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Efnb's do not affect canonical Reelin signaling

Theresa Pohlkamp¹, Lei Xiao^{2,4}, Rukhsana Sultana^{2,4}, Asim Bepari^{2,4}, Hans H. Bock⁶, Mark Henkemeyer^{2,4}, and Joachim Herz^{1,2,3,5}

¹Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

²Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

³Department of Neurology and Neurotherapeutics, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

⁴Department of Developmental Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

⁵Department of Center for Translational Neurodegeneration Research, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

⁶Clinic for Gastroenterology, Hepatology and Infectiology, Heinrich-Heine-University, Düsseldorf, Germany

Abstract

Reeler mice exhibit loss of the signaling protein Reelin (Reln) which affects radial pyramidal neuron migration and results in cortical layer malformation¹. During cortical development Eph receptor/ephrin ligand (Eph/Efn) cell-cell interactions are important for neuronal migration, axonal/dendritic growth, synaptogenesis, and pruning. Recently it has been claimed that Ephb/Efnb and Reln pathways interact genetically and layering defects in triple *Efnb1;2;3*^{-/-} mutants and *Efnb3*^{-/-}; *Reln*^{+/-} compound mice are similar to those present in *reeler*². Here we demonstrate that contrary to this previous report the compound mutant *Efnb3*^{-/-}; *Reln*^{+/-} as well as the triple mutant *Efnb1;2;3*^{-/-} do not show defects of cortical layering or elevated Dab1 levels. While protein-protein interactions between Efnbs and Reln have been confirmed, they do not affect the radial positioning of migrating neocortical pyramidal neurons.

The similarity of the *reeler*-like phenotype in mice lacking the Reln receptors Very low density lipoprotein receptor (Vldlr) and Apolipoprotein E receptor Apoer2, the intracellular kinases Src/Fyn, or the adaptor protein Disabled 1 (Dab1) led to the identification of the canonical Reln pathway: Reln binding to its receptors induces Dab1 phosphorylation (pDab1) via Src/Fyn to regulate actin and tubulin dynamics of the cytoskeleton necessary for orderly radial migration of cortical neurons³. pDab1 is degraded through ubiquitination, consequently unphosphorylated Dab1 levels in *reeler* mice are elevated^{4,5}. Dab1 binds via its phosphotyrosine binding (PTB) domain to the intracellular NPXY motif of Vldlr/Apoer2, yet it is unclear how Src/Fyn are recruited to phosphorylate Dab1. Sentürk et al. suggested that this role was filled by Ephrin ligands (ephrins/Efns), which like their protein tyrosine kinase receptors (Ephs) are membrane-anchored proteins and control axon guidance and

other forms of cell-cell communication including at the synapse. Cellular communication is bidirectional through kinase activation in the Eph expressing cell and effector recruitment on the Efn expressing cell⁶. Sentürk et al. reported that the ectodomains of Efnb2 and Efnb3 bind to Reln². We confirmed that Efnb3 binds to Reln while Efnb1 did not⁷. In addition genetic interaction of *Efnbs* with *Reln* was implicated through the analysis of compound *Efnb3*^{-/-};*Reln*^{+/-} animals which, by Sentürk et al., was reported to exhibit a phenotype similar to the *reeler* mouse². Based on their results they concluded that Src/Fyn recruitment requires Efnbs, making Efnbs essential for canonical Reln signaling and neuronal migration during brain development². We have been unable to reproduce key elements of the mechanism reported by Sentürk et al.⁷.

We previously reported that Reln also directly binds to Ephb receptors⁷. Furthermore, Reln, even though less efficient than Efnb1, could induce Ephb2 clustering, tyrosine phosphorylation, and degradation. This signaling mechanism was independent of the canonical Reln receptors Apoer2/Vldlr or Dab1 phosphorylation⁷. To determine if Ephb receptors play a similar role in radial migration of cortical pyramidal neurons as described for Efnbs (particularly for *Efnb3*^{-/-};*Reln*^{+/-})², we examined the brains of *Ephb2*^{-/-};*Reln*^{+/-} and *Efnb3*^{-/-};*Reln*^{+/-} mice (*Efnb3*^{-/-} mice have been described in detail before⁸). In contrast to Sentürk et al., we were unable to detect any malformations of cortical pyramidal neuron layering in either of these genotypes. Nissl staining (Fig.1a-d), NeuN (Fig.2a), Brn1 (Fig.1i-l,q-v), and Tbr1 (Fig.1m-p) immunoreactivity and the analysis of the distribution/polarity of Thy1-GFP-M positive neurons (Fig.1e-h) failed to reveal any obvious cortical layer malformation. In addition we did not find the described difference in numbers of NeuN positive neurons² in the cortical marginal zone (Fig.2a,c), the hippocampal CA1 stratum oriens (Fig.2b,c), nor the cerebellar molecular layer (Fig.2b,c), when we compared *Efnb3*^{-/-};*Reln*^{+/-} mice with their *Efnb3*^{-/-};*Reln*^{+/+} littermates.

During development Cajal Retzius cells secrete Reln and express a set of different Ephbs and Efnbs on their surface. Early in cortical development, Cajal Retzius cells in *Ephb1;2;3*^{-/-} mutant mice disperse less efficiently, resulting in large gaps between neighboring Cajal Retzius cells in the marginal zone, most prominently in the caudal region of the hemispheres⁹. We thus analyzed the layering of the caudal cortex of *Efnb3*^{-/-};*Reln*^{+/-} mice, but could not find any differences in this anatomical locale either (Fig.1I,k,m,o,q-s). However, we recorded a non-Mendelian inheritance pattern in *Efnb3*^{-/-};*Reln*^{+/-} X *Efnb3*^{-/-};*Reln*^{+/-} and *Ephb2*^{-/-};*Reln*^{+/-} X *Ephb2*^{-/-};*Reln*^{+/-} crosses at weaning age with only 8% (12 of 150, p<1.5*10⁻⁶, chi-square test) or 16% (27 of 174, p<0.005, chi-square test) of double knockout mice, respectively. The survival of mice homozygous for the *reeler* mutation in the *Reln* compound *Efnb3*^{-/-} line was significantly lower than in the *Ephb2*^{-/-} line (p<0.05, two-tailed Fisher's exact test).

We next attempted to reproduce the reported *reeler*-like phenotype of the *Efnb1;2;3*^{-/-} triple knockouts². To generate *Efnb1;2;3*^{-/-} triple knockouts we used a *Nestin-Cre* transgenic line, as was used by Sentürk et al.², and combined it with *Efnb1*^{lox/y} and *Efnb2*^{lox/lox} conditional alleles and an *Efnb3* null allele. In the developing CNS the *Nestin* promoter is active from E7.75 on¹⁰, *Nestin-Cre* driven recombination was detected as early as E11^{11,12}. Our analysis of *Efnb1;2;3*^{-/-} triple knockouts did not reveal abnormal cortical pyramidal neuron

migration (Fig.1s,v;2a) or canonical, ApoER2/VLDLR-dependent Reln pathway disruption (Fig.2d,e).

Analysis of *Ephb1;2;3*^{-/-} triple knockouts for the three predominant Ephb receptors expressed in the brain also failed to reveal cortical layering malformations (Fig.1s,v;2a) or canonical Reln pathway disruption (Fig.2d,e).

During the phase of pyramidal neuron migration (E11-17) Reln is produced by Cajal-Retzius cells at the pial surface, while Apoer2/Vldlr are expressed by migrating pyramidal neurons. Temporal expression of *Ephbs/Efnbs* mRNA in the neocortex as revealed by the Allen Brain “Developing Mouse Brain” atlas¹³ showed expression of *Efnb1*, *Efnb2*, *Ephb1* and *Ephb2* from E11.5-18.5. *Efnb3* and *Ephb3* expression was extremely low in the cortex. In contrast almost all *Ephbs* and *Efnbs* were highly expressed in the developing hippocampus, where they are essential for proper layering and axonal/dendritic outgrowth^{3,6,14,15}.

The *Efnb3*^{-/-} mutant mouse strains used by Sentürk et al. and our study were generated by different knockout strategies. Sentürk et al. used the *Efnb3*^{-/-} mutant described in Kullander et al.¹⁶, in which a LacZ knockin at the initiating methionine in the first exon of *Efnb3* generates a true null allele. By contrast, the *Efnb3* mutation used in the current study involves the insertion of a neo-cassette into *Efnb3*-intron 4 (*Efnb3*^{neo/neo}⁸), which causes the loss of any detectable protein expression¹⁷. Phenotypic expression of the gene defect in both strains is indistinguishable, including a unique locomotor defect, corticospinal projection defect, and impaired long-term potentiation^{8,16,18,19}. Taken together the data are consistent with the *Efnb3* mutant developed by Yokoyama et al. being a null-allele, rather than hypomorphic. There is ample of evidence that differences in genetic background can contribute to the severity of mutant phenotypes. However, the effects of *Reln* knockout on neuronal layering and the effects of *Efnb3* knockout on locomotion and mossy fiber pruning are very robust in a variety of genetic backgrounds. While it is possible that Sentürk et al. saw phenotypic variations in their *Efnb3*^{-/-}; *Reln*^{+/-} and *Efnb1;2;3*^{-/-} mice that depend on genetic background, this would merely reaffirm that Efnbs are in fact not essential for Reln pathway activation.

Our findings show that Efnb ligands and Ephb receptors are not required for Reln guided primary radial migration in the neocortex, while possible roles in pyramidal neuron axonal/dendritic growth, pruning, synaptogenesis and/or synaptic plasticity remain to be determined.

Material and Methods

The source of mutant mouse lines, their breeding, as well as immunohistochemical and biochemical techniques are described in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Efnbs are not essential for Reln guided radial pyramidal neuron migration during corticogenesis

a-d: Nissl staining of the somatosensory cortex of different knockout mice (>1 month) as indicated. **e-h:** The Thy1-GFP-M reporter was used to visualize layer V pyramidal cells (>1 month). As indicated with double arrows, GFP labeled cells were mainly located in layer V, which is a typical characteristic of the Thy1-GFP transgenic line²⁰. Some ectopic cells were found close to the midline in the upper layers (II-IV) of the retrosplenial area. In the hippocampus the infrapyramidal bundle of the mossy fibers is prolonged (arrowheads) in mice lacking *EfnB3*, as we described before¹⁴. Mice lacking all *EfnBs* have a dispersion of pyramidal cells in the CA3 (cornus ammonis) of the hippocampus (asterisk) and reduced volume of the dentate gyrus granule cell layer (pound key), similar to what we described for

mice lacking *Ephb1/Ephb2*^{7,15}. There was agenesis (acc) of the corpus callosum (cc) in several brains like reported for different *Efnb* and *Ephb* knockout mice²¹, which did not seem to be aggravated by *Reln* phenotype, but *Efnb* triple mutants consequently had this phenotype. **i-p:** Brn1 positive neurons in P3 brains of *Efnb3* mutants are located in the upper layers without (i,j) or with (k,l) heterozygosity for the *reeler* allele, as are the Tbr1 positive neurons located in the lower layers (mp). **q-v:** Brn1 immunoreactivity in the adult cortex of triple mutants shows the same pattern as in wildtype. i,k,m,o and q-s are more caudal, j,l,n,p,t-v more rostral.

Figure 2. *Efnb3*^{-/-};*Reln*^{+/-} mice lack typical features of *reeler* mice such as overmigration of pyramidal neurons and elevated Dab1 expression

a: NeuN staining in different knockout mice (>1 month) as indicated, double arrow illustrates the less densely packed layer V, which, like the marginal zone (layer I), is present in all brains except for *reeler* (*Ephb2*^{-/-};*Reln*^{-/-}, 2nd panel). **b:** NeuN labeled neurons in the cerebellum and hippocampus in *Efnb3*^{-/-} mice with two or one *Reln* allele. **c:** Quantification of NeuN labeled neurons in the somatosensory cortical marginal zone of *Efnb3*^{-/-} with or without *Reln* heterozygosity or *Efnb1,2,3*^{-/-} mice did not reveal any differences when compared to their wt controls. *Efnb3*^{-/-} knockout mice with or without *Reln* heterozygosity had similar numbers of NeuN labeled cells in the cerebellar molecular layer and the stratum oriens of the hippocampal CA1 layer. **d/e:** Western blotting of whole brain lysates (1 month old mice) and quantification (with standard deviation) did not show significantly altered Dab1 protein concentrations between the *Efnb3* mutants that were either wild type or heterozygous for the *reeler* mutant. In the *Efnb1,2,3*^{-/-} or *Ephb1,2,3*^{-/-} triple knockout mice Dab1 was slightly decreased. In the *Reln* homozygote (*Reln*^{-/-}) Dab1 levels were about 3-fold increased (p<0.0005). There was no significant difference between Dab1 levels in *Efnb3*^{-/-} or *Ephb2*^{-/-} with or without *Reln* heterozygosity, respectively. Wild type Dab1

levels were normalized to 1 for each group of genotypes. n.s.= not significant; *= $p<0.05$; **= $p<0.005$; ***= $p<0.001$; two tailed student's t-test.

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