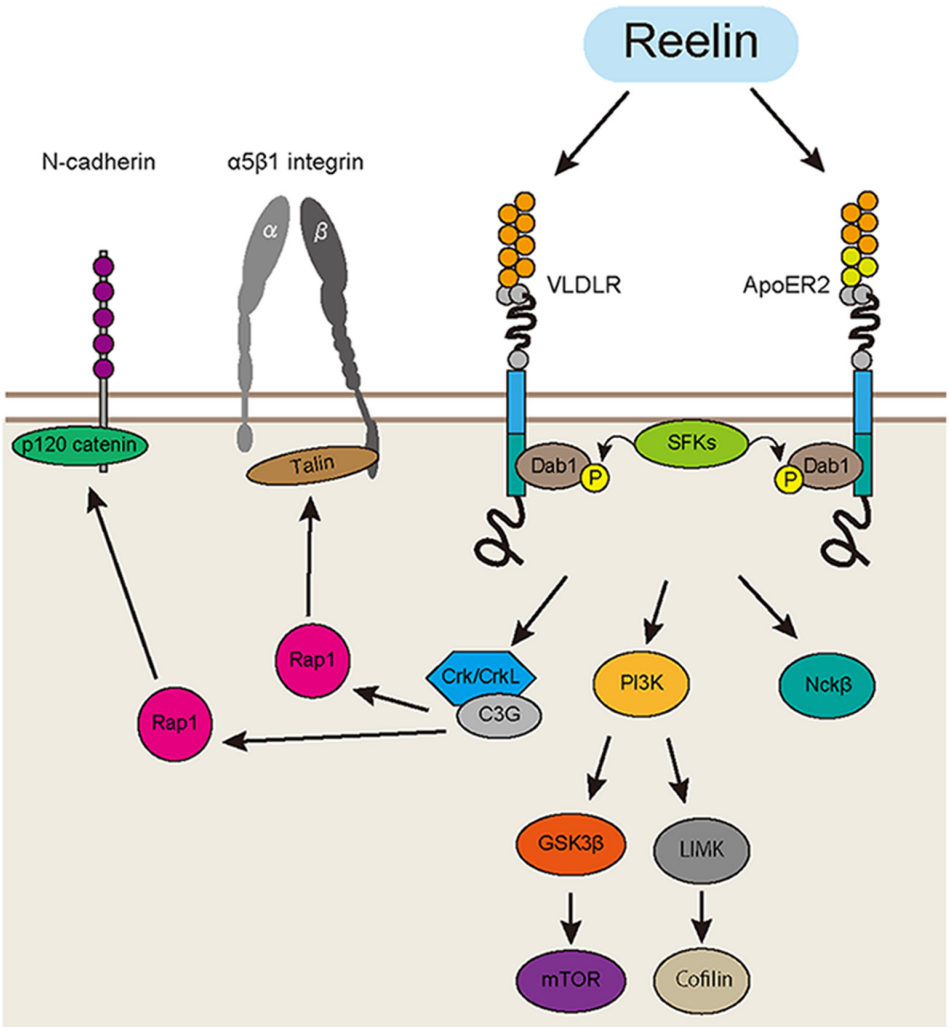

FIGURE 1 | Reelin signaling pathway in the developing cortex. The Reelin protein binds to very-low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2). Then, Dab1 is phosphorylated by the Src-family tyrosine kinases (SFKs). Phosphorylated Dab1 recruits downstream molecules such as Crk/CrkL, phosphatidylinositol 3-kinase (PI3K) and Nckβ. CrkL binds to C3G, an effector protein. C3G promotes the formation of Rap1-GTP, which activates cell adhesion molecules, including α5β1 integrin and N-cadherin. There are also Dab1-PI3K-mTOR, Dab1-PI3K-cofilin and Dab1-Nckβ pathways, which regulate the actin cytoskeleton relevant to dendrite formation and neuronal migration in the developing cortex.

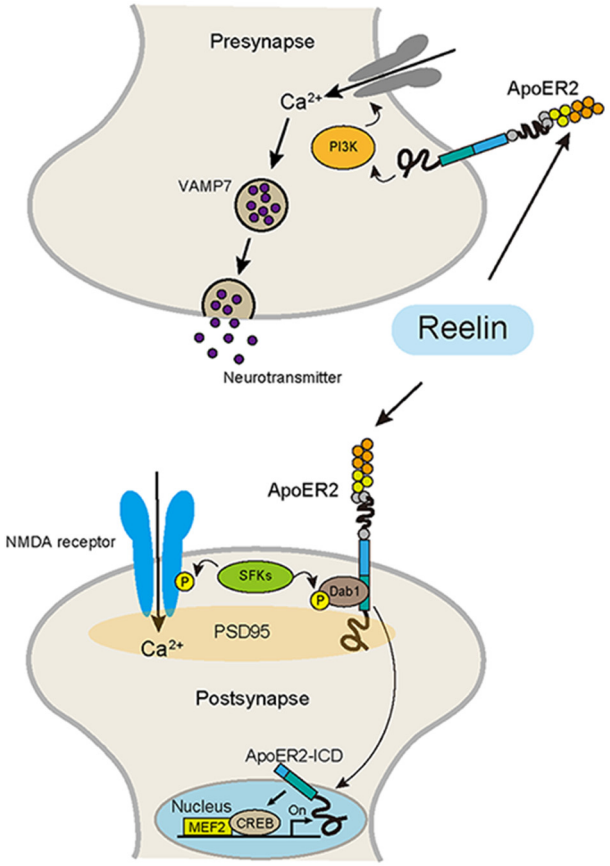

FIGURE 2 | Reelin signaling pathway in the adult brain, which regulates synaptic function. Reelin binds to ApoER2 and activates SFKs, which phosphorylate a tyrosine of the NR2 subunit of the N-methyl-D-aspartate (NMDA) receptor, resulting in the potentiation of NMDA receptor-mediated Ca^{2+} influx. ApoER2 binds to PSD-95, a scaffolding protein in the post synapse. ApoER2 is proteolytically cleaved by γ-secretase activity, which is activated by NMDA receptor signaling. Then, the intracellular domain of ApoER2 (ApoER2-ICD) is released from the cell membrane and translocates into the nucleus and activates transcriptional factors, including MEF2 and CREB. In the presynapse, the Reelin-ApoER2-PI3K pathway promotes Ca^{2+} influx, which leads to the selective mobilization of VAMP7-enriched synaptic vesicles, promoting neurotransmitter release.

neuropsychiatric disorders associated with mutations in the *RELN* gene locus. Then, we focus on how Reelin dysfunction leads to the behavioral abnormalities relevant to neuropsychiatric diseases in mouse models. Finally, we discuss the basis for selecting Reelin and molecules in its downstream signaling pathway as potential therapeutic targets for neuropsychiatric disorders.

REELIN HAS AN ESSENTIAL ROLE IN CORTICAL DEVELOPMENT

In mammals, cortical expansion is thought to contribute to the acquisition of higher brain functions during the course of evolution (Molnar et al., 2006). The neocortex is composed of a well-organized six-layer structure, including excitatory and inhibitory neurons (Rakic, 2009). The excitatory neurons, born directly or indirectly from the radial glia (Tabata et al., 2009; Sekine et al., 2013), which are neural progenitor cells located in the ventricular zone, migrate radially towards the brain surface, and eventually reach their final destinations (Rakic, 1972). Since the late-born neurons migrate past their predecessors, the earlier-born neurons are placed at a deeper position and the later-born neurons at a more superficial position in the cortical plate (CP; Caviness, 1982; Takahashi et al., 1999). This pattern of cell alignment is called a birth-date-dependent "inside-out" pattern. By contrast, inhibitory interneurons are born in the ganglionic eminences (GEs) in the ventral telencephalon and preoptic area in the rostral diencephalon that are located far from their final destination, and migrate tangentially for a long distance towards the dorsal pallium (Anderson et al., 1997; Tamamaki et al., 1997; Yozu et al., 2005; Kanatani et al., 2008, 2015; Gelman et al., 2009, 2011; Marin et al., 2010). Correct neuronal migration and laminar formation are essential for the establishment of proper brain functions. Indeed, disruption of the cortical architecture has been observed in various neuropsychiatric disorders (Arnold, 2000; Wegiel et al., 2010).

Reelin is a glycoprotein that is mainly secreted from the Cajal-Retzius cells located in the marginal zone (MZ), and a subpopulation of GABAergic interneurons (Rice and Curran, 2001). Reelin-deficient mice, *reeler*, show largely inverted cortical layers, which strongly supports the notion that Reelin is a key regulator of cortical development. A recent study showed that cortical laminar formation in *reeler* brains exhibit a more complex pattern than previously thought. Boyle et al. (2011) reported that the layer formation is severely disorganized in the *reeler* cortex, with layer II/III neurons located in the middle of the cortex and cells from other layers (layer IV, V and VI) split between the deep and superficial layers in a mirror image fashion. The secreted Reelin protein binds to its receptors, apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR), which are mainly expressed on the cell membrane of cortical neurons (Trommsdorff et al., 1999; Hirota et al., 2015). Then, disabled homolog 1 (Dab1), an intracellular adaptor protein, is phosphorylated via Fyn and Src, the Src-family tyrosine kinases (SFKs; Hiesberger et al., 1999; Howell et al., 1999). Phosphorylated Dab1 recruits and binds with several downstream molecules, including Crk, phosphatidylinositol 3-kinase (PI3K), and Nckβ (Beffert et al., 2002; Pramatarova et al., 2003; Park and Curran, 2008). The Crk family proteins (Crk and CrkL) are adaptor proteins that bind to phosphorylated Dab1 and recruit their effector proteins (Chen et al., 2004). Park and Curran (2008) reported that conditional double-knockout mice of *Crk* and *CrkL* showed the major anatomic features of *reeler*, including disruption of layer formation in the cerebral cortex and hippocampus, absence of preplate splitting, impaired dendrite formation, and cerebellar hypofoliation. The brains of mice deficient in C3G, an effector protein of CrkL, also show impaired preplate splitting (Voss et al., 2008). These findings indicate

the Reelin-Dab1-Crk/CrkL pathway plays a critical role in controlling layer formation in the cortex, including preplate splitting. Sekine et al. (2012) recently reported that Reelin activates integrin α5β1 through an intracellular Dab1-Crk/CrkL-C3G-Rap1 pathway. Furthermore, they showed that activated integrin α5β1 controls the terminal translocation, a final mode of neuronal movement beneath the MZ (Nadarajah et al., 2001; Sekine et al., 2011, 2012). Other groups reported that Reelin signaling regulates the function of another cell adhesion molecule, N-cadherin. Franco et al. (2011) reported that Dab1 acts on the migratory neurons to stabilize their leading processes in a Rap1-dependent manner. They showed that Rap1 regulates the function of N-cadherin and finally controls the somal translocation (Franco et al., 2011). The same group also reported that N-cadherin activated through the Reelin-Rap1 pathway enhances heterotypic cell-cell contact between the Cajar-Retzius cells and migratory neurons (Gil-Sanz et al., 2013). Jossin and Cooper (2011) demonstrated that the Rap1-N-cadherin pathway also regulates the transition of migratory neurons from multipolar cells to bipolar cells beneath the CP; this mode change is crucial for the migratory neurons to enter the CP (**Figure 1**).

Reelin signaling plays a role in the processes of dendrite development (Olson et al., 2006; Jossin and Goffinet, 2007). Olson et al. (2006) found that Dab1-suppressed migrating neurons showed simplified leading processes that were less likely to attach to the MZ and exhibited abnormal cell positioning. Jossin and Goffinet (2007) reported that Reelin promoted dendritogenesis through activation of mammalian target of rapamycin (mTOR) and S6 kinase 1 (S6K1) in a Dab1-, PI3K- and Akt-dependent manner. In regard to axon formation, by ablation of Cajal-Retzius cells in a slice and analysis of *reeler* mice, Del Rio et al. (1997) demonstrated abnormalities in the development of axon fibers from the entorhinal cortex to the hippocampus. However, using *reeler* cortical explants, Jossin and Goffinet (2001) found that Reelin itself did not exhibit any significant attraction or repulsion to cortical axons.

In the adult brain, the main source of Reelin is no longer the Cajal-Retzius cells, but a subpopulation of GABAergic interneurons, a change that is also associated with a change in the distribution pattern of Reelin (Alcántara et al., 1998; Pesold et al., 1998). These findings suggest that the roles of Reelin in the adult brain may also be different from those in the developing brain. We shall review and discuss the functions of Reelin in the adult brain in "Reelin Regulation of Brain Function and Behavior" Section.

GENETIC STUDY OF *RELN* IN NEUROPSYCHIATRIC DISORDERS

In humans, the mutations of the *RELN* have been shown to be associated with autosomal recessive lissencephaly with cerebellar hypoplasia (Hong et al., 2000). The brain phenotypes in these patients are similar to those found in the *reeler* mice, including abnormal laminar formation and cerebellar hypoplasia. In addition to this severe brain malformation, mutations in the *RELN* locus are also associated with neuropsychiatric disorders, with no apparent abnormalities in the brain structure. In this section, we provide an overview of the recent genetic studies carried out to examine the association of *RELN* with neuropsychiatric disorders.

Schizophrenia

SZ is a devastating psychiatric disease that affects approximately 1% of the population and is characterized by hallucinations, delusions and cognitive disturbances. The first clinical features of SZ typically emerge between early childhood and adolescence, with many patients experiencing chronic SZ symptoms (Sawa and Snyder, 2002). The clinical symptoms are based on brain dysfunctions attributed to genetic and environmental factors (Insel, 2010). As SZ shows high heritability, the risk genes with the greatest impact on the predisposition to SZ have been pursued by many researchers (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). As for the *RELN* gene, Li et al. (2013) conducted a case-control association study and identified six single-nucleotide polymorphisms (SNPs; rs2237628, rs362626, rs362814, rs362813, rs362731, rs362726) which are located in the intron 29 of the *RELN* gene that were significantly associated with the risk of SZ in the Chinese population. Several groups have reported that the existence of a gender-specific (women) association between SNPs in *RELN* and SZ (Shifman et al., 2008; Kuang et al., 2011; Li et al., 2011). Li et al. (2011) also reported the association of another SNP (rs12705169) in the *RELN* locus with SZ. Although rs12705169 is located in the intron of *RELN* and does not lead to any change of the protein structure or function, it may be involved in alternative RNA splicing and miRNA generation. When they divided the subjects by gender, they found that this SNP was positively associated with SZ only in women. Similarly, Kuang et al. (2011) demonstrated an association of rs362719 of *RELN*, which is located in the exon 42 and contributes to the EGF-like domain of the fifth Reelin repeat, with susceptibility to SZ in their female, but not male, participants. Shifman et al. (2008) performed a genome-wide association study (GWAS) for SZ in Ashkenazi Jews and showed that rs7341475, a SNP in the intron four of the *RELN*, was associated with SZ only in women. However, the association of this SNP (rs7341475) with SZ was not replicated in any other study in the Chinese population (Liu et al., 2011). Subsequently, Ben-David and Shifman (2010) performed a meta-analysis of four GWAS studies to assess the association of rs7341475 with SZ, and suggested this locus might have a small effect on the SZ risk. Therefore, the association of rs7341475 with SZ may remain inconclusive. Wedenoja et al. (2008) replicated the previous linkage of SZ on 7q22 in an independent subject. They also studied four candidate genes, including *RELN*. Although the selected candidate genes showed no association to the clinical diagnosis of SZ, the quantifiable trait component analysis revealed that allelic variants of *RELN* contributed to the endophenotypes of SZ, including working memory and executive function (Wedenoja et al., 2008).

Other Neuropsychiatric Disorders and Neurological Diseases

ASD is characterized by impairments of social interaction and communication, as well as repetitive behaviors and restricted interests (Maenner et al., 2014). Twin and family studies have revealed the important role of genetic factors in ASD, with a heritability value of as high as 90% (Freitag et al., 2007). Although many studies have been conducted to explore the genetic association between *RELN* and ASD, the results have been inconclusive. Persico et al. (2001) was the first to report that polymorphic GGC repeats located in the 5′ untranslated region (5′ UTR) of the *RELN* were associated with autistic disorder, finding that was subsequently replicated in three studies (Zhang et al., 2002; Skaar et al., 2005; Dutta et al., 2007). However, other groups have failed to show an association between the triplet repeats in the 5′ UTR of the *RELN* and autism (Krebs et al., 2002; Bonora et al., 2003; Devlin et al., 2004; Li et al., 2004). In the family-based association analyses carried out by Serajee et al. (2006), the most significant results were the apparent association of autism, in a broad diagnosis of the disease, with rs736707 in intron 59 and rs362691 in exon 22 of the *RELN*. Li et al. (2008) also showed a significant genetic association between rs736707 in intron 59 and ASD in a Han Chinese population. On the other hand, other groups have failed to detect these associations (Dutta et al., 2008; He et al., 2011). Dutta et al. (2008) carried out case-control and family-based association studies for six SNPs (rs727531, rs2072403, rs2072402, rs362691, rs362719, rs736707), and found that these SNPs of *RELN* were unlikely to be associated with ASD. Recently, a Chinese group conducted a meta-analysis for case-control and transmission disequilibrium test (TDT) studies published from 2001 to 2013 and concluded that rs362691 might contribute significantly to the risk of ASD (Wang et al., 2014).

There are only a few reports that suggested the existence of an association between the *RELN* and BP (Goes et al., 2010; Ovadia and Shifman, 2011). Goes et al. (2010) found that the rs362719 of *RELN* was associated with susceptibility to BP, particularly in females.

Several groups have also reported an association between AD and the *RELN* gene (Seripa et al., 2008; Antoniades et al., 2011; Kramer et al., 2011; Bufill et al., 2015; Fehér et al., 2015). AD is a neurodegenerative disease characterized by the formation of neurofibrillary tangles and beta-amyloid plaques in the brain, and patients clinically manifest progressive impairments of memory and cognition (Scheltens et al., 2016). Several SNPs in the *RELN* gene have been reported to be associated with the risk of AD. Seripa et al. (2008) showed an association between a triplet tandem repeat in the 5′UTR and rs607755 with AD, particularly in females. Another group also showed significant association between rs607755 and the risk of AD (Fehér et al., 2015). However, their results were inconsistent with the conclusion by Seripa et al. (2008) that rs607755 was significantly associated with AD only in males (Fehér et al., 2015). Antoniades et al. (2011) reported that an SNP in exon 22 of *RELN* (rs362691) was significantly associated with the risk of AD in a Greek population. Bufill et al. (2015) reported that SNPs in the *RELN* gene (rs528528 and rs2299356) and two genes (*PLK2* and *CAMK2A*) related to the Reelin signaling pathway were associated with AD and mild cognitive impairment (MCI).

REELIN REGULATION OF BRAIN FUNCTION AND BEHAVIOR

Expression Level of Reelin and Vulnerability to Neuropsychiatric Disorders

Many studies have provided evidence for altered Reelin expression in rodents with cognitive dysfunction, which may relate to neuropsychiatric diseases. Heterozygous *reeler* mice (HRM), in which the amount of Reelin protein is approximately 50% as compared to that in the wild type mice, exhibit behavioral abnormalities (Costa et al., 2002). Thus, HRM has been of interest as an animal model of psychiatric diseases. In spite of the initial inconsistent findings on the occurrence of behavioral deficits in HRM (Salinger et al., 2003; Podhorna and Didriksen, 2004), many groups have reported behavioral traits associated with *Reln* haploinsufficiency in HRM (Tueting et al., 1999; Qiu et al., 2006a; Barr et al., 2007, 2008; Teixeira et al., 2011; Kutiyanawalla et al., 2012; Iafrati et al., 2014). Several groups have also reported defect in the prepulse inhibition (PPI), an impairment of sensory motor gating that is associated with SZ, in HRM (Tueting et al., 1999; Barr et al., 2008; Teixeira et al., 2011; Kutiyanawalla et al., 2012). Iafrati et al. (2014) showed that Reelin deficiency in HRM caused defects in the juvenile morphofunctional properties of excitatory synapses in the prefrontal cortex (PFC) and behavioral dysfunction in prefrontal circuits. Furthermore, Barr et al. (2007) showed that the Reelin receptors VLDLR and ApoER2 regulated sensory motor gating. They demonstrated that acoustic PPI was intact in both *Vldlr*- and *Apoer2*-mutant mice. However, *Vldlr*-homozygous knockouts mice exhibited deficits in crossmodal PPI, while *Apoer2*-heterozygous and homozygous knockouts mice exhibited increased crossmodal PPI (Barr et al., 2007). Qiu et al. (2006a) demonstrated that HRM exhibited hippocampus-dependent learning deficit underlying the impairment of hippocampal plasticity. There are several interesting studies to show that mouse behavioral alterations are manifested as a consequence of gene-environment interaction, similar to many psychiatric disorders in humans (Laviola et al., 2009; Romano et al., 2014; Howell and Pillai, 2016). There have also been a number of reports indicating that in humans, the amount of Reelin protein in the brain, plasma and cerebrospinal fluid (CSF) are associated with neuropsychiatric conditions. Almost all studies have shown that the reduced amount of Reelin in the brain and blood are associated with a high risk of development of neuropsychiatric disorders (Guidotti et al., 2000; Fatemi et al., 2005; Eastwood and Harrison, 2006). Interestingly, however, increased expression levels of Reelin in the CSF (Sáez-Valero et al., 2003) and frontal cortex (Botella-López et al., 2006) were found in case of AD. Accumulation of Reelin has also been reported in amyloid-like plaques, with a decline in

Reelin-positive neurons, which might represent a risk factor for AD (Knuesel et al., 2009). Reelin protein itself is thought to be protective against AD-like neuropathology, since the reduced Reelin expression in a transgenic AD background markedly elevated amyloid-β plaque formation (Kocherhans et al., 2010).

The Expression Level of Reelin is Regulated by Multiple Mechanisms

In addition to genetic mutations/polymorphisms, various mechanisms may lead to aberrant expression of Reelin. In the postnatal brain, the main source of Reelin is shifted from Cajal-Retzius cells to a subpopulation of GABAergic interneurons (Alcántara et al., 1998; Pesold et al., 1998). Using *in situ* hybridization analysis combined with immunohistochemistry, Pesold et al. (1998) showed that Reelin is preferentially expressed in GABAergic interneurons in the adult rat cortex and hippocampus. Furthermore, they found Reelin immunoreactivity not only in neurons, but also in the extracellular space, suggesting that the GABAergic interneurons secrete Reelin into the extracellular matrix (Pesold et al., 1998). However, how Reelin is secreted by these specialized interneurons in the postnatal brain remains an unresolved question, although a constitutive mechanism dependent on a specific sequence of positively charged amino acids in the Reelin carboxy terminus domain has been reported (Rodriguez et al., 2000). Thus, in mature brains, the amount of secreted Reelin protein may depend on the number and distribution of Reelin-positive interneurons.

Several studies have shown the involvement of epigenetic mechanisms in the transcriptional regulation of *RELN* (Abdolmaleky et al., 2005; Grayson et al., 2005). Grayson et al. (2005) analyzed the pattern of methylation within the CpG island of the *RELN* promoter in human SZ brains obtained from a brain bank. They showed hypermethylation of the promoter region of *RELN* in the SZ brains (Grayson et al., 2005). Abdolmaleky et al. (2005) also demonstrated hypermethylation of a CpG island containing CRE and an SP1-binding site in the promoter region of *RELN* in post-mortem examination of the brains of SZ patients. However, Tochigi et al. (2008) reanalyzed this *RELN* promoter region by using the same brain samples as in the previous study, and showed that the extent of methylation in this promoter region did not differ significantly between the SZ patients and controls. Furthermore, Mill et al. (2008) prepared a microarray-based comprehensive epigenomic scan and found epigenetic changes in the loci associated with glutamatergic and GABAergic neurotransmitter pathways. However, they did not detect any association between hypermethylation in the promoter region of *RELN* and major psychosis. Recently, a systematic review of DNA methylation in SZ and BP has been reported (Teroganova et al., 2016). Although a number of differentially methylated genes were detected, including *RELN*, diverse methodologies used across studies hampered the reliability of the meta-analysis (Teroganova et al., 2016). Thus, in regard to the epigenetic regulation of *RELN* expression, further investigation is needed to confirm the association of hypermethylation in the *RELN* promoter region with the predisposition to major mental illnesses.

Perturbation of transcriptional regulation could affect the Reelin protein distribution as well as expression levels. Baek et al. (2015) reported that *RELN* transcription was derepressed in ectopic cortical regions, mediated by activation of the transcription factor FOXG1 in human focal cortical malformations. They found that misexpression of Reelin in the progenitor cells caused non-cell autonomous neuronal migration defects, resulting in focal cortical malformations. These results may, at least to some extent, resemble the effects of ectopic Reelin overexpression in the mouse brain (Kubo et al., 2010).

Reelin Signaling is Involved in Synaptic Functions and Behavior

During cortical development, the main source of Reelin is the Cajal-Retzius cells located in the MZ. Subsequently, these cells gradually disappeared, and the main source of Reelin finally shifts to a subtype of GABAergic interneurons (Miyoshi et al., 2010). Thus, during this period, the expression pattern of Reelin changes dynamically. This suggests that the role of Reelin in the postnatal brain also changes dramatically. Indeed, in adulthood, there is much evidence to indicate that Reelin modulates NMDA receptor-mediated synaptic functions (Beffert et al., 2005; Iafrati et al., 2014; **Figure 2**). Dysfunction of NMDA receptor signaling plays a key role in the pathogenesis of major neuropsychiatric disorders, including SZ and AD (Zhou and Sheng, 2013). Recently, it has been reported that hundreds of genetic loci were associated with SZ in a large-scale GWAS (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). In this analysis, the glutamatergic neurotransmission-related genes including *GRIN2A*, a NMDA receptor subunit, were highly associated with SZ. NMDA dysfunction has also been reported to be related to cognitive impairment in AD. In addition to the widely used drug, cholinesterase inhibitors, for AD, a new drug that modulates glutamate signaling has also become available recently for AD (Scarpini et al., 2003). Also, it has been shown that Reelin acts on NMDA receptor signaling via ApoER2, a Reelin receptor (Beffert et al., 2005; Chen et al., 2005; Herz and Chen, 2006). Beffert et al. (2005) demonstrated that an alternative splicing variant of ApoER2 modulated the NMDA receptor activity through Reelin signaling. They found that alternatively spliced exon 19, which encodes amino acids in the intracellular domain of ApoER2 (ApoER2-ICD), is necessary for the stimulation of tyrosine phosphorylation of the NMDA receptor subunit NR2 in hippocampal slices in response to Reelin. Furthermore, they confirmed that the mice expressing a mutant ApoER2 lacking exon 19 showed severe cognitive disturbances related to the NMDA receptor function (Beffert et al., 2005). Interestingly, birds do not have exon 19 (Brandes et al., 1997), suggesting that adding this new exon may partly contribute to the acquisition of higher brain functions in mammals. Chen et al. (2010) further showed that the

Reelin-ApoER2-NMDA receptor pathway is involved in the pathophysiology of AD. They demonstrated that ApoE4, which is associated with an earlier average age at onset of dementia, depletes ApoER2 from the neuronal surface after ligand-induced endocytosis by Reelin, which impairs synaptic plasticity and NMDA phosphorylation induced by Reelin (Chen et al., 2010). Moreover, Lane-Donovan et al. (2015) demonstrated that Reelin signaling protects against the Aβ toxicity that induces synaptic dysfunction in the early stages of aging. Using Reelin conditional knockout mice, they found that the loss of Reelin rendered excitatory synapses susceptible to functional suppression by Aβ, which resulted in impaired learning and memory. Iafrati et al. (2014) demonstrated that *in vivo* injection with ketamine, an NMDA receptor antagonist, or Ro25-6981, an inhibitor of GluN2B-NMDA receptors, in the juvenile period rescued the behavioral abnormalities, reduced the dendritic spine density, and anomalous long-term potentiation (LTP) in the PFC of HRM. Their results suggest Reelin is essential for proper functional and behavioral development of juvenile prefrontal circuits through modulating the NMDAR-mediated signaling pathway.

Reelin signaling is also involved in the presynaptic functions (**Figure 2**). Reelin acts presynaptically in mature neurons to rapidly enhance neurotransmitter release. This role of Reelin depends on the function of VAMP7, a vesicular SNARE protein (Bal et al., 2013). Telese et al. (2015) reported interesting findings; they reported that the Reelin pathway controls learning and memory through activation of the transcriptional factors. Proteolytic cleavage of ApoER2 is a crucial component of the synapse-to-nuclear signaling triggered by Reelin. When Reelin binds to its receptor ApoER2, nuclear translocation of the ApoER2-ICD is triggered. Then, ApoER2-ICD binds to the transcriptional factors MEF2 and CREB, and as a result, expressions of some genes involved in synaptic plasticity are activated (Telese et al., 2015).

Observations in animal studies using *reeler* and Reelin receptor mutant mice support the notion that Reelin-synapse dysfunction leads to cognitive dysfunction and neuropsychiatric symptoms (Weeber et al., 2002; Qiu et al., 2006a,b; Pujadas et al., 2010; Trotter et al., 2013). Several groups have reported that Reelin plays an important role in synaptic plasticity, mainly dependent on the NMDA receptor dysfunction in the hippocampal region (Weeber et al., 2002; Qiu et al., 2006b; Pujadas et al., 2010). Weeber et al. (2002) reported that Reelin signaling is crucial for memory formation and synaptic plasticity in the hippocampal CA1 region. They demonstrated using hippocampal slices, that mice with KO of each of the Reelin receptors, VLDLR and ApoER2, exhibited defects in LTP. They further showed that treatment of the hippocampal slices obtained from wild-type mice with Reelin significantly enhanced the LTP in the CA1 region, which was abolished in mice deficient in either of the receptors. Qiu et al. (2006b) replicated *Weeber*'s findings by using HRM. They also observed defect of LTP in the CA1 region by electrophysiological analysis. In addition, according to a report by Pujadas et al. (2010), overexpression of Reelin enhanced the synaptic function in the hippocampus. They generated transgenic mice that overexpressed Reelin under the control of the CaMKIIα promoter. The mice showing Reelin overexpression exhibited increased spine hypertrophy and LTP in the hippocampus (Pujadas et al., 2010). Trotter et al. (2013) generated *Dab1*-conditional knockout mice and examined the spine morphology and synaptic functions. They conditionally deleted Dab1 protein from the excitatory neurons of the adult forebrain using $Dab1^{flox/flox}$; *CaMKII-Cre* mice. This Cre driver line exhibits Cre expression in the forebrain, starting at approximately P19. They showed that loss of Dab1 led to a reduction of the spine size, suppression of Akt and ERK signaling, loss of hippocampal LTP, and deficits in hippocampus-dependent learning and memory. These results obtained using transgenic mice are consistent with the previous results obtained using *reeler* mice (Pujadas et al., 2010; Trotter et al., 2013). Furthermore, recently, Imai et al. (2016) generated dorsal forebrain-specific *Dab1* conditional knockout mice ($Dab1^{flox/flox}$; *Emx1-Cre* mice), in which Cre is expressed specifically in the dorsal forebrain from the beginning of corticogenesis, and performed behavioral tests. These *Dab1*-conditional knockout mice showed normal motor functions, but exhibited hyperactivity, decreased anxiety-like behavior, and deficits in spatial reference and working memory (Imai et al., 2016). In these mice, in addition to the deficient Reelin-Dab1 signaling, the disorganized laminar structure in the cerebral cortex might also have contributed to the observed behavioral abnormalities. The best way to understand Reelin function in adulthood is to analyze conditional *Reelin* gene (*Reln*) knockout mice. Lane-Donovan et al. (2015) generated inducible conditional *Reln* knockout mice and analyzed them. After Reelin inactivation at 2 months of age, they found that while the mice had no apparent abnormalities of the cortical structure, they did exhibit mild behavioral changes and electrophysiological phenotype. Importantly, the behavioral phenotypes in the conditional *Reln* knockout mice may be somewhat different from those observed in *Reln*-deficienct (homozygous and heterozygous *reeler*) mice, which may be based on the degree of structural abnormalities. In the mature brain, the structure would not be seriously affected by Reelin deficiency. By contrast, Reelin deficiency during the developmental and early postnatal stages would severely affect the brain structure, which may be related to the development of some types of young-onset mental disorders. Further investigations in conditional *Reln* knockout mice are needed to clarify this issue.

In humans, dysfunction of the PFC, which plays crucial roles in cognitive functions, has been implicated in many psychiatric disorders (Weinberger et al., 1988). Analogous to the human PFC, the medial PFC (mPFC) is involved in various cognitive functions and social behaviors in rodents (Ishii et al., 2015a). Brosda et al. (2011) demonstrated that Reelin knockdown in the mPFC resulted in behavioral alterations in young adult rats, including disruption of sensorimotor gaiting and deficits in spatial working memory as well as object recognition. Sui et al. (2012) also reported that the expression level of Reelin was related to the regulation of LTP in the rat mPFC. They found that epigenetic regulation of *Reln* was involved in the induction of

LTP in the mPFC, which resulted in behavioral alterations. Iafrati et al. (2014) also reported that HRM showed reduced dendritic spine density and abnormal LTP in the PFC. Interestingly, Ishii et al. (2015b) demonstrated dominant expression of VLDLR in both excitatory pyramidal neurons and GABAergic inhibitory interneurons, except for the migrating interneurons in the rostral migratory stream in the postnatal mPFC. As compared to the findings in the hippocampus, there is not much evidence to support the importance of Reelin signaling in the synaptic functions in the mPFC. Further investigations are needed to elucidate the role of Reelin in the functions of the mPFC relevant to mental illnesses.

REELIN AND ITS DOWNSTREAM SIGNALING MOLECULES AS A TARGET OF THERAPEUTIC INTERVENTION

The findings described in the above sections suggest that Reelin pathways could be potentially useful as targets of therapeutic interventions for neuropsychiatric disorders. Indeed, several groups have directly assessed this possibility by examining the effects of administration of Reelin protein into the mouse brain (Rogers et al., 2011, 2013; Hethorn et al., 2015; Ishii et al., 2015b). Ishii et al. (2015b) demonstrated that Reelin exerted a preventive effect on phencyclidine (PCP)-induced behavioral deficits. They injected a conditioned medium containing Reelin protein into the mouse cerebral ventricles before administering PCP, and assessed the behavior of the animals. The group of mice that had received prior administration of Reelin showed normal cognitive and sensory-motor gating, indicating that the PCP-induced brain dysfunction was prevented by the Reelin injection. This study is based on their previous findings that prior transplantation of GABAergic neuronal progenitors into the mPFC of mice prevented the behavioral deficits induced by PCP and that the transplanted progenitors preferentially differentiated into Reelin/somatostatin-double positive GABAergic neurons specifically in the mPFC (Tanaka et al., 2011). Another group demonstrated that *in vivo* injection of Reelin into the mouse cerebral ventricle affected the synaptic functions and cognitive functions in wild-type mice and HRM (Rogers et al., 2011, 2013). The same group further showed that Reelin administration ameliorated both the synaptic plasticity and the cognitive behavioral deficits in a mouse model of Angelman syndrome, which is characterized by mental retardation, absence of speech, seizures and motor dysfunction (Hethorn et al., 2015).

Although we cannot directly apply this strategy of directly injecting Reelin into the human brain, modulating the activities of the signaling molecules downstream of Reelin could be a potential therapeutic approach. Reelin has been shown to activate PI3K and Akt (protein kinase B), to inhibit glycogen synthase kinase 3β (GSK3 β; Beffert et al., 2002) and activate the mTOR-S6K1 pathway (Jossin and Goffinet, 2007). Among them, GSK3 is already known as a target for mood stabilizers (De Sarno et al., 2002) and antipsychotics (Emamian et al., 2004; Li et al., 2007). In addition, animal models with disrupted mTOR signaling exhibit cognitive and behavioral deficits mimicking neuropsychiatric symptoms (Gururajan and van den Buuse, 2014). On the other hand, rapamycin, a well-established mTORC1 inhibitor, has been demonstrated to rescue cognitive impairments in several neurodevelopmental models of neuropsychiatric disorders, such as those of serotonin receptor ($5\text{-}HT_6$) activation, neonatal PCP treatment, and post-weaning social isolation (Meffre et al., 2012), indicating overstimulation of mTOR signaling in these models. Surprisingly, rapamycin treatment also rescues lamination defects in tuberous sclerosis complex (TSC) 2-deficient mice, in which loss of TSC2 leads to activation of mTOR signaling and aberrant regulation of Reelin-Dab1 signaling (Moon et al., 2015). This inconsistency in the mTOR activities between different models might be explained by the varied activities of mTOR signaling as well as Reelin signaling among different brain regions and/or neuronal populations. Since such variation of Reelin (and its downstream) signaling activities could underlie the complex behavioral phenotypes of human neuropsychiatric disorders, further studies are required to distinguish clinical phenotypes and/or subpopulations that reflect disrupted Reelin signaling.

CONCLUSION

In the developing cortex, Reelin is a key regulator of neuronal migration and laminar formation, which are essential for achieving higher brain functions in mammals. Intriguingly, however, the expression pattern of Reelin and its functions are dramatically different in the postnatal brain as compared to the embryonic brain. Indeed, Reelin modulates synaptic functions, which may also be based on the structural changes of the dendrites and spines in the postnatal period, which are closely related to cognitive behaviors and predisposition to neuropsychiatric symptoms. Although genetic studies have lent support to the notion of an association between the *RELN* gene and neuropsychiatric disorders, recent large-scale genome-wide analyses have revealed that the contribution of *RELN* mutations/polymorphisms to the development of neuropsychiatric disorders is not much higher than previously thought. However, many studies have indicated multiple mechanisms underlying the regulation of Reelin production, e.g., epigenetic regulation, cleavage events, and subtype specification of GABAergic interneurons. Perturbation of these regulatory mechanisms can also lead to brain dysfunction. The findings of animal experiments suggest that Reelin and its downstream signaling are closely related to the synaptic functions that underlie the mouse behaviors relevant to neuropsychiatric disorders. Therefore, the Reelin pathway is a potential therapeutic intervention target for neuropsychiatric disorders. Further investigations are needed to clarify how Reelin signaling regulates the higher brain functions and how it is involved in the development of neuropsychiatric disorders.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the ideas and editing of the manuscript.

ACKNOWLEDGMENTS

We thank members of the Nakajima Laboratory for valuable discussions. This work supported by grants form the MEXT/JSPS KAKENHI (JP 16H06482, JP 15H01586, JP 15H02355, JP 15H01293, JP 26430075, JP 15K09723, JP 16K09997), Keio Gijuku Academic Development Funds, Keio Gijuku Fukuzawa Memorial Fund for the Advancement of Education and Research, Program for the Advancement of Keio Next Generation Research Projects, Takeda Science Foundation, and The Naito Foundation.

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