

Reelin induces EphB activation

Elisabeth Bouché^{1,2,*}, Mario I Romero-Ortega^{3,*}, Mark Henkemeyer⁴, Timothy Catchpole⁴, Jost Leemhuis^{1,8}, Michael Frotscher^{1,5}, Petra May^{1,2,6}, Joachim Herz^{1,7}, Hans H Bock^{1,2,6}

¹Center for Neuroscience, Albert-Ludwigs-University, Albertstrasse 23, D-79104 Freiburg, Germany; ²Department of Medicine II, Albert-Ludwigs-University, Freiburg, Germany; ³Department of Biomedical Engineering, University of Texas at Arlington, Arlington, TX 76019, USA; ⁴Department of Developmental Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA; ⁵Center for Molecular Neurobiology, Hamburg, Germany; ⁶Clinic for Gastroenterology, Hepatology and Infectiology, Heinrich-Heine-University Düsseldorf, Moorenstrasse 5, D-40225 Düsseldorf, Germany; ⁷Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

The integration of newborn neurons into functional neuronal networks requires migration of cells to their final position in the developing brain, the growth and arborization of neuronal processes and the formation of synaptic contacts with other neurons. A central player among the signals that coordinate this complex sequence of differentiation events is the secreted glycoprotein Reelin, which also modulates synaptic plasticity, learning and memory formation in the adult brain. Binding of Reelin to ApoER2 and VLDL receptor, two members of the LDL receptor family, initiates a signaling cascade involving tyrosine phosphorylation of the intracellular cytoplasmic adaptor protein Disabled-1, which targets the neuronal cytoskeleton and ultimately controls the positioning of neurons throughout the developing brain. However, it is possible that Reelin signals interact with other receptor-mediated signaling cascades to regulate different aspects of brain development and plasticity. EphB tyrosine kinases regulate cell adhesion and repulsion-dependent processes via bidirectional signaling through ephrin B transmembrane proteins. Here, we demonstrate that Reelin binds to the extracellular domains of EphB transmembrane proteins, inducing receptor clustering and activation of EphB forward signaling in neurons, independently of the ‘classical’ Reelin receptors, ApoER2 and VLDLR. Accordingly, mice lacking EphB1 and EphB2 display a positioning defect of CA3 hippocampal pyramidal neurons, similar to that in Reelin-deficient mice, and this cell migration defect depends on the kinase activity of EphB proteins. Together, our data provide biochemical and functional evidence for signal integration between Reelin and EphB forward signaling.

Keywords: signal transduction; signaling crosstalk; tyrosine kinase; ephrin; lipoprotein receptor; hippocampus; neural development

Cell Research (2013) 23:473–490. doi:10.1038/cr.2013.7; published online 15 January 2013

Introduction

In the developing brain, postmitotic neurons migrate in a highly coordinated manner from the proliferative zone towards their final position, where they differentiate and

form synaptic contacts with neighboring cells to establish functional neural circuits [1]. Reelin, a large glycoprotein secreted by Cajal-Retzius cells in the developing brain and mainly by interneurons in the mature brain, controls several of these processes, including the positioning of principal neurons in laminated brain structures, the differentiation of neurites, as well as the formation of spines and synapses. Furthermore, Reelin modulates synaptic glutamatergic transmission and potentiation by regulating glutamate receptor maturation, surface expression and recycling, indicating a role in memory and learning and a likely involvement in the pathogenesis of Alzheimer’s disease and schizophrenia (reviewed in [2, 3]). Reelin signaling involves binding to the extracellular domains

*These two authors contributed equally to this work.

Correspondence: Hans H Bock

Tel: +49-761-203 8421; Fax +49-761-203 8477

E-mail: hans.bock@zfn.uni-freiburg.de; HansHenrich.Bock@med.uni-duesseldorf.de

⁸Present address: Roche Pharma AG, D-79639 Grenzach-Wyhlen, Germany

Received 27 April 2012; revised 28 September 2012; accepted 9 November 2012; published online 15 January 2013

of the lipoprotein receptors ApoER2 and VLDL receptor (VLDLR), 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 [4-6]. Binding of Reelin to other transmembrane receptors including integrins and amyloid precursor protein (APP) has been reported, although the physiological functions of these interactions remain poorly understood [7, 8]. Recently, it has been proposed that transmembrane ephrin B proteins are required for the recruitment of Src family kinases to the Reelin-lipoprotein receptor-Dab1 signaling complex at the plasma membrane [9].

Ephrin B proteins bind to the EphB family of transmembrane receptor tyrosine kinases and transduce bidirectional tyrosine kinase-mediated signals to both the Eph-expressing (forward signaling) and the ephrin-expressing cell (reverse signaling). These bidirectional signals initiated by Eph-ephrin contact control diverse cellular adhesion/repulsion responses, such as axon guidance, and dendritic spine and synapse morphogenesis [10, 11].

Both Reelin and EphB signaling regulate synaptic plasticity by inducing Fyn-dependent phosphorylation of NMDA receptor subunits and subsequent potentiation of synaptic calcium influx [3, 12-14]. In addition, both pathways have been shown to modulate the toxic effects of amyloid- β oligomers on NMDA receptor-dependent synaptic transmission [15, 16], and to control common intracellular effector molecules, including Src family kinases, the Rho GTPase Cdc42 or the actin-binding protein cofilin [17-20]. Moreover, both EphB2 and the Reelin receptors ApoER2 and VLDLR have been reported to be proteolytically processed by the γ -secretase complex [21, 22]. The parallels between Reelin receptor and Eph protein signalings prompted us to examine a possible direct link between both signaling pathways. We found that Reelin induces clustering of EphB proteins independently of its interaction with lipoprotein receptors, by binding directly to the extracellular portion of EphB proteins. Furthermore, we demonstrated that treatment with Reelin induces autophosphorylation and subsequent proteolytic degradation of EphB proteins, indicating that the Reelin-EphB interaction can trigger EphB forward signaling in responsive cells. We also observed that stimulation of endogenous EphB2 receptors with Reelin leads to Cos-1 cell retraction and rounding, indicative of the deadhesive cytoskeletal changes after activation of EphB forward signaling. Finally, we showed that mice lacking EphB1 and EphB2 display a dispersion of the medial hippocampal CA3 region. This migration defect depends on the kinase activity of EphB receptors and cannot be explained

solely by defective ephrin B-mediated forward signaling. Altogether, our results suggest a functional role for the interaction between Reelin and EphB, which is involved in the shaping of the developing hippocampus.

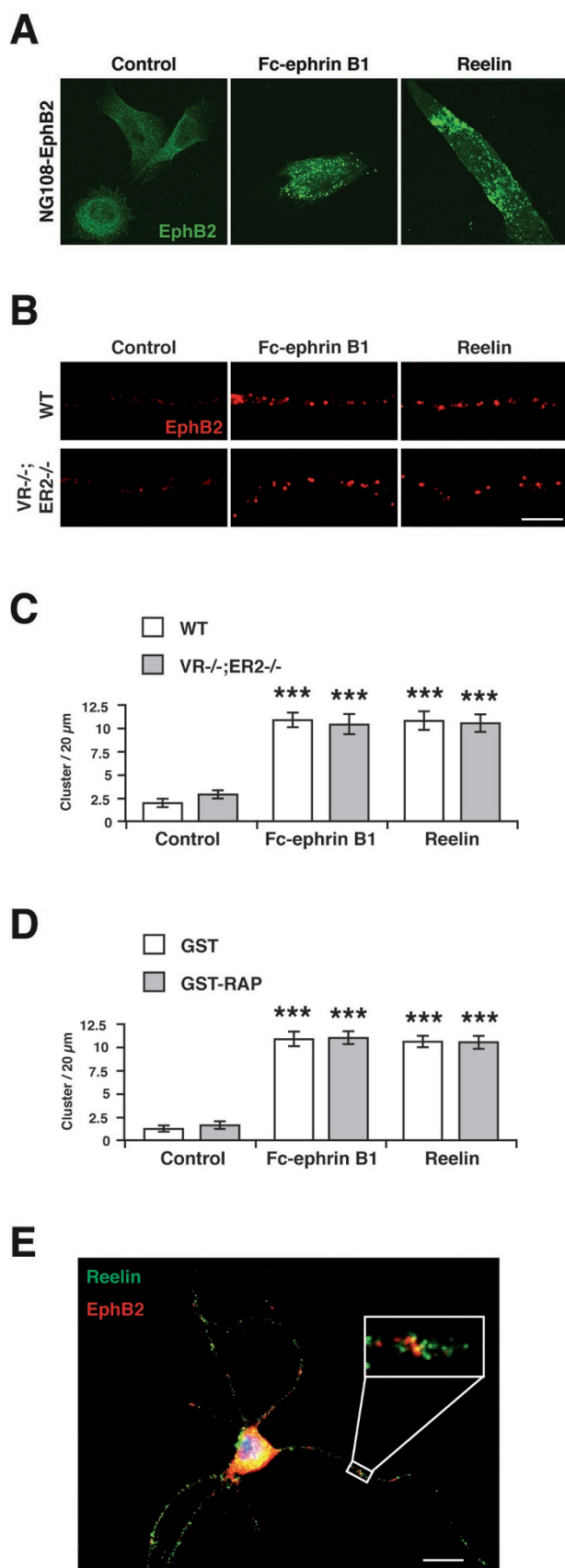
Results

The secreted glycoprotein Reelin induces clustering of EphB2

To test a potential crosstalk of Reelin with Eph class tyrosine kinases, we analyzed the ability of Reelin to bind and activate EphB proteins. When Eph-expressing cells are treated with soluble, preclustered Fc-tagged ephrin ectodomain, the Eph proteins become clustered to distinct spots at the cell surface [23]. We found that recombinant Reelin was also able to induce clustering of EphB2 ectopically expressed in the neuronal cell line NG108-15 [24], similar to that elicited by preclustered soluble ephrin B1 ligand (Figure 1A). We also showed that EphB2 became clustered in response to Reelin in primary embryonic cortical and hippocampal neurons, including neurons from compound *Vldlr* and *Apoer2* knockout mice [25] (Figure 1B and 1C). In addition, the lipoprotein receptor antagonist RAP did not block the effect of Reelin on EphB2 clustering (Figure 1D and Supplementary information, Figure S1A), suggesting that Reelin might induce receptor clustering independently of ApoE receptors, by binding directly to EphB2. In support of this, we observed that Reelin and EphB2 clusters colocalized on the dendrites of cultured cortical neurons (Figure 1E).

Reelin directly interacts with the extracellular domain of EphB receptors

To test whether Reelin directly binds to EphB receptors, we incubated recombinant Reelin with the recombinant Fc-tagged ectodomain of EphB2 and performed a pull-down experiment. We found that Reelin interacted with the EphB2 ectodomain (Figure 2A). To ensure specificity of the observed binding, we conducted a control experiment with the Fc-tagged soluble ectodomain of another receptor tyrosine kinase, the platelet-derived growth factor receptor- β (PDGFR β) subunit, which did not bind Reelin (Figure 2A). Similar pull-down experiments using Fc-tagged EphB family ectodomains showed that Reelin binds to EphB1, EphB2 and EphB3 with similar affinities (Figure 2B). The recently reported interaction of Reelin with the extracellular domain of the EphB ligand ephrin B3 [9], but not with ephrin B1, was also confirmed (Figure 2B). Coincubation with receptor-associated protein (RAP), an ER chaperone that acts as a lipoprotein receptor antagonist [26], or the calcium



chelator EDTA have been shown to block Reelin binding to the ectodomains of ApoER2 (Figure 2C) or VLDLR [27]. However, neither treatment blocked the interaction of Reelin with EphB2 (Figure 2D).

Mapping of the EphB-interacting Reelin domain

Full-length Reelin is a large secreted glycoprotein of > 400 kDa that consists of an amino-terminal signal peptide followed by an F-spondin homology domain, a unique segment of ~400 amino acids, eight so-called ‘Reelin repeats’ of 300-350 amino acids (R1-R8) and a short basic carboxy-terminal region (Figure 3A). To examine which Reelin domain interacts with EphB2, we performed pull-down assays using conditioned media containing comparable amounts of myc-tagged Reelin fragments carrying various deletions [28] (Figure 3B). Binding of the lipoprotein receptors ApoER2 and VLDLR involves the central fragment of Reelin comprising repeats 3-6, whereas the amino- and carboxy-terminal domains of Reelin are dispensable for this interaction [28] (Figure 3C). For the fragment N-R2, comprising the amino-terminal domain and the Reelin repeats R1-R2, binding to the EphB2 ectodomain was observed (Figure 3D). Fragments R3-R6 and R7-R8 did not coprecipitate with Fc-EphB2 (Figure 3D), mapping the interaction site to the amino-terminal region of Reelin. These results indicate that full-length Reelin in principle can interact with lipoprotein receptors and EphB2 simultaneously.

Figure 1 Reelin induces clustering of EphB2 proteins. **(A)** Stimulation with preclustered ephrin B1 extracellular domain or Reelin induces clustering of EphB2 (green) in stably transfected NG-108 cells. **(B)** Clustering of endogenous EphB2 (red) in primary cortical neurons after stimulation with preclustered ephrin B1 or Reelin. Clustering is observed in wild-type (WT) neurons as well as in neurons lacking both ApoER2 and VLDLR (VR^{-/-};ER2^{-/-}). Scale bar, 5 μm. **(C)** Quantification of EphB2 cluster density in ephrin B1- or Reelin-treated primary neurons prepared from WT or ApoER2/VLDLR double knockout embryos (VR^{-/-};ER2^{-/-}) (S.E.M., ****P* < 0.001 compared with control, *n* = 15 per condition, 3 knockout embryos). **(D)** Quantification of EphB2 cluster density in ephrin B1- or Reelin-treated primary neurons preincubated with GST or GST-RAP, a lipoprotein receptor antagonist (S.E.M., ****P* < 0.001 compared with control, *n* = 15 per condition). **(E)** Co-localization of EphB2 and Reelin in cortical neurons. Cultured cortical neurons were treated with recombinant Reelin, washed twice, fixed and immunostained at DIV 6 for EphB2 and Reelin. Scale bar, 10 μm. A higher magnification of a dendrite is shown (boxed area). Co-localization of EphB2 (red) and Reelin (green) appears as yellow puncta.

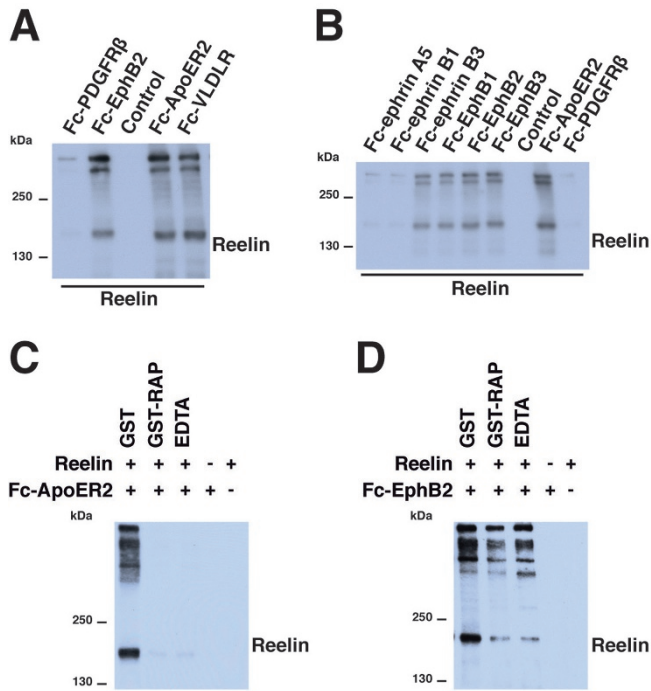


Figure 2 Reelin binds to the extracellular domain of EphB proteins. **(A)** Supernatant of Reelin-expressing HEK-293 cells was incubated with the ectodomains of different transmembrane receptors. The Fc-fused ectodomains were precipitated with protein A/G-agarose beads. Reelin binds to the ectodomains of the lipoprotein receptors ApoER2 and VLDLR (positive controls), and to the ectodomain of EphB2. The ectodomain of platelet-derived growth factor receptor- β (Fc-PDGFR β) and protein A/G-agarose beads without Fc-coupled ectodomain (control) served as negative controls. **(B)** Biochemical interaction of Reelin with members of the EphB and ephrin B gene families. Recombinant Reelin was incubated with the ectodomains of ephrin A5, ephrin B1 and ephrin B3, EphB1, EphB2 and EphB3, and of PDGFR β as a negative control. The Fc-fused ectodomains were precipitated with protein A/G-beads, and bound Reelin was detected by immunoblotting. Fc-ApoER2 served as a positive control. Reelin was bound by the ectodomains of EphB1-3. Moderate binding was also observed for Fc-ephrin B3. **(C)** GST-RAP (30 μ g/ml) and the calcium chelator EDTA (30 mmol/l) blocked binding of Reelin to its lipoprotein receptors, shown here for ApoER2. **(D)** The EphB2-Reelin interaction was not blocked by GST-RAP or EDTA.

Activation of EphB forward signaling by Reelin treatment

The ability of Reelin to bind to EphB receptors and to induce clustering of EphB2 to distinct membrane spots in stably transfected NG-108 cells and primary neurons prompted us to examine whether Reelin might activate Eph protein forward signaling. We stimulated primary cortical neurons with Reelin or preclustered ephrin B1, and cell lysates were immunoprecipitated with an anti-

body against EphB2 and probed by western blotting with an anti-phosphotyrosine antibody. Stimulation with preclustered ephrin B1 resulted in a robust increase in EphB2 phosphorylation (Figure 4A), indicating activation of the receptor kinase domain. We observed that Reelin treatment also induced receptor phosphorylation, albeit to a lesser extent than the cognate EphB2 ligand ephrin B1 (Figure 4A). This activation was not inhibited by coinubation with the lipoprotein receptor antagonist RAP (Figure 4B), although treatment with GST-RAP blocked activation of the lipoprotein receptor-dependent Reelin signaling cascade as indicated by the inhibition of Reelin-induced Dab1 tyrosine phosphorylation (Figure 4B, middle blot, lane 5). In line with this, Reelin-induced EphB2 phosphorylation was also observed in neurons lacking both ApoER2 and VLDLR (Figure 4D). To further exclude the possibility that the Reelin-induced increase in EphB2 phosphorylation is the result of trans-phosphorylation via Src kinases, we used Dab1-deficient neurons (Figure 4C). As Dab1 acts as a Src family kinase (SFK) switch [29], the lack of Dab1 prevents Reelin-induced SFK activation [30]. However, Reelin-mediated EphB2 phosphorylation was also observed in the Dab1-deficient neurons (Figure 4C), indicating that this effect is mediated directly via the interaction of Reelin with EphB2.

To further validate our observation that Reelin binding can activate EphB forward signaling, we analyzed whether Reelin activates proteolytic processing of EphB2, as has been shown in response to other stimuli, including ephrin B ligand binding [21, 31, 32]. In HEK-293 cells transfected with full-length EphB2 receptor, treatment with preclustered Fc-ephrin B1 for 4 h resulted in a significant decrease of full-length EphB2, which was also seen after treatment with recombinant Reelin (Figure 5A). A moderate ligand-dependent decrease in full-length EphB2 levels in response to treatment with Fc-ephrin B1 or Reelin was also observed in primary cortical neurons, which reached its maximum after an incubation period of 8 h (Figure 5B-5D). Quantification of *Ephb2* mRNA by real-time PCR in 6 h Reelin-treated neurons indicated that the Reelin-induced decrease of EphB2 is not caused by changes at the transcriptional level (Figure 5E). It has previously been shown that the ephrin B ligand can induce endocytosis and subsequent endosomal processing of its receptor EphB2 [21]. In line with this, inhibition of endosomal acidification by bafilomycin A1 blocked the ligand-induced decrease of full-length EphB2 by preclustered ephrin B1 or Reelin in transfected HEK-293 cells (Figure 5F), which do not endogenously express ApoER2, VLDLR or EphB2 (Supplementary information, Figure S1B). In addition,

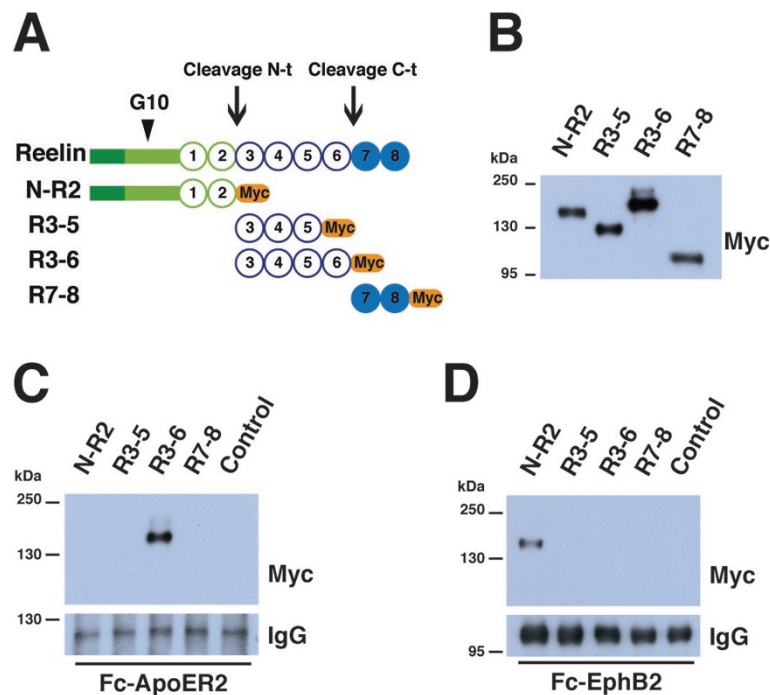


Figure 3 EphB2 interacts with the amino-terminal domain of Reelin. **(A)** Schematic diagram of partial Reelin constructs. The dark- and bright-green rectangles indicate the signal peptide, the F-spondin homology region and the unique region that is recognized by the G10 anti-Reelin monoclonal antibody (arrowhead). The circles with numbers represent the eight Reelin repeats. The carboxy-terminal myc-tags of the partial Reelin constructs are highlighted in orange. Arrows indicate sites of proteolytic Reelin cleavage. **(B)** The Reelin constructs shown in **A** were transfected into HEK-293 cells. Supernatants were separated by SDS-PAGE and proteins were detected with an anti-myc antibody. Comparable amounts of the constructs were used for the coprecipitation experiments shown in **C** and **D**. **(C)** Fc-tagged ApoER2 or **(D)** EphB2 ectodomains were incubated with supernatants containing similar amounts of the different myc-tagged partial Reelin polypeptides and precipitated with protein A/G-sepharose. Coprecipitated Reelin constructs were visualized by immunoblotting with an anti-myc antibody. The central fragment containing Reelin repeats R3-6 binds to the lipoprotein receptor ectodomains **(C)**, whereas EphB2 binds to the amino-terminal Reelin fragment N-R2 **(D)**.

we observed accumulation of an EphB2 fragment of ~50 kDa after stimulation with ephrin B1 or Reelin, using an antibody that recognizes a cytoplasmic epitope of the receptor in EphB2-overexpressing cells pretreated with the proteasome inhibitors lactacystin (Figure 5G) or epoxomicin (data not shown), consistent with the concept that Reelin, like ephrin B1, promotes the ligand-induced degradation of EphB receptors. This fragment (CTF2) is generated by the γ -secretase complex, as pretreatment with the γ -secretase inhibitor DAPT in combination with lactacystin led to the accumulation of a fragment with a slightly higher molecular weight (CTF1), which serves as a γ -secretase substrate [21] (Figure 5G). Finally, we demonstrated that the Reelin-induced degradation of EphB2 does not depend on the interaction of Reelin with its lipoprotein receptors ApoER2 and VLDLR, either by blocking neuronal lipoprotein receptors with GST-RAP (Figure 5H) or by using neurons that were prepared from

embryos lacking both receptors (Figure 5I).

Reelin induces EphB2-dependent deadhesive cytoskeletal changes in Cos cells

To address functional effects of Reelin-mediated activation of EphB forward signaling, we investigated whether Reelin was able to induce deadhesive cytoskeletal changes in Cos-1 cells. These cells, which endogenously express EphB2 but not the Reelin lipoprotein receptors or Dab1 (Figure 6A), undergo cytoskeletal changes resulting in the rounding and detachment of cells after prolonged exposure to preclustered ephrin B1 or the alternative EphB2 ligand ephrin A5 [33, 34] (Figure 6B and 6C). Similarly, cells retracted and lost attachment to the coverslip after treatment with recombinant Reelin (Figure 6B and 6C). To demonstrate the requirement for EphB2 forward signaling in mediating this Reelin effect, we used WHI-P180, a pharmacological compound that

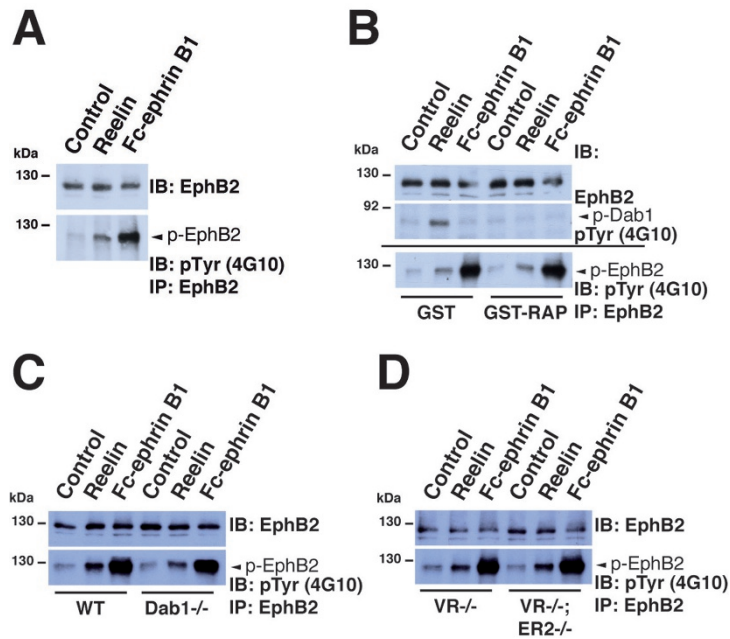


Figure 4 Reelin induces EphB2 tyrosine phosphorylation in cortical neurons. **(A)** Primary neurons were treated with recombinant Reelin or preclustered Fc-ephrin B1. Before immunoprecipitation with an anti-EphB2 antibody, the cell lysates were analyzed by western blotting with the same antibody (IB: EphB2; input). The immunoprecipitated samples (IP: EphB2) were analyzed by western blotting with a phosphotyrosine-specific monoclonal antibody (IB: pTyr) to determine tyrosine phosphorylation levels of the EphB2 receptor in Reelin- or Fc-ephrin B1-treated neurons compared to control treatment. **(B)** Primary neurons were preincubated with GST or GST-RAP before stimulation with Reelin or preclustered soluble ephrin B1. The lipoprotein receptor antagonist RAP prevented the Reelin-induced tyrosine phosphorylation of Dab1 as detected by immunoblotting with the phosphotyrosine-specific antibody (middle blot, lane 5), but did not block the induction of EphB2 tyrosine phosphorylation (bottom). Shown are representative blots of at least three independent experiments **(A-B)**. **(C)** Primary neurons lacking the adapter protein Dab1 (*Dab1^{-/-}*) were treated with Reelin or preclustered Fc-ephrin B1 (*n* = 2 knockout embryos). Deficiency in the Src family kinase switch protein Dab1 did not prevent Reelin- or ephrin B1-induced EphB2 phosphorylation. **(D)** Primary neurons deficient in VLDLR or both Reelin receptors ApoER2 and VLDLR were treated with Reelin or preclustered soluble ephrin B1. Lack of VLDLR (*VR^{-/-}*) or VLDLR and ApoER2 (*VR^{-/-};ER2^{-/-}*) did not block the induction of EphB2 tyrosine phosphorylation by Reelin or ephrin B1. Neurons were prepared from three different double knockout embryos.

has been shown to act as an ATP-competitive inhibitor of the EphB2 receptor tyrosine kinase [35] (Supplementary information, Figure S1C). Pretreatment of Cos-1 cells with this substance effectively inhibited the cell retraction induced by ephrin B1 or Reelin treatment (Figure 6D), suggesting that EphB2 receptor autophosphorylation is required for the ligand-induced cell detachment. As WHI-P180 targets other kinases as well, we sought for a more specific approach to confirm the involvement of EphB2 forward signaling in the Reelin-mediated detachment of Cos-1 cells. To block EphB2 receptor activation directly, we utilized a peptide (SNEWIQRLPQH) that selectively binds to EphB2 and antagonizes binding of its cognate ligand ephrin B1 [34]. Preincubation of Cos-1 cells with the SNEW peptide blocked the effect of preclustered ephrin B1 or Reelin on cell rounding (Figure 6E), whereas the lipoprotein receptor antagonist RAP had no effect (Figure 6B and 6C). As a third independent

approach to inactivate EphB2 signaling, we used siRNA-mediated knockdown of EphB2 (Supplementary information, Figure S1D), which prevented the Reelin- and ephrin B1-mediated effect on Cos-1 cell rounding (Figure 6F). Altogether, these data provide functional evidence that Reelin binds to and activates EphB proteins, thereby inducing receptor forward signaling and functional reorganization of the cytoskeleton in responsive cells.

Defective hippocampal development in Ephb1;Ephb2 compound mutant mice

The role of Reelin-mediated EphB forward signaling during neurodevelopment was tested through the evaluation of EphB receptor double knockout mice. Adult mice simultaneously lacking EphB1 and EphB2 have a reduced number of neural progenitors in the subgranular proliferation zone of the hippocampus and a significantly reduced dentate gyrus volume. This phenotype is largely

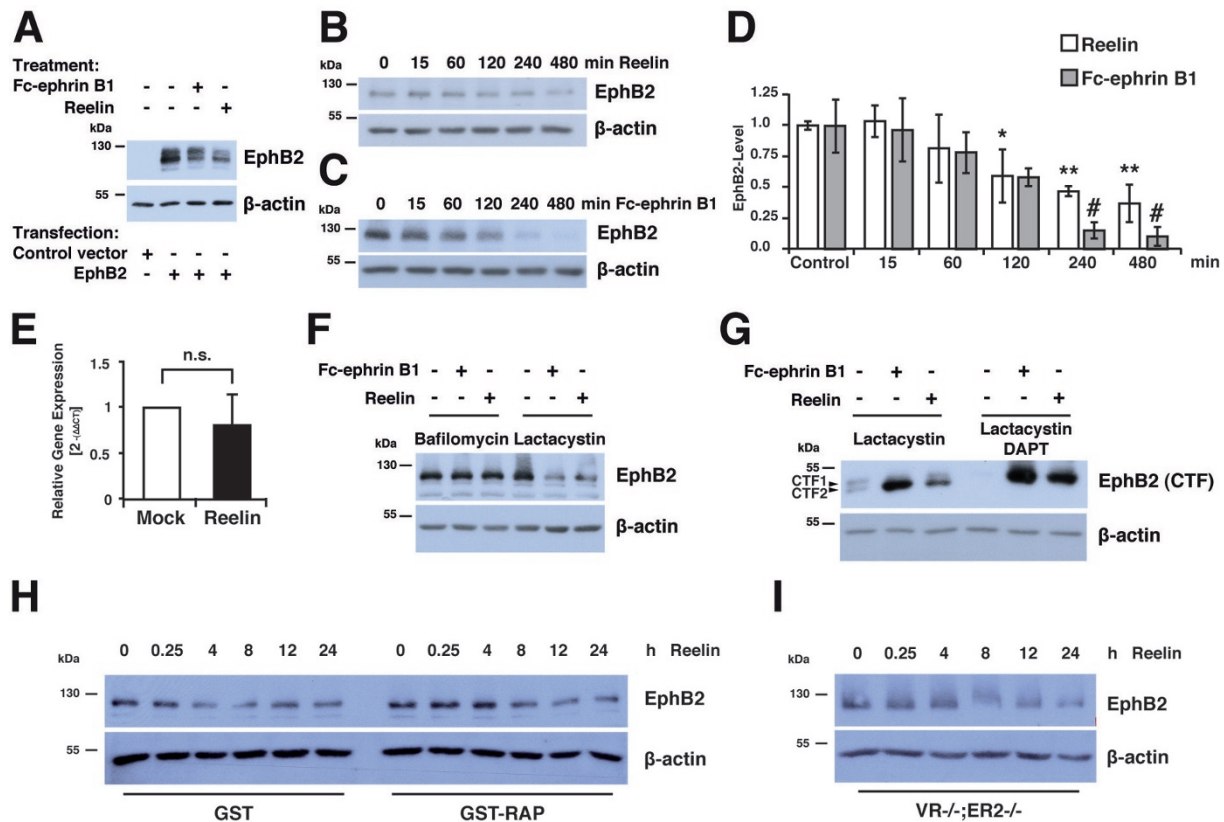


Figure 5 Reelin induces proteolytic processing of EphB2. **(A)** Treatment with Reelin induces decrease of full-length EphB2 in transfected HEK-293 cells. HEK-293 cells were transiently transfected with a plasmid encoding full-length EphB2 and treated with preclustered soluble ephrin B1 or Reelin for 4 h. Cell lysates were analyzed by western blotting. Treatment with both preclustered ephrin B1 or Reelin led to a significant decrease of full-length EphB2 protein levels. Actin served as a loading control. **(B, C)** Decrease of EphB2 levels in cortical neurons after stimulation with Reelin **(B)** or Fc-ephrin B1 **(C)**. Cortical neurons (E15.5, DIV5) were treated for the indicated times, lysed in RIPA buffer and analyzed by western blotting for EphB2 protein levels. Treatment with Reelin induced a moderate decrease in the levels of full-length EphB2 after 2–8 h. Actin served as a loading control. **(D)** Quantification of the EphB2 signal intensity after treatment with Reelin (white bars) or Fc-ephrin B1 (grey bars) is shown (S.D., * $P < 0.05$, ** $P < 0.01$ for Reelin and # $P < 0.005$ for Fc-ephrin B1 as compared to controls, $n = 3$). **(E)** Quantification of relative *Ephb2* mRNA levels in primary neurons treated for 6 h with recombinant Reelin ($n = 3$) as determined by qRT-PCR. n.s., non-significant. **(F)** Cortical neurons were preincubated with bafilomycin A1 or lactacystin for 30 min and then treated with Reelin or preclustered Fc-ephrin B1 for 7.5 h. The endosomal inhibitor bafilomycin prevented degradation of the full-length form of EphB2, whereas the proteasomal inhibitor lactacystin had no effect. **(G)** EphB2-transfected HEK-293 cells were preincubated with lactacystin or lactacystin + DAPT, a γ -secretase inhibitor, and then treated with Reelin or preclustered Fc-ephrin B1 for 8 h. Inhibition of the proteasome led to an increase of a carboxy-terminal fragment of EphB2 (CTF2), whereas pretreatment with lactacystin and DAPT leads to the accumulation of CTF1, a slightly larger carboxy-terminal fragment of EphB2 that serves as a substrate for the γ -secretase complex. **(H)** Cortical neurons were pretreated with GST or GST-RAP (30 $\mu\text{g}/\text{ml}$), then stimulated with Reelin for the indicated times, lysed in RIPA buffer and analyzed by western blotting for EphB2 protein levels. GST-RAP did not prevent the Reelin-induced EphB2 degradation. Actin served as a loading control. One experiment representative of 3 is shown. **(I)** Cortical neurons were prepared from embryos lacking both VLDLR and ApoER2 ($VR^{-/-};ER2^{-/-}$), stimulated with Reelin for the indicated times and analyzed as described above. Shown is one out of two independent experiments.

attributable to defective ephrin B ligand-like activity [36]. Moreover, mice lacking only EphB2 display a circumscribed defect in the development of the lateral subregion of the suprapyramidal granule cell layer of the dentate gyrus. This phenotype is recapitulated in mice that do not

express the ephrin B1 ligand [37]. However, compared with wild-type mice and single EphB mutants, histological evaluation of the medial cornu ammonis area 3 (CA3) in the hippocampus of *Ephb1*;*Ephb2* compound null mice revealed abnormally broader localization, and thus

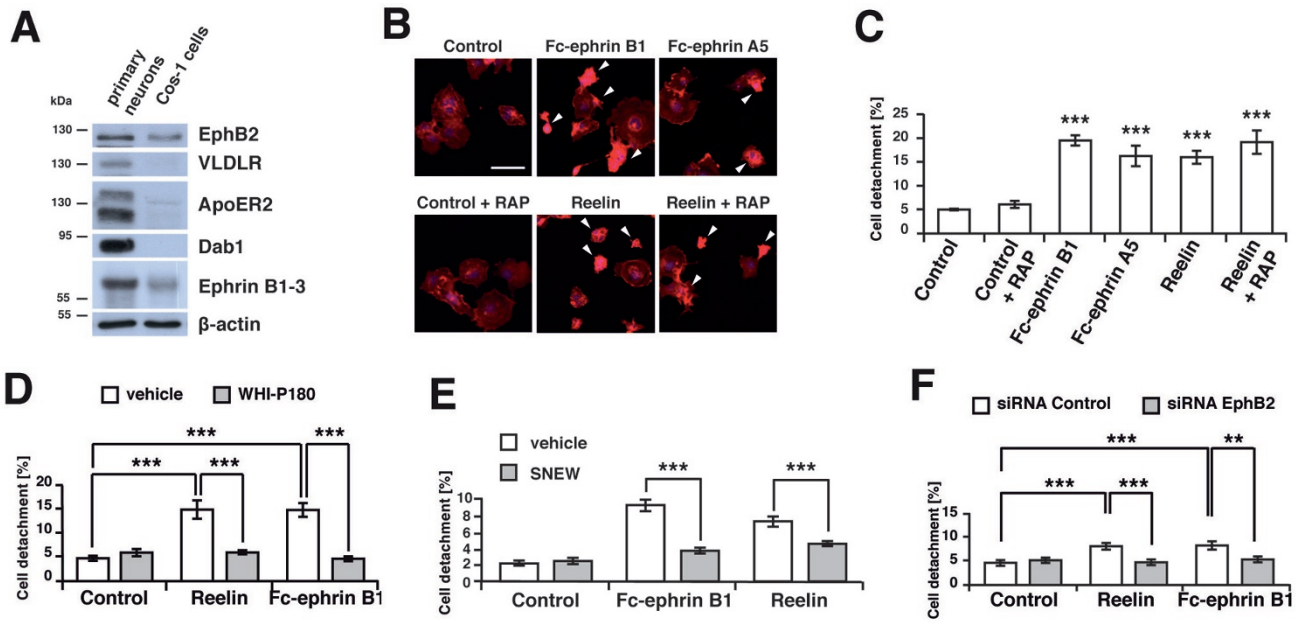


Figure 6 Reelin-dependent activation of EphB2 induces deadhesive cytoskeletal changes in Cos cells. **(A)** Expression of components of the Reelin and EphB2 signaling cascades in primary neurons and Cos-1 cells was analyzed by western blotting. Cos-1 cells express EphB2 and ephrin B proteins but do not express detectable levels of VLDLR, ApoER2 or Dab-1. Actin was used as a loading control. **(B)** Treatment with preclustered ephrin B1 (4 μ g/ml), ephrin A5 (20 μ g/ml) or Reelin but not with control medium induces deadhesive cytoskeletal changes in Cos-1 cells, resulting in rounding and loss of attachment (arrowheads) after 2.5 h of treatment. The effect of Reelin was not blocked by the lipoprotein receptor antagonist RAP, and treatment with RAP alone has no effect. **(C)** Quantification of the deadhesive changes in Cos-1 cells shown in **B** as compared with control (S.E.M., $***P < 0.001$, 3 independent experiments, $n = 10$ fields of view with a minimum of 1 000 cells per group analyzed). **(D)** Pharmacological EphB2 kinase inhibition with WHI-P180 (10 μ mol/l) blocks the Reelin- or Fc-ephrin B1-induced detachment of Cos-1 cells. Quantification of deadhesive changes in Cos-1 cells (S.E.M., $n = 10$ fields of view per group analyzed, $***P < 0.001$) is shown. **(E)** Pretreatment with the EphB2-selective antagonistic 12-mer peptide SNEW (400 μ mol/l) blocks the Reelin- or Fc-ephrin B1-induced detachment of Cos-1 cells. Quantification of the deadhesive changes in Cos-1 cells is shown (S.E.M., $n = 10$ fields of view per group analyzed, $***P < 0.001$). **(F)** Knockdown of endogenous EphB2 in Cos-1 cells by exogenous siRNA against human EphB2 (siRNA EphB2) prevented the Reelin- and ephrin B1-mediated detachment of Cos-1 cells, as compared with control-treated cells (siRNA control). Quantification of the cell detachment is shown (S.E.M., $n = 10$ fields of view per group analyzed, $***P < 0.001$, $**P < 0.01$).

abnormal migration of pyramidal neurons in this region but not in CA1 (boxed area, Figure 7A). To confirm the need for EphB forward signaling in cell migration, we evaluated the CA3 cell density in animals in which the intracellular domain of the EphB2 receptor was replaced with β -galactosidase [38]. Compound *Ephb1*^{-/-};*Ephb2*^{lacZ/lacZ} (and *Ephb1*^{lacZ/lacZ};*Ephb2*^{-/-}, data not shown) mutant mice showed a dispersion of the medial CA3 area, which was comparable to that of *Ephb1*;*Ephb2* double knockout mice (Figure 7A and 7B). Similarly, mice expressing a kinase-dead EphB2 receptor (K661R) [39] on an EphB1-deficient background also displayed CA3 heterotopia, whereas deletion of the three carboxy-terminal amino acids of EphB2, which disrupts its PDZ domain-binding motif (*Ephb1*^{-/-};*Ephb2*^{dVEV/dVEV}) [39], had no effect (Figure 7A and 7B). Hence, EphB forward signaling is required

for proper development of the CA3 subfield. Some cell dispersion was also quantified in animals lacking ephrin B1 (*Efnb1*). However, the duplication of the pyramidal cell layer in the medial CA3, adjacent to the hilar region, is not fully recapitulated in *Efnb1* knockout mice (Figure 7A and 7B), and the cellular dispersion is mild compared with that observed in *Ephb1/2* double knockout mice. In sharp contrast, no cell lamination defects were observed in the CA1 region in all animals examined (Figure 7A and 7B). The CA3 heterotopia in animals with impaired EphB1/2 kinase activity is similar to that observed in the *reeler* hippocampus (Figure 7C), suggesting the possibility of a combined role for Reelin and EphB receptors in orchestrating CA3 formation. Using β -galactosidase reporter mice, we then confirmed that both EphB1 and EphB2 receptors, but not their ephrin B2 and B3 ligands

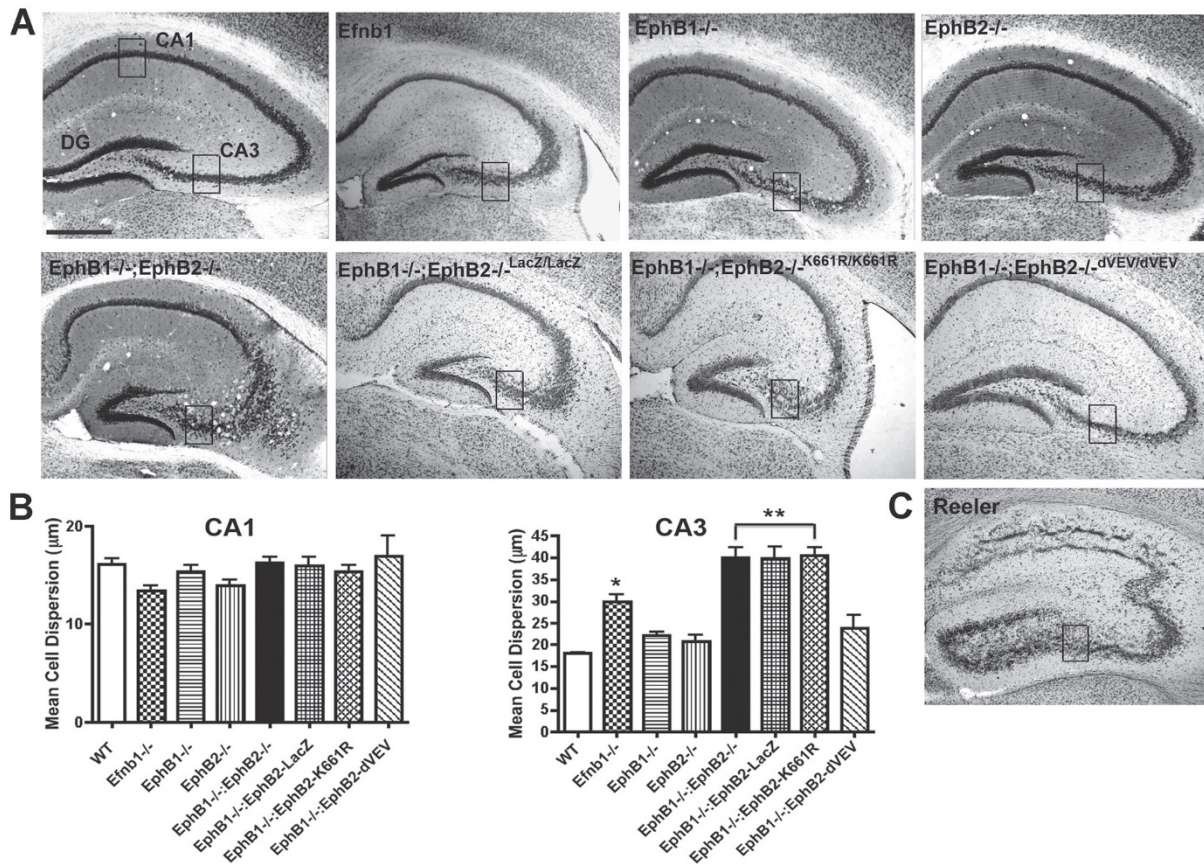


Figure 7 Defective forward signaling leads to a neuronal migration defect in the hippocampus CA3 region of EphB1/2-deficient mice. **(A)** H&E staining of coronal hippocampal sections of WT, Ephrin B1 (*Efnb1*) knockout, EphB1 knockout, EphB2 knockout and compound *Ephb1*^{-/-};*Ephb2*^{-/-} knockout mice, those with a lacZ reporter motif replacing the EphB2 kinase domain (EphB2-lacZ) and those with either a point mutation disrupting EphB2 kinase activity (K661R) or with inactivation of the PDZ domain-binding motif of EphB2 (dVEV994) on an EphB1-deficient background. Scale bar, 500 µm. CA1 and CA3, cornu ammonis subfields 1 and 3 of the hippocampus proper; DG, dentate gyrus. **(B)** Quantification of the mean cell dispersion (in µm) in the CA1 vs CA3 hippocampal subfield (boxed areas in **A**; mean ± standard deviation (S.D.), *n* = 4-8 animals per genotype; **P* ≤ 0.01 for *Efnb1* knockout mice vs control, ***P* ≤ 0.001 for compound KO mice vs other genotypes; ANOVA followed by Newman-Keuls multiple comparison test). The compound knockout mice lacking EphB2 or expressing either carboxy-terminally truncated EphB2-lacZ or EphB2 carrying point mutation K661R that inactivates the tyrosine kinase catalytic domain on an EphB1-deficient background display an increased dispersion of the CA3 pyramidal layer that is not seen in the CA1 region. **(C)** H&E staining of *reeler* hippocampus showed cellular dispersion in all CA subfields. Specifically, the cellular defects of the medial CA3 region in *reeler* mice (boxed area) are comparable to those observed in the *Ephb1*^{-/-};*Ephb2*^{-/-} mice.

are expressed at E16.5 during active hippocampus cell migration (Figure 8A). Furthermore, double immunolabeling of NeuroD-expressing hippocampal precursors and β-galactosidase in *EphB1*^{lacZ/lacZ} animals showed that cells migrating towards the future CA3 area expressed EphB1 receptors (Figure 8B, middle column). Although dentate gyrus granule cells are NeuroD positive as well, only a small proportion of those at the subventricular zone are known to express EphB1 receptors [36], and most cells migrating towards the dentate gyrus lacked an EphB1-lacZ signal (Figure 8B, right column). Moreover, β-galactosidase labels almost exclusively the CA3

subfield of adult *EphB1*^{lacZ/lacZ} mice, whereas only few granule cells are stained (Figure 8C). This supports the notion that migrating CA3 precursors express EphB receptors during active migration, and at their final destination, suggesting that the EphB receptors in those cells interact with Reelin during migration, which is expressed at high levels in the developing hippocampus (Figure 8D, left). It has been described that Reelin expression above the lateral suprapyramidal blade of the dentate gyrus is slightly reduced in EphB2-defective mice during late prenatal stages [37]. However, near the developing CA3 area, Reelin is highly expressed, both in the hilar

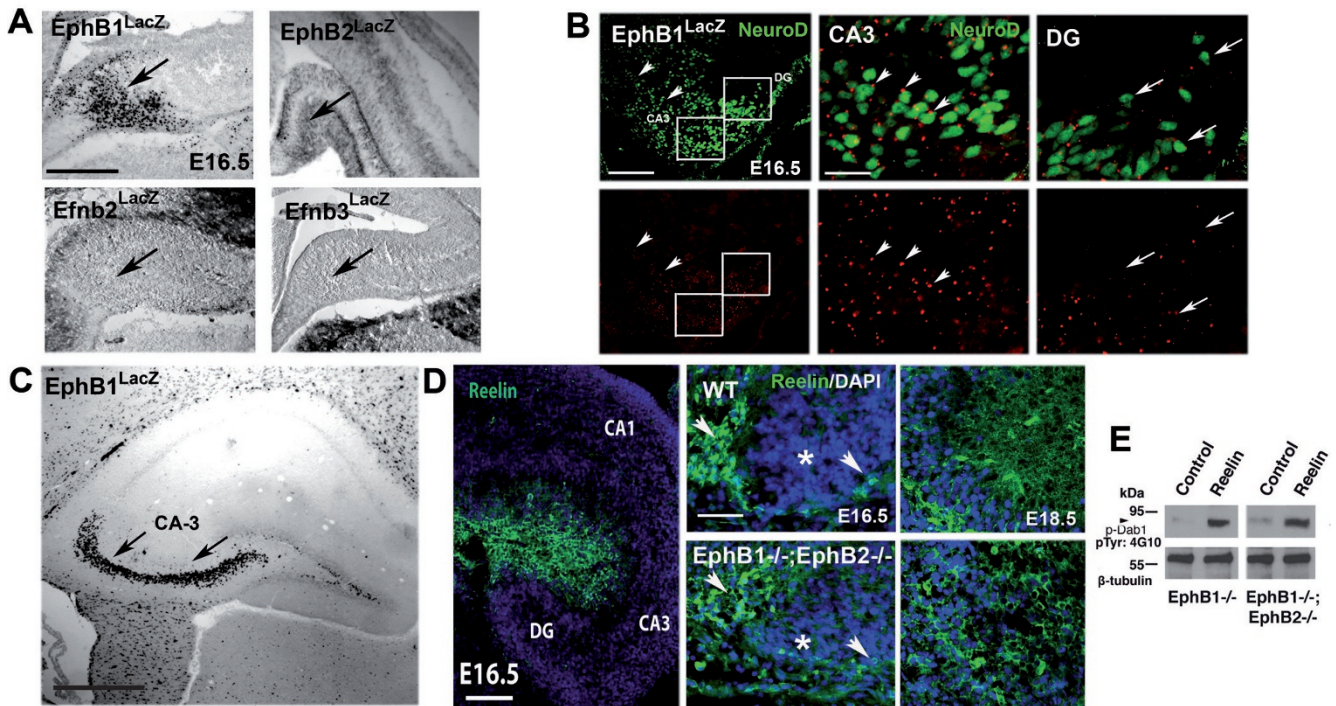


Figure 8 Reelin and EphB/ephrin B expression in the hippocampus. **(A)** Expression of EphB1, EphB2 and their ligands ephrin B2 (Efnb2) and ephrin B3 (Efnb3) in the developing hippocampus. Using animals in which most of the intracellular domains of the EphB and ephrin B transmembrane proteins is substituted by the *lacZ* gene, we determined their expression patterns at E16.5 by β -galactosidase staining. EphB1 protein is almost exclusively expressed by migratory CA3 pyramidal cell precursors. EphB2-expressing cells were also located throughout the CA3 field (arrow). Both Efnb2 and Efnb3 are not expressed in this region prenatally. **(B)** Double immunofluorescence of β -galactosidase and NeuroD, a specific marker for CA3 and DG neuronal precursors, in the developing EphB1^{lacZ} brain demonstrated the neural expression of EphB1 in CA3 precursor cells. Arrowheads in the left panel showed β -galactosidase-positive cells throughout the CA3 subfield. Middle, higher magnification of the CA3 region (boxed area) showed robust NeuroD and β -galactosidase co-localization, confirming the expression of EphB1 (arrowheads) by CA3 migrating neurons. In contrast, NeuroD-positive cells in the dentate gyrus area (right, higher magnification of boxed area) showed reduced EphB1 expression (arrows). **(C)** β -galactosidase staining in adult EphB1^{lacZ} hippocampus further confirmed that EphB1 is expressed almost exclusively in the CA3 region (arrows). **(D)** Reelin (green) is expressed medially to the developing CA3 region prenatally (left). The levels of Reelin expression (green, arrowheads) in both WT (top) and Ephb1^{-/-};Ephb2^{-/-} mice (bottom) are comparable at both E16.5 (left) and E18.5 (right). Counterstain with DAPI (blue). **(E)** Normal Dab1 phosphorylation in primary neurons from mice lacking EphB1 (left) or both EphB1 and EphB2 (right) after treatment with Reelin for 15 min. β -tubulin served as a loading control. Scale bars, 100 μ m **(A)**, 100 μ m **(B, left)**, 20 μ m **(B, middle and right)**, 500 μ m **(C)** and 200 μ m **(D, left)** and 50 μ m **(D, middle and right)**.

region at E16.5 (Figure 8D, left and middle) and in the lateral CA3 area at E18.5 (Figure 8D, right) in both wild-type and compound EphB1/2-deficient mice, excluding the possibility that the described CA3 heterotopia in EphB1/2-deficient mice is merely caused by a reduction in Reelin expression or signaling. We also showed that the Reelin-dependent phosphorylation of Dab1 via lipoprotein receptors is not affected in neurons lacking both EphB1 and EphB2 (Figure 8E). Together, these data suggested that EphB receptors and Reelin might interact during neurodevelopment to contribute to the formation of the CA3 hippocampal subfield, and that EphB forward

signaling is required for proper migration of the neuronal precursors in this hippocampus subfield.

Interaction of ephrin B ligands with EphB receptors does not activate Dab1 signaling

Another possible mechanism that might influence the observed phenotype could be a reciprocal crosstalk of Reelin and EphB receptor signaling, i.e., tyrosine phosphorylation of Dab1 by activation of EphB receptors through their cognate ephrin B ligands. The cytoplasmic adapter protein Disabled-1 (Dab1) is essential for the transmission of Reelin lipoprotein receptor-dependent

signals and has been reported to interact with other transmembrane receptors like APP, LRP1 or Notch as well (reviewed in [5]). Therefore, we examined whether Dab1 interacts with EphB receptors. Using a pull-down assay with a recombinant GST-fusion protein containing the protein interaction (PI/PTB) domain of Dab1, which mediates the interaction with the NPXY tetra-amino acid motif in the cytoplasmic tails of different transmembrane receptors, we could coprecipitate EphB2 from a neuronal lysate (Supplementary information, Figure S2A). In the reverse experiment, Dab1 coprecipitated with the intracellular domain of the EphB2 receptor tyrosine kinase fused to GST (Supplementary information, Figure S2B). The cytoplasmic tail of EphB2 does not contain a NPXY motif. However, we found a DPXY sequence (amino acids 777-780 of murine EphB2) that is highly conserved among different EphB family members and various species. Inactivation of this motif by mutating it to AAAA weakened the binding of neuronal Dab1 (Supplementary information, Figure S2B), indicating that this motif is involved in the interaction of EphB2 with Dab1.

Next, we analyzed whether the activation of EphB receptors might induce tyrosine phosphorylation of Dab1, which is a key step in the activation of the Reelin-Dab1 signaling cascade [40]. We first coexpressed Dab1 with EphB2 receptor tyrosine kinase in transiently transfected HEK-293 cells, which led to a robust increase in Dab1 tyrosine phosphorylation (Supplementary information, Figure S3A). However, treatment with preclustered ephrin B1 in primary cortical neurons led to a less than 2-fold increase in Dab1 tyrosine phosphorylation after 1-8 h (Supplementary information, Figure S3B and S3C), and we did not observe increased degradation of Dab1 protein after a prolonged treatment with ephrin B1, which is the result of ubiquitination and proteasomal degradation of phosphorylated Dab1 in Reelin-treated neurons [41, 42] (Supplementary information, Figure S3B). These results indicated that the induction of EphB2 forward signaling cannot sufficiently substitute for the activation of the lipoprotein receptor-mediated Reelin signaling cascade. Moreover, costimulation with preclustered ephrin B1 neither augmented nor inhibited the effect of Reelin on Dab1 phosphorylation (Supplementary information, Figure S3D and S3E).

To explore the possibility that the previously described interaction of Reelin with ephrin B ligands [9, 43] might influence the Reelin-mediated EphB forward signaling, we re-examined the effect of a treatment of cortical neurons with preclustered Fc-EphB3 ectodomain, which has been reported to induce Dab1 phosphorylation [9]. Using different treatment periods, we did not observe a significant increase in Dab1 tyrosine phosphorylation in lysates

of cortical neurons by immunoblotting (Supplementary information, Figure S4A and S4B) or after Dab1 immunoprecipitation (Supplementary information, Figure S4C). In addition, we did not see a decrease in total Dab1 levels after prolonged treatment with preclustered EphB3 (Supplementary information, Figure S4D and S4E), which would be expected if Fc-EphB3 could mimic Reelin's actions on Dab1 phosphorylation. These results suggest a more complex interplay between Reelin and ephrin B signaling than previously discussed, which also has to take into account the direct interaction between Reelin and EphB receptors reported here (Figure 9).

Discussion

Our study identifies EphB proteins as receptors for the neuronal signaling molecule Reelin. We found that Reelin directly binds to the extracellular domain of EphB receptor tyrosine kinases, which induces EphB receptor autophosphorylation and forward signaling leading to cytoskeletal changes in responsive cells. Our analysis of mice lacking both EphB1 and EphB2 suggests that Reelin-induced forward signaling in concert with cognate ephrin B ligands participates in the formation of the CA3 region during hippocampal development. Together, our data provide biochemical and functional evidence for a novel Reelin-EphB receptor crosstalk, which has potentially important implications for the role of both signaling systems in the adult and aging brain as well as in peripheral organs, and during tumorigenesis.

Reelin is a pleiotropic molecule with different context-dependent roles. For most of its functions, signal transduction via the lipoprotein receptor Dab1 cascade is required. However, some phenotypical characteristics in the reeler mouse have been reported to be independent of the 'canonical' Reelin receptors ApoER2 or VLDLR, or do not require Dab1 [44, 45], indicating that alternative Reelin receptors must exist. Indeed, binding of Reelin to other transmembrane receptors has been reported, including the amyloid precursor protein, β -class integrins, the LDL receptor and ephrin B proteins [7, 9, 43, 46, 47]. The sharing of common signaling pathways targeting the cytoskeleton, the requirement of ligand-mediated receptor oligomerization for the initiation of Reelin or Eph signaling [23, 48], and the ligand-induced proteolytic processing of ApoER2 and EphB2 by the γ -secretase complex [21, 22] prompted us to investigate whether Reelin and EphB receptors directly interact. We found that Reelin, like preclustered soluble ephrin B ligand, induces the oligomerization of EphB receptors, a prerequisite for EphB receptor activation and forward signaling. In principle, this oligomerization might be indirect, medi-

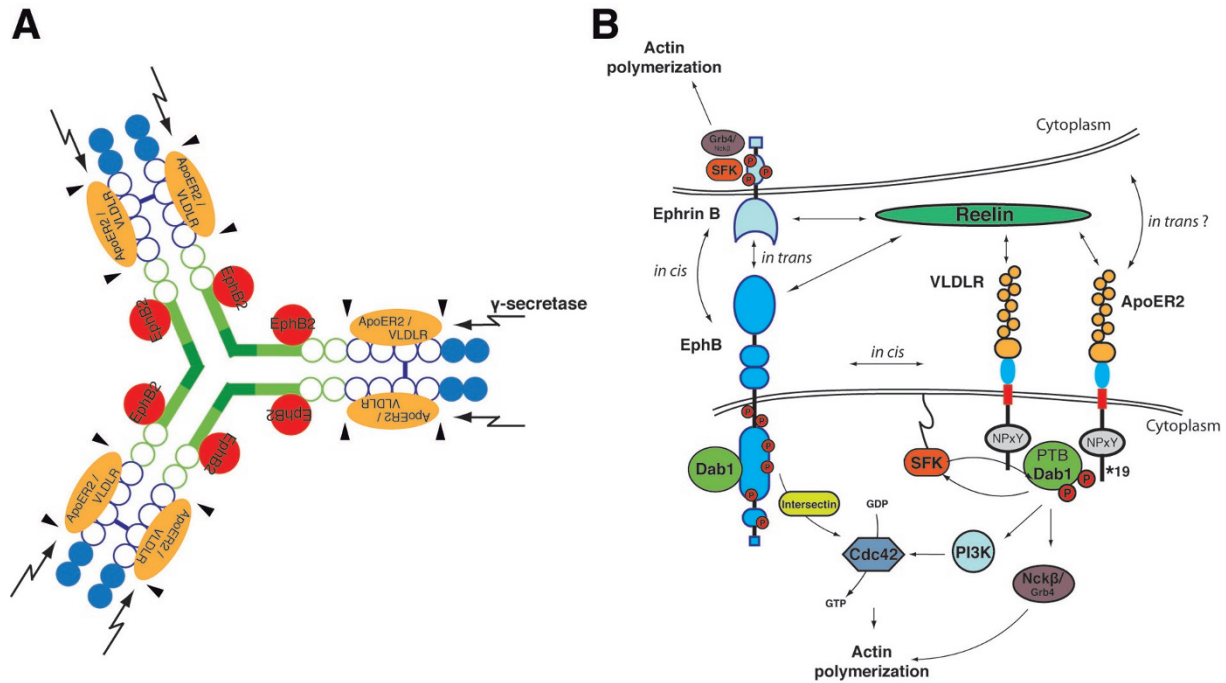


Figure 9 Model of Reelin-EphB signaling crosstalk. **(A)** Oligomerized Reelin binds to the lipoprotein receptors ApoER2 or VLDLR (orange) with its central fragment (Reelin repeats 3-6), whereas the amino-terminal domain (green) interacts with EphB2 (red). The composition and turnover of the Reelin-receptor supramolecular complex and thereby its signaling output are dynamically regulated, depending on the developmental stage, tissue expression and activity of additional molecules interacting with Reelin and its receptors, and the proteolytic cleavage of Reelin and its receptors by metalloproteases (arrowheads) and the intramembrane γ -secretase complex (arrows). **(B)** Model of how Reelin-induced ApoE receptor and Eph/ephrin-dependent signaling cascades may interact to regulate cellular behavior in responsive neurons. Reelin might activate different receptor systems individually or recruit them into larger macromolecular complexes, either *in cis* or *in trans*, which would differentially modulate the signaling output of its components. Abbreviations: SFK, Src family kinase; PTB: phosphotyrosine binding domain; NPXY, Asn-Pro-X-Tyr tetra-amino-acid motif; PI3K, phosphatidylinositol-3-kinase; *19, alternatively spliced exon 19 of ApoER2.

ated by an interaction of EphB receptors with ApoER2 or VLDLR. Alternatively, the interaction of both receptors might depend on a shared interacting partner, e.g., a third transmembrane protein like the NMDA receptor [12, 49], or a cytoplasmic adapter protein bridging both receptor systems. However, we observed Reelin-mediated EphB2 clustering in ApoER2/VLDLR-deficient neurons. Moreover, using a coprecipitation assay where the secreted extracellular domain of EphB2 fused to the Fc region of human IgG bound recombinant Reelin, we could demonstrate a direct interaction between Reelin and EphB receptors. Interaction of Reelin with Fc-EphB1 and Fc-EphB3 but not Fc-ephrin A5 was also detected. The interaction site was mapped to the amino-terminal domain of Reelin, whereas lipoprotein receptors interact with the central fragment comprising the Reelin repeats R3-R6 [28]. Thus, Reelin would be able to simultaneously recruit ApoE receptors and EphB proteins into a macromolecular complex, which might involve components *in cis*

or *in trans*, respectively (Figure 9). As the EphA subclass of Eph transmembrane proteins share a high degree of sequence homology with EphB proteins in their extracellular region, we also tested whether Reelin can bind to the EphA4 extracellular domain, which was the case (Supplementary information, Figure S5). This suggests that Reelin may function as an extracellular modulator of transmembrane receptor signaling, which orchestrates diverse cellular signaling responses depending on different receptor binding affinities, tissue expression patterns and availability of complementary ligands in a highly complex manner (Figure 9).

The recruitment of EphB transmembrane proteins to distinct spots in the cell membrane by exogenous soluble ligands indicates the initiation of EphB forward signaling, which depends on receptor autophosphorylation [50]. Accordingly, we observed that Reelin was able to induce EphB2 tyrosine phosphorylation in cultured neurons, albeit to a lower extent than preclustered Fc-ephrin B1,

its ‘canonical’ ligand. EphB clustering precedes receptor internalization by endocytosis, which is a prerequisite for its ligand-induced intramembrane proteolytic cleavage by γ -secretase [21]. Indeed, we also observed Reelin-induced EphB2 degradation, again to a somewhat lower degree than that by soluble preclustered ephrin B1. This was impaired by the endosomal/lysosomal inhibitor bafilomycin A1, providing further evidence that Reelin can activate EphB forward signaling in responsive cells. The Reelin-induced degradation is dependent on the γ -secretase system, as treatment with the γ -secretase inhibitor DAPT led to an accumulation of a truncated EphB2 fragment (CTF1), whose generation was blocked by an inhibitor of β -secretase (data not shown). Recently, depletion of EphB2 by soluble amyloid- β oligomers has been suggested as a mechanism contributing to the cognitive decline in Alzheimer patients [16]. Direct binding of amyloid- β to EphB2 induced proteasomal degradation of EphB2, which impairs EphB2-dependent tyrosine phosphorylation and surface recruitment of NMDA receptors, ultimately leading to an impairment of NMDA receptor-dependent synaptic plasticity [16]. The deleterious effects of toxic concentrations of oligomeric amyloid- β on synaptic strength are partly counteracted by Reelin-dependent activation of Src family kinases, which is mediated by lipoprotein receptors and modulated in an apolipoprotein E isoform-dependent manner [15, 51]. It is tempting to speculate that the activation of EphB forward signaling by Reelin shown here modulates the interplay between amyloid- β , Reelin and EphB signaling in the regulation of NMDA receptor-dependent functions at the synapse. As Reelin binds ApoE receptors with its central portion and EphB proteins with its amino-terminal domain, a large multifunctional signaling complex can be assembled at the synapse, whose turnover and therefore its signaling output is determined by the proteolytic processing of Reelin itself and of its receptors, including Eph proteins and ApoE receptors [21, 31, 52, 53] (Figure 9A). An additional level of complexity arises from the fact that the transmembrane components (including the amyloid- β precursor APP) are connected via the adaptor protein Dab1 (Figure 9B). It will be important to determine how these interactions, which may also involve Reelin-ephrin B signaling [9], contribute to synaptic transmission, neural network activity and cognitive functions.

During neurodevelopment, most of Reelin’s functions are mediated by the ApoE receptor-Dab1 signaling cascade. In mice lacking both EphB1 and EphB2, we found a localized cell migration defect in the developing CA3 subfield of the hippocampus, which is involved in the rapid encoding of contextual memory [54, 55]. This

phenotype depends on EphB forward signaling activity, as mice expressing EphB2 transmembrane proteins with intracellular truncations or carrying a point mutation that selectively inactivates the receptor tyrosine kinase domain on an EphB1-deficient background bear similar CA3 abnormalities. Although dispersion of the pyramidal cell layer in the lateral region of CA3 was also seen in mice lacking ephrin B1, which is the only ephrin B protein expressed in this region during its ontogenesis, the displacement in the medial CA3 was larger in the EphB compound mutant mice, suggesting involvement of an additional EphB ligand. Of note, the medial CA3 is in part populated by late-generated neurons that use a radial instead of a tangential migration mode [56]. As Reelin is expressed in the developing hilar region adjacent to the CA3 area when EphB1/EphB2-expressing neural precursors are actively migrating to the same hippocampal location (Figure 8), it seems plausible that the lack of Reelin-mediated EphB forward signaling results in the specific CA3 migration abnormalities observed in EphB1/2-deficient mice. Heterotopia of CA3 neurons in Dab1- and ApoER2/VLDLR-deficient mutants is completely consistent with defects in canonical Reelin signaling, which is dominant over other signaling pathways regulating CA3 formation. This opens up the possibility that the observed CA3 dispersion in the EphB1/2 knockout mice might be the result of a reduced production of Reelin in these mice. Although we cannot completely exclude this possibility, our evaluation showed no significant reduction of Reelin levels adjacent to the CA3 region (Figure 8). Moreover, *reeler* heterozygous mice show no CA3 dispersion. Altogether, our data are consistent with and support the notion that Reelin-EphB2 interaction is directly involved in CA3 pyramidal cell migration. Further studies including *in vivo* mutagenesis of the involved genes will be required to fully dissect the extent to which the canonical Reelin and EphB pathways are integrated with Reelin/EphB signaling in orchestrating the migration of pyramidal precursor cells in the developing hippocampus.

The canonical EphB ligand ephrin B3 has recently been described to act as a Reelin receptor [9], a finding that we have confirmed (Figure 2B). It was also reported that stimulation of ephrin B reverse signaling by EphB3 could substitute for Reelin’s actions on Dab1 tyrosine phosphorylation and Src kinase activation. However, preclustered EphB3 ectodomain did not induce Dab1 phosphorylation in our hands. In addition, the commercial p-(Y232)Dab1 antibody used in the previous study [9] did not detect the Reelin-induced tyrosine phosphorylation of Dab1 in primary neurons in our hands (Supplementary information, Figure S4F and S4G). Alto-

gether, our data do not support a crucial role for ephrin B reverse signaling in mediating Reelin's effects on Dab1 activation. We propose an alternative model where the soluble ligand Reelin can simultaneously or exclusively bind to ApoE receptors, Eph receptor tyrosine kinases and ephrin B proteins, thereby acting like a 'distribution board' that coordinates the output of different signaling cascades in a context-dependent manner (Figure 9B). Proteolytic cleavage of Reelin or its receptors allows further fine-tuning of this signaling network in response to external and internal cues. It is possible that this Reelin-dependent receptor crosstalk also influences signaling from heterooligomeric EphA/EphB clusters [57], which could be relevant for the proposed function of Reelin as a tumor suppressor [58].

Materials and Methods

Animals

EphB1 [59] and EphB2 [38] knockout mice as well as EphB1-lacZ [60] and EphB2-lacZ [38] mice encoding fusion proteins of the respective EphB proteins linked to β -galactosidase replacing the tyrosine kinase and carboxy-terminal domains have been previously described, as have been ephrin B2-lacZ [61] and ephrin B3-lacZ mice [62]. The generation of mice carrying point mutations in the EphB2 intracellular domain disrupting its kinase activity (K661R), its PDZ domain (Δ VEV994) or both (Δ VEVK) has been described elsewhere [39]. Mutant mice were maintained in a heterozygous state on a Sv129 \times C57BL/6 background and genotypes of all mice were confirmed by PCR analysis of genomic DNA as described. The animals were housed in the Animal Resource Centers of the University of Freiburg, Germany and of the University of Texas Southwestern Medical Center at Dallas, USA, and were maintained in accordance with the institutional guidelines of the respective Institutional Animal Care and Use Committees.

Preparation and treatment of primary cortical neurons

Cortical neurons were prepared as described [30] from E15.5 NMRI wild-type mice, with E0.5 defined as the morning of plug detection. Briefly, cortical lobes were trypsinized in Hanks' Balanced Salt Solution (HBSS) (Invitrogen) supplemented with 0.05% trypsin-EDTA (Invitrogen) at 37 °C for 15 min. After adjusting the suspension to 5% FCS, the tissue was collected by a brief centrifugation at 500 \times g and triturated in HBSS supplemented with 0.025% DNase I, 0.4 mg/ml trypsin inhibitor, 3 mg/ml BSA and 12 mM MgSO₄. The dissociated cells were resuspended in DMEM (4.5 g/l glucose) plus 8% FCS and plated on glass coverslips coated with 0.1 mg/ml poly-L-lysine (Biochrom AG) or on 60 or 100 mm plastic cell culture dishes coated with 0.05 mg/ml poly-D-lysine (Sigma). After 4 h, the medium was changed and replaced by serum-free Neurobasal medium supplemented with 2% B27 and 1 mM GlutaMax (Invitrogen) after 24 h. After 5 days in culture (DIV5) at 37 °C in a 5% CO₂ atmosphere, the neurons were stimulated and further analyzed by immunocytochemistry, immunoblotting or immunoprecipitation. Stimulation with Reelin or control-conditioned supernatant was performed as described [30]. Preclustered chimeric recombinant Eph or ephrin ectodomains fused to Fc (R&D

Systems) were used at a final concentration of 4 μ g/ml (Fc-ephrin A5: 20 μ g/ml) for the treatment of cultured neurons.

Plasmids, cell lines and transfection

The cloning of plasmids encoding Fc-tagged ectodomains of murine ApoER2 and human VLDLR [27] and of the amino-terminal, central and carboxy-terminal fragments of murine Reelin [28] have been described. A cDNA encoding full-length murine PDGFR β was obtained from Philippe Soriano and cloned into the pcDNA3.1 vector. To construct an expression vector of the PDGFR β ectodomain fused to the constant human IgG domain, the sequence encoding the first 530 amino-terminal amino acids was cut out of the full-length cDNA and cloned into the *Hind*III and *Xba*I restriction sites of a modified pcDNA3.1-V5-His vector, where the 6His tag had been replaced by a sequence encoding Fc as described [27]. The expression plasmids encoding full-length EphB2 [63], Disabled-1 and Src [30] and the GST-fusion plasmids of the protein interaction domain of Dab1 and of the intracellular tail of murine Apoer2 [64] have been described before. A GST fusion plasmid of the murine Ephb2 intracellular domain was prepared by amplifying the tail sequence by PCR from the full-length expression plasmid with the following primer pair: 5'-ATTC-TAGACTTTGAGCGTGCCGACTCAGAGTACACGGAC-3' (including an artificially introduced *Xba*I restriction site) and 5'-GGCCCTCTAGATGCATGCTCGAGCGGCCGC-3' (including an artificially introduced *Xho*I restriction site). After subcloning into the pCR2.1-TOPO vector (Invitrogen) the EphB2 fragment was verified by sequencing, excised by *Xba*I-*Xho*I digestion and cloned into the pGEX-KG plasmid.

HEK-293 cells and NG-108 cells were maintained in DMEM (4.5 g/l glucose) with GlutaMax and 8% FCS, transfected with polyethylenimine [65] or FuGene (Roche) according to standard protocols and harvested for immunoblotting or analyzed by immunocytochemistry after 36 h. Stably transfected NG108 cells expressing EphB2 [24] were maintained in DMEM containing 10% FBS and 1 \times hypoxanthine-aminopterin-thymidine (HAT) (Invitrogen).

Cell detachment assay

Cos-1 cells were grown in DMEM (4.5 g/l glucose) with GlutaMax and 8% FCS as described in [33, 66]. After stimulation with Reelin or preclustered Fc-Ephrin for 150 min, the cells were fixed with 4% paraformaldehyde in PBS for 15 min and stained with phalloidin-tetramethylrhodamine B isothiocyanate (Sigma-Aldrich) and DAPI for 1 h. To determine the number of detaching cells, characterized by rounding and intensely fluorescent somata, in relation to the total number of cells, 10 microscopic fields of view (\times 10 objective) per condition were analyzed in a blinded manner ($n \geq 3$ independent experiments). Statistical significance of differences between mean values of treatment conditions was evaluated by one-way ANOVA followed by Dunnett's *post-hoc* test.

EphB2 knockdown

Cos-1 cells were changed to serum-free medium and transfected with fluorescein-conjugated control siRNA or siRNA directed against human EphB2 (Santa Cruz) according to the manufacturer's instructions (final siRNA concentration 60 nM in a volume of 1 ml) using siRNA Transfection Reagent (Santa Cruz). After 8 h, DMEM (4.5 g/L glucose) containing FCS was added, and

changed to 1% FCS-containing medium after 18 h. According to the fluorescein fluorescence of the control siRNA-transfected cells, a transfection efficiency of > 90% was obtained. After 2 days, the cells were treated and stimulated as required for the cell detachment assay, fixed and analyzed as described above.

Reagents and recombinant proteins

WHI-P180 (Calbiochem), a Cdk2 inhibitor that also inhibits EphB2 activation in a cell-based autophosphorylation assay [35], was dissolved in DMSO and used at a final concentration of 10 μ M. The other inhibitors were used at the indicated concentrations: bafilomycin A1 (Axxora, 1 μ M in DMSO), lactacystin (Axxora, 10 μ M in DMSO), DAPT (N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester, Merck, 5 μ M in DMSO). The SNEW peptide [34] (SNEWIQPRLPQH, purity > 95%) was synthesized using Fmoc solid phase chemistry (Genecust, Dudelange, Luxembourg). A stock solution in PBS was prepared and the peptide was used at the indicated concentration. Fc-tagged unclustered recombinant murine ectodomains of ephrins and Eph receptors (20 μ g, R&D Systems) were preclustered with 2.5 μ g goat anti-human IgG (Jackson ImmunoResearch) in 0.2 ml at 4 °C overnight. For the preparation of full-length Reelin or control-conditioned supernatant, HEK-293 cells were transfected with the pCRL full-length Reelin expression plasmid [67] or pcDNA control vector and stable transfectants were selected with Geneticin (G418, AppliChem) at a concentration of 400 μ g/ml followed by subcloning of selected colonies. Stably transfected cells were grown to subconfluency, and incubated with DMEM (1.0 g/l glucose) containing 0.1% bovine serum albumin (IgG-free, low endotoxin, Sigma) for 36 h. The conditioned media were centrifuged and stored at -80 °C after partial purification by 10-fold concentration with 100 kDa ultrafiltration discs (Millipore). To prepare myc-tagged partial Reelin fragments (N-R2, R3-5, R3-6, and R7-8), HEK-293 cells were grown to subconfluency and transiently transfected with the respective expression vectors. One day after transfection the medium was replaced by serum-free DMEM (1.0 g/L glucose). After 36 h, conditioned medium was collected and the supernatants were analyzed by western blotting with anti-myc antibody (Cell Signaling, 1:1 000). Comparable amounts of different Reelin fragments were used for the receptor binding experiments. Receptor-associated protein fused to glutathione-transferase (GST-RAP) [26], other GST-fusion proteins and GST control protein were produced as described previously [64, 26].

In vitro binding assays

In vitro receptor binding assay Supernatants containing equal amounts of Fc-tagged receptor ectodomains (2 μ g of Fc-EphB, Fc-ephrin and Fc-PDGFR β ectodomains, ~0.5 μ g of Fc-ApoER2 and Fc-VLDLR) were mixed with protein A/G PLUS agarose (Santa Cruz) and recombinant Reelin and incubated for 4 h at 4 °C. The beads were collected by a brief centrifugation, washed (1 \times buffer-1, 2 \times buffer-2: both 30 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂ with 1% (buffer-1) and 0.1% Triton X-100 (buffer-2)), resuspended in loading buffer and analyzed by immunoblotting, which was performed as described [30]. For immunoprecipitation, cells were lysed in ice-cold buffer-1 including protease and phosphatase inhibitor mixtures (Sigma-Aldrich), and 1 mg protein was incubated with the respective primary antibody (5 μ l rabbit serum or 2 μ g mouse IgG) in a total volume of 1 ml

followed by protein A/G agarose precipitation. After washing with buffer-1 (1 \times) and buffer-2 (2 \times), the beads were resuspended in loading buffer, boiled under reducing conditions and subjected to western blot analysis.

GST pull-down assay GST pull-down assays were performed as described [68]. Cells were lysed in ice-cold buffer-1. After a preclearing step with GST protein coupled to glutathione-agarose, the lysates were incubated with 10 μ g GST-fusion protein for 1 h and glutathione-agarose beads for 4 h at 4 °C. After washing with lysis buffer (1 \times) followed by wash buffer (2 \times ; 10 mM Tris pH 7.5, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂), the beads were resuspended in loading buffer and subjected to western blot analysis.

Immunoblotting

Neurons were harvested in cell lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate and 1% Nonidet P-40 with phosphatase and protease inhibitor mixtures). For immunoblotting, cell or tissue lysates were cleared by centrifugation (20 min at 20 000 \times g, 4 °C), adjusted for protein content using a detergent-compatible colorimetric protein assay (Bio-Rad), and mixed with 4 \times concentrated loading buffer (125 mM Tris pH 6.8, 50% glycerol, 4% SDS and 5% β -mercaptoethanol). Heat-denatured samples were separated by SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare) and incubated with the respective primary and secondary HRP-coupled antibodies (GE Healthcare, 1:10 000). The primary antibodies used were: rabbit anti-Dab1 antibody raised against the 13 carboxyl-terminal amino acids of murine Dab1 [64] (1:2 000), rabbit anti-Dab1 (1:1 000, Millipore) raised against residues 400-555 of mouse Dab1, mouse anti-VLDLR (6A6, 1:200, Santa Cruz), rabbit monoclonal anti-ApoER2 (1:1 000, Epitomics), mouse anti-Reelin (G10) (1:2 000, Millipore), goat anti-EphB2 (1:1 000, R&D Systems), rabbit anti-ephrin-B1/2/3 (C-18, 1:500, Santa Cruz), rabbit anti-actin (1:20 000, Sigma), mouse anti-myc (1:1 000, Cell Signaling), mouse anti-p-tyrosine (4G10) (1:3 000, Millipore), mouse anti-GST (1:1 000, Merck), mouse anti-EphB2 (1:500, Invitrogen) and goat anti-human IgG (1:5 000, Santa Cruz). Bound IgG was detected using enhanced chemiluminescence and Fuji Super RX films. Densitometric quantification of western blots was done with ImageJ. Differences between different groups were tested for statistical significance with one-way ANOVA followed by Dunnett's *post-hoc* test.

Quantitative real-time PCR

Total RNA was prepared from E15.5, DIV5 Reelin- or control-treated mouse primary neuronal cultures ($n = 3$) with the RNeasy kit (Qiagen, Germany) as described [68]. Contaminating DNA was removed by DNase I treatment (Fermentas). 2 μ g of total RNA was reverse transcribed using 500 ng/ μ l oligo(dT) primer (Promega), 0.5 mM of each dNTP (Genaxxon), 20 U of ribonuclease inhibitor (Promega) and 200 U of M-MLV reverse transcriptase (Promega) in a total volume of 30 μ l. For quantitative real-time PCR analyses, 35 μ l of the amplification mixture (QuantiTect SYBR Green kit, Qiagen) containing 20 ng of reverse-transcribed RNA and 300 nmol/l forward and reverse primers was used. Reactions were incubated in triplicate on an MX3000P detector (Agilent Technologies, Germany). The cycling conditions were: 15 s at 95 °C followed by 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. Expression values were normalized

to Rps29 (40S ribosomal protein S29-like). Primers used were murine EphB2 forward 5'-GTGTGGAGCTATGGCATCGT-3', reverse 5'-TGGGCGGAGGTAGTCTGTAG-3' [16], Rps29 forward 5'-ATGGGTCACCAGCAGCTCTA-3' and reverse 5'-AGCCTATGTCCTTCGCGTACT-3'. The $\Delta\Delta C_t$ method was used to compare increased or decreased expression.

Histochemistry, cytochemistry and imaging

Animals were perfused transcardially under anesthesia with 0.9% normal saline followed by 4% w/v paraformaldehyde in 0.1 M phosphate buffer, and postfixed by immersion at 4 °C overnight. Coronal brain sections were cut on a cryostat (30 μ m) or on a vibratome (50 μ m) and were Nissl- or H&E-stained. For the β -galactosidase staining, the sections were incubated in a solution containing 2 mM MgCl₂, 40 mg/ml X-gal and 5 mM potassium ferri/ferrocyanide in PBS at 37 °C overnight [69]. Cell dispersion in the adult hippocampus was determined by measuring the linear distribution of CA1 and CA3 pyramidal neurons. For immunohistochemistry, antibodies against β -galactosidase (rabbit 1:500; Millipore), NeuroD (goat 1:50; SantaCruz) and Reelin (mouse 1:100; G10 Millipore) were used. After overnight fixation, brains were embedded in 4% agarose (vibratome) or OCT (cryotome) in PBS. Afterwards, sections were blocked (5% donkey normal serum and 0.3% Triton X-100 in PBS) for 2 h at room temperature (RT). The primary antibodies were incubated at 4 °C overnight in blocking solution followed by 3 washing steps in PBS for 10 min. Donkey secondary antibodies (1:1 000 AF488; 1:400 Cy2 and Cy3, Jackson ImmunoResearch) were incubated at RT for 2 h followed by 3 washing steps in PBS for 10 min. Some sections were counterstained using DAPI, and mounted on microscopic slides with ProLong-antifading solution (Invitrogen) [70].

NG108-EphB2 cells were fixed by pre-cooled 100% methanol at -20 °C for 10 min. Immunocytochemistry (ICC) of 4% PFA-fixed neurons and Cos-1 cells was performed essentially as described [71] using the indicated primary antibodies: EphB2 (1:250, R&D Systems), Reelin (G10, 1:1 000, Millipore), MAP2 (1:500, Leinco) and phalloidin-tetramethylrhodamine B isothiocyanate to label F-actin, and the appropriate Alexa-488 or -555-coupled secondary antibodies (Invitrogen). Images were acquired with a Zeiss Axioplan-2 Imaging microscope using 4 \times , 5 \times , 10 \times , 40 \times and 63 \times objectives.

Acknowledgments

We thank Jonathan Göldner and Aileen Koch (University of Freiburg, Germany), Wen-Ling Niu and Rebekah Hewitt (UT Southwestern Medical Center at Dallas, USA) for technical assistance, André Goffinet (Catholic University of Leuven, Belgium) for providing plasmids encoding partial Reelin fragments, Brian Howell (SUNY Upstate Medical University, USA) for helpful discussions, Philippe Soriano (Mt. Sinai School of Medicine, USA) for the PDGFR β cDNA, Hubert E Blum (University Hospital, Freiburg, Germany) for continuous support, Carsten Schwan and Kai Zurhove (University of Freiburg, Germany) and Pingsheng Liu (Institute of Biophysics, Chinese Academy of Sciences, China) for discussions. This study was supported in part by the Landesgraduiertenkolleg Baden-Württemberg (to EB) and the Deutsche Forschungsgemeinschaft (DFG; BO1806/3-1, SFB780/B5, MA2410/1-3). JH is supported by grants from the NIH (HL20948

and HL63762), the American Health Assistance Foundation, the Consortium for Frontotemporal Dementia Research and SFB780. MH receives support by the NIH (R01-MH066332). MF is Senior Research Professor for Neuroscience of the Hertie Foundation.

References

- 1 Ayala R, Shu T, Tsai L-H. Trekking across the brain: the journey of neuronal migration. *Cell* 2007; **128**:29-43.
- 2 Rice DS, Curran T. Role of the reelin signaling pathway in central nervous system development. *Annu Rev Neurosci* 2001; **24**:1005-1039.
- 3 Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. *Nat Rev Neurosci* 2006; **7**:850-859.
- 4 Tissir F, Goffinet AM. Reelin and brain development. *Nat Rev Neurosci* 2003; **4**:496-505.
- 5 Stolt PC, Bock HH. Modulation of lipoprotein receptor functions by intracellular adaptor proteins. *Cell Signal* 2006; **18**:1560-1571.
- 6 Leemhuis J, Bock HH. Reelin modulates cytoskeletal organization by regulating Rho GTPases. *Commun Integr Biol* 2011; **4**:254-257.
- 7 Hoe HS, Lee KJ, Carney RS, et al. Interaction of reelin with amyloid precursor protein promotes neurite outgrowth. *J Neurosci* 2009; **29**:7459-7473.
- 8 Dulabon L, Olson EC, Taglienti MG, et al. Reelin binds alpha3beta1 integrin and inhibits neuronal migration. *Neuron* 2000; **27**:33-44.
- 9 Senturk A, Pfennig S, Weiss A, Burk K, Acker-Palmer A. Ephrin Bs are essential components of the Reelin pathway to regulate neuronal migration. *Nature* 2011; **472**:356-360.
- 10 Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell* 2008; **133**:38-52.
- 11 Klein R. Eph/ephrin signaling in morphogenesis, neural development and plasticity. *Curr Opin Cell Biol* 2004; **16**:580-589.
- 12 Dalva MB, Takasu MA, Lin MZ, et al. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell* 2000; **103**:945-956.
- 13 Takasu MA, Dalva MB, Zigmond RE, Greenberg ME. Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. *Science* 2002; **295**:491-495.
- 14 Chen Y, Beffert U, Ertunc M, et al. Reelin modulates NMDA receptor activity in cortical neurons. *J Neurosci* 2005; **25**:8209-8216.
- 15 Durakoglulugil MS, Chen Y, White CL, Kavalali ET, Herz J. Reelin signaling antagonizes beta-amyloid at the synapse. *Proc Natl Acad Sci USA* 2009; **106**:15938-15943.
- 16 Cissé M, Halabisky B, Harris J, et al. Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. *Nature* 2011; **469**:47-52.
- 17 Leemhuis J, Bouche E, Frotscher M, et al. Reelin signals through apolipoprotein E receptor 2 and Cdc42 to increase growth cone motility and filopodia formation. *J Neurosci* 2010; **30**:14759-14772.
- 18 Irie F, Yamaguchi Y. EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. *Nat Neurosci* 2002; **5**:1117-1118.

- 19 Chai X, Forster E, Zhao S, Bock HH, Frotscher M. Reelin acts as a stop signal for radially migrating neurons by inducing phosphorylation of n-cofilin at the leading edge. *Commun Integr Biol* 2009; **2**:375-377.
- 20 Shi Y, Pontrello CG, DeFea KA, Reichardt LF, Ethell IM. Focal adhesion kinase acts downstream of ephb receptors to maintain mature dendritic spines by regulating cofilin activity. *J Neurosci* 2009; **29**:8129-8142.
- 21 Litterst C, Georgakopoulos A, Shioi J, *et al.* Ligand binding and calcium influx induce distinct ectodomain/gamma-secretase-processing pathways of EphB2 receptor. *J Biol Chem* 2007; **282**:16155-16163.
- 22 May P, Bock HH, Nimpf J, Herz J. Differential glycosylation regulates processing of lipoprotein receptors by gamma-secretase. *J Biol Chem* 2003; **278**:37386-37392.
- 23 Davis S, Gale NW, Aldrich TH, *et al.* Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* 1994; **266**:816-819.
- 24 Holland SJ, Gale NW, Gish GD, *et al.* Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J* 1997; **16**:3877-3888.
- 25 Trommsdorff M, Gotthardt M, Hiesberger T, *et al.* Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* 1999; **97**:689-701.
- 26 Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J Biol Chem* 1991; **266**:21232-21238.
- 27 Hiesberger T, Trommsdorff M, Howell BW, *et al.* Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* 1999; **24**:481-489.
- 28 Jossin Y, Ignatova N, Hiesberger T, Herz J, Lambert de Rouvroit C, Goffinet AM. The central fragment of Reelin, generated by proteolytic processing *in vivo*, is critical to its function during cortical plate development. *J Neurosci* 2004; **24**:514-521.
- 29 Feng L, Cooper JA. Dual functions of Dab1 during brain development. *Mol Cell Biol* 2009; **29**:324-32.
- 30 Bock HH, Herz J. Reelin activates Src family tyrosine kinases in neurons. *Curr Biol* 2003; **13**:18-26.
- 31 Lin KT, Sloniowski S, Ethell DW, Ethell IM. Ephrin-B2-induced cleavage of EphB2 receptor is mediated by matrix metalloproteinases to trigger cell repulsion. *J Biol Chem* 2008; **283**:28969-28979.
- 32 Attwood BK, Bourgognon JM, Patel S, *et al.* Neuropsin cleaves EphB2 in the amygdala to control anxiety. *Nature* 2011; **473**:372-375.
- 33 Himanen JP, Chumley MJ, Lackmann M, *et al.* Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nat Neurosci* 2004; **7**:501-509.
- 34 Koolpe M, Burgess R, Dail M, Pasquale EB. EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. *J Biol Chem* 2005; **280**:17301-17311.
- 35 Slon-Usakiewicz JJ, Ng W, Foster JE, *et al.* Frontal affinity chromatography with MS detection of EphB2 tyrosine kinase receptor. 1. Comparison with conventional ELISA. *J Med Chem* 2004; **47**:5094-5100.
- 36 Chumley MJ, Catchpole T, Silvany RE, Kernie SG, Henkemeyer M. EphB receptors regulate stem/progenitor cell proliferation, migration, and polarity during hippocampal neurogenesis. *J Neurosci* 2007; **27**:13481-13490.
- 37 Catchpole T, Henkemeyer M. EphB2 tyrosine kinase-dependent forward signaling in migration of neuronal progenitors that populate and form a distinct region of the dentate niche. *J Neurosci* 2011; **31**:11472-11483.
- 38 Henkemeyer M, Orioli D, Henderson JT, *et al.* Nuk controls pathfinding of commissural axons in the mammalian central nervous system. *Cell* 1996; **86**:35-46.
- 39 Genander M, Halford MM, Xu NJ, *et al.* Dissociation of EphB2 signaling pathways mediating progenitor cell proliferation and tumor suppression. *Cell* 2009; **139**:679-692.
- 40 Howell BW, Herrick TM, Hildebrand JD, Zhang Y, Cooper JA. Dab1 tyrosine phosphorylation sites relay positional signals during mouse brain development. *Curr Biol* 2000; **10**:877-885.
- 41 Arnaud L, Ballif BA, Cooper JA. Regulation of protein tyrosine kinase signaling by substrate degradation during brain development. *Mol Cell Biol* 2003; **23**:9293-9302.
- 42 Bock HH, Jossin Y, May P, Bergner O, Herz J. Apolipoprotein E receptors are required for Reelin-induced proteasomal degradation of the neuronal adaptor protein disabled-1. *J Biol Chem* 2004; **279**:33471-33479.
- 43 Senturk A, Pfennig S, Weiss A, Burk K, Acker-Palmer A. Ephrin Bs are essential components of the Reelin pathway to regulate neuronal migration. *Nature* 2011; **478**:274.
- 44 Cariboni A, Rakic S, Liapi A, Maggi R, Goffinet A, Parnavelas JG. Reelin provides an inhibitory signal in the migration of gonadotropin-releasing hormone neurons. *Development* 2005; **132**:4709-4718.
- 45 Rossel M, Loulier K, Feuillet C, Alonso S, Carroll P. Reelin signaling is necessary for a specific step in the migration of hindbrain efferent neurons. *Development* 2005; **132**:1175-1185.
- 46 Schmid RS, Jo R, Shelton S, Kreidberg JA, Anton ES. Reelin, integrin and dab1 interactions during embryonic cerebral cortical development. *Cereb Cortex* 2005; **15**:1632-1636.
- 47 D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T. Reelin is a ligand for lipoprotein receptors. *Neuron* 1999; **24**:471-479.
- 48 Strasser V, Fasching D, Hauser C, *et al.* Receptor clustering is involved in Reelin signaling. *Mol Cell Biol* 2004; **24**:1378-1386.
- 49 Beffert U, Weeber EJ, Durudas A, *et al.* Modulation of synaptic plasticity and memory by reelin involves differential splicing of the lipoprotein receptor apoer2. *Neuron* 2005; **47**:567-579.
- 50 Binns KL, Taylor PP, Sicheri F, Pawson T, Holland SJ. Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. *Mol Cell Biol* 2000; **20**:4791-4805.
- 51 Chen Y, Durakoglugil MS, Xian X, Herz J. ApoE4 reduces glutamate receptor function and synaptic plasticity by selectively impairing ApoE receptor recycling. *Proc Natl Acad Sci*

- USA 2010; **107**:12011-12016.
- 52 May P, Bock HH, Herz J. Integration of endocytosis and signal transduction by lipoprotein receptors. *Sci STKE* 2003; **2003**:PE12.
 - 53 Jossin Y, Gui L, Goffinet AM. Processing of reelin by embryonic neurons is important for function in tissue but not in dissociated cultured neurons. *J Neurosci* 2007; **27**:4243-4252.
 - 54 Kesner RP. Behavioral functions of the CA3 subregion of the hippocampus. *Learn Mem* 2007; **14**:771-781.
 - 55 Nakashiba T, Young JZ, McHugh TJ, Buhl DL, Tonegawa S. Transgenic inhibition of synaptic transmission reveals role of CA3 output in hippocampal learning. *Science* 2008; **319**:1260-1264.
 - 56 Nakahira E, Yuasa S. Neuronal generation, migration, and differentiation in the mouse hippocampal primordium as revealed by enhanced green fluorescent protein gene transfer by means of *in utero* electroporation. *J Comp Neurol* 2005; **483**:329-340.
 - 57 Janes PW, Griesshaber B, Atapattu L, *et al.* Eph receptor function is modulated by heterooligomerization of A and B type Eph receptors. *J Cell Biol* 2011; **195**:1033-1045.
 - 58 Smith DI, Zhu Y, McAvoy S, Kuhn R. Common fragile sites, extremely large genes, neural development and cancer. *Cancer Lett* 2006; **232**:48-57.
 - 59 Williams SE, Mann F, Erskine L, *et al.* Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. *Neuron* 2003; **39**:919-935.
 - 60 Chenux G, Henkemeyer M. Forward signaling by EphB1/EphB2 interacting with ephrin-B ligands at the optic chiasm is required to form the ipsilateral projection. *Eur J Neurosci* 2011; **34**:1620-1633.
 - 61 Dravis C, Yokoyama N, Chumley MJ, *et al.* Bidirectional signaling mediated by ephrin-B2 and EphB2 controls urorectal development. *Dev Biol* 2004; **271**:272-290.
 - 62 Yokoyama N, Romero MI, Cowan CA, *et al.* Forward signaling mediated by ephrin-b3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. *Neuron* 2001; **29**:85-97.
 - 63 Henkemeyer M, Marengere LE, McGlade J, *et al.* Immunolocalization of the Nuk receptor tyrosine kinase suggests roles in segmental patterning of the brain and axonogenesis. *Oncogene* 1994; **9**:1001-1014.
 - 64 Trommsdorff M, Borg JP, Margolis B, Herz J. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J Biol Chem* 1998; **273**:33556-33560.
 - 65 Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* 2002; **30**:E9.
 - 66 Dail M, Richter M, Godement P, Pasquale EB. Eph receptors inactivate R-Ras through different mechanisms to achieve cell repulsion. *J Cell Science* 2006; **119**:1244-1254.
 - 67 D'Arcangelo G, Nakajima K, Miyata T, Ogawa M, Mikoshiba K, Curran T. Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J Neurosci* 1997; **17**:23-31.
 - 68 Zurhove K, Nakajima C, Herz J, Bock HH, May P. γ -Secretase limits the inflammatory response through the processing of LRP1. *Sci Signal* 2008; **1**:ra15.
 - 69 Ma L, Harada T, Harada C, *et al.* Neurotrophin-3 is required for appropriate establishment of thalamocortical connections. *Neuron* 2002; **36**:623-634.
 - 70 Brunne B, Zhao S, Derouiche A, *et al.* Origin, maturation, and astroglial transformation of secondary radial glial cells in the developing dentate gyrus. *Glia* 2010; **58**:1553-1569.
 - 71 Campo CG, Sinagra M, Verrier D, Manzoni OJ, Chavis P. Reelin secreted by GABAergic neurons regulates glutamate receptor homeostasis. *PLoS One* 2009; **4**:e5505.

(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)



This work is licensed under the Creative Commons Attribution-NonCommercial-No Derivative Works 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0>