Reelin-dependent ApoER2 downregulation uncouples newborn neurons from progenitor cells

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Summary
Reelin and its receptor machinery are well known to be required for the migration and positioning of neocortical projection neurons. More recently, reelin has been shown both necessary and sufficient to determine the rate of neocortical neurogenesis. The molecular links underlying its seemingly distinct proliferative and post-proliferative functions remain unknown. Here we reveal an enriched expression of functional reelin receptors, largely of Apolipoprotein E Receptor 2 (ApoER2), in radial glia basal processes and intermediate progenitor cells during mid/late cortical development. In vivo, ApoER2 overexpression inhibits neuronal migration. In contrast, precluding excessive levels of ApoER2 in reelin-deficient cortices, by either ApoER2 knock-down or the transgenic expression of reelin in neural progenitor cells, improves neuronal migration and positioning. Our study provides groundwork for the highly orchestrated clearance of neocortical neurons from their birth site, suggesting that a reelin-dependent ApoER2 downregulation mechanism uncouples newborn neurons from progenitor cells, thereby enabling neurons to migrate.

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Introduction
Excitatory long distance projecting neurons originate from neural progenitor cells (NPCs) locally arranged in the ventricular (VZ) and subventricular (SVZ) zones of the dorsal pallium. Newborn postmitotic neurons migrate out from their birth sites in the basal direction to form the cortical plate (CP), the cerebral cortex anlage, where they end forming highly organized connections within the cortex or with subcortical targets. The mechanisms regulating NPCs proliferation also regulate total numbers of neurons produced at specific developmental periods and destined to a specific neocortical layer (Caviness et al., 2009). The mechanisms underlying the tight spatiotemporal link between neurogenesis and neuronal migration remain, however, less well understood (Ge et al., 2006; Nguyen et al., 2006; Pawlisz et al., 2008).

The gene mutated in reeler mouse encodes reelin, a glycoprotein whose function is required for laminar positioning of cortical neurons. Reelin, synthesized and secreted by Cajal-Retzius cells in the marginal zone, is thought to deliver a signal to young migrating projection neurons instructing them to reach their right position. It is well established that reelin binding to Apolipoprotein E Receptor 2 (ApoER2) or Very Low Density Lipoprotein Receptor (Vldlr) induces phosphorylation of the cytoplasmic adaptor protein Disabled 1 (Dab1), a tyrosine kinase signal transduction cascade, and Dab1-regulated turnover. Indeed, the reeler mice and the mutant null mice for Dab1 or for both ApoER2 and Vldlr show similar positioning defects (Cooper, 2008; Rice and Curran, 2001; Tissir and Goffinet, 2003). However, despite numerous proposals (quite often incompatible), a coherent model of reelin action to position neurons does not yet exist (Luque, 2007). Reelin signaling decoding is often assumed to occur exclusively in neurons but it is even unclear whether neuronal migration per se is influenced by reelin (Mayer et al., 2006). Cells in the VZ, where primary NPCs reside, are indeed competent to respond to exogenous reelin by phosphorylating Dab1 and the reelin pathway is normally activated in these cells at the preplate stage of development. Likewise, ectopic expression of reelin in the VZ of reeler mice promoted migration of a subset of neurons past the subplate so rescuing preplate splitting during CP formation. Nevertheless, reelin in the VZ was found not enough to establish correct cortical lamination (Magdaleno et al., 2002). Moreover, reelin receptors are much more abundant in the reeler CP, suggesting that reelin induces their downregulation and that...
downregulation of reelin receptors begins at early/pre-migratory stages, well before neurons reach the CP (Uchida et al., 2009). More recently, reelin (cooperating with Notch signaling) has been shown both required and sufficient to determine the rate of neocortical neurogenesis (Lakomá et al., 2011). Such interaction is also required for neuronal migration and positioning (Hashimoto-Torii et al., 2008). Thus, the early function of reelin in the proliferative compartment might underlie its post-proliferative requirement raising the possibility that acts coupling neurogenesis and neuronal migration, perhaps akin to a permissive rather than instructive signal for cellular migration.

We address this question by investigating the significance of reelin receptors regulation during mid/late neocortical development.

### Materials and Methods

#### Mice

Heterozygous reeler mice were purchased from Jackson Laboratory (Bar Harbor, ME). The nestin-reeler transgenic mice were the generous gift from T. Curran (Magdaleno et al., 2002). The day of vaginal plug appearance was considered to be embryonic day 0 (E0) and genotyping performed as previously described (Lakomá et al., 2011). Animals were handled according to protocols approved by the European Union and NIH guidelines, the Nagoya University and the Instituto de Neurociencias Animal Care and Use Committees.

#### AP-RR36 in situ staining and densitometric analysis

An Alkaline Phosphatase (AP)-fusion probe of the receptor-binding region of reelin (repeats 3 to 6, AP-RR36) was used to detect functional reelin receptors during mid/late cortical development. Validation of the probe, and standard binding and staining procedures were previously described (Uchida et al., 2009). Here, we first avoid endogenous AP inactivation steps and conventional substrate reagents. Then, AP-RR36 in situ binding was detected using AP fluorescent substrates or, after careful fixation of free-floating slices, using anti-AP antibodies in combination with cell markers. A semi-quantifying densitometric analysis of standard AP-RR36 signal was performed using the Quantity One Software on 3 equal 2412 arbitrary square units per section randomly distributed through the CP region above (+/−rl and +/−rl ne-reelin), below (+/− rl) or both above and below (+/− rl ne-reelin) the estimated position of the subplate.

#### Immunohistochemistry and data analysis

Immunostaining was performed as previously described (Lakomá et al., 2011). Primary and secondary antibodies were acquired and diluted as indicated: rabbit anti-Dab1 (B3, 1:500, gift from B. Howell), rabbit anti-Tbr1 (1:1000, Chemicon), mouse anti-nestin (1:10 DSHB), rat anti-BrdU (crossreaction with CldU, 1:100, Serotec), mouse anti-BrdU (crossreaction with IdU, 1:100, BD), mouse anti-human Alkaline Phosphatase (1:250, Abcam), guinea pig anti-Vglut1 (1:10000, Chemicon), rat anti-GFP (1:500 Nacalni), Cy2-goat anti-mouse, Cy3-goat anti-rabbit (1:400, Jackson Immunoresearch), biotin-donkey anti-guinea pig (1:500, Jackson Immunoresearch) and Cy5-streptavidin (1:800, Jackson Immunoresearch). Unconjugated Fab fragments were used when anti-Thr1 and anti-Cux1 were combined. Nuclei were counterstained with DAPI. Images were captured on a Leica TCS SP2 AOS inverted Laser Scanning Confocal Microscope or a NIKON fluorescent microscope equipped with a confocal structured light system (Optigrid). Velocity 5.2, Image J (NIH, http://rsb.info.nih.gov/ij), and Adobe Photoshop software were used for image capturing and analysis.

#### Thymidine analogs double-labeling

Chlorodeoxyuridine (CldU, Sigma, 41.66 mg/kg) and Iododeoxyuridine (IdU, Fluka, 58.33 mg/kg) were administered intraperitoneally in pregnant reelin heterozygous ne-reelin mice. CldU was injected on the 13th day of embryonic development and IdU 24 hours later. The litter was sacrificed at P0, genotyped and further processed for immunofluorescence adding a DNA denaturating step.

#### Electroporation and data analysis

Electroporation-mediated in utero gene transfer was carried out on E14 mice embryos that were harvested at E18 or P0 as previously described (Tabata and Nakajima, 2001) on wild type (WT), heterozygous or reeler backgrounds. On an entire litter of animals 90 ng/μl of a plasmid encoding EGFP protein was injected into the brain lateral ventricle, whereas on another two litters, the same amount of EGFP plasmid was co-electroporated with either 1 μg/μl of a siRNA targeting the AP-RR36 gene (AMBION s69322, validated siRNA) in order to knock-down the expression of ApoER2, or the same concentration of a plasmid encoding ApoER2 proteins (pMSCV-puro-mmApoER2, a kind gift from J. Nimpf; Hibi et al., 2009) to induce ApoER2 overexpression. For ApoER2 overexpression experiments the position of 599 EGFP+ cells was analyzed, while 2412 EGFP+ cells were analyzed for ApoER2 siRNA experiments. Quantification of Dab1 signal intensity in 1332 electroporated cells was done with the NIH software ImageJ. SPSS was used to perform Student t test for statistical significance and GraphPad Prism to build up the graphs. The values represent means ± standard errors. Further methodological details and specifications are available upon request.

### Results and Discussion

**Localization of functional ApoER2 in NPCs**

An Alkaline Phosphatase (AP)-fusion probe of the receptor-binding region of reelin (repeats 3 to 6, RR36) (Uchida et al., 2009) was employed to assess the localization of functional reelin receptors (i.e., those in the plasma membrane as mature forms) during the neocortical neurogenic phase. AP-RR36 in situ binding was performed using AP fluorescent substrates (Fig. 1A,B) or followed by double immunofluorescence labeling of AP and several NPCs markers (Fig. 1A,C–N). Radial glial cells, the primary NPCs in the VZ, were detected as cell expressing Brain Lipid Binding Protein [BLBP] (Anthony et al., 2004). By embryonic day (E) 15 radial glial cell bodies in the basal-most part of the VZ and processes spanning the CP expressed functional reelin receptors (Fig. 1C–H). Tbr2 immunostaining was used to examine secondary NPCs in the SVZ, so called intermediate progenitor cells (IPCs) (Kriegstein et al., 2011). The day of vaginal plug appearance was considered to be embryonic day 0 (E0) and genotyping performed as previously described (Lakomá et al., 2011). Animals were handled according to protocols approved by the European Union and NIH guidelines, the Nagoya University and the Instituto de Neurociencias Animal Care and Use Committees.

**Fig. 1. Neocortical NPCs express functional ApoER2.** In situ binding with AP-RR36, an Alkaline Phosphatase-fusion probe of the receptor binding region of reelin, reveals the presence of functional reelin receptors, largely ApoER2, in the developing cortex at E15 (A,B). AP-RR36 staining is observed in several BLBP+ radial glia processes spanning the cortical wall (C–E) as well as in subpopulations of radial glia cell bodies in the basal most VZ (F–H). Most Tbr2+ IPCs in the SVZ (I–K) and some BrdU+ cycling progenitors (L–N) stain also with AP-RR36. Scale bar: 200 μm in B; 150 μm in C–E; 50 μm in F–N.
ApoER2 overexpression inhibits neuronal migration

ApoER2 is much more abundant in the reeler CP as compared to wild type, suggesting that reelin induces its downregulation (Uchida et al., 2009). To investigate the significance of ApoER2 regulation, WT cortices were electroporated at E14 with either enhanced Green Fluorescent Protein (GFP, 4 embryos), with a full-length ApoER2 plasmid (ApoER2-full-GFP, 4 embryos) or a plasmid with a truncated form of the receptor lacking the cytoplasmic domain (ApoER2-CΔCD-GFP, 2 embryos) and examined the brains at postnatal day (P) 0. The alignment of a layer of cells beneath the marginal zone was apparent through this temporal window in the GFP-electroporated cortices (Fig. 2A). In contrast, in the ApoER2-CΔCD-GFP-electroporated cortices fewer cells appeared aligned beneath the marginal zone and many of them were down dispersed through the CP (Fig. 2B). More dramatically, in the ApoER2-full-GFP-electroporated cortex, most cells appeared dispersed through the CP, the intermediate and the SVZ with very little, if any, cells positioned just beneath the marginal zone (Fig. 2C). A quantification of this effect is shown in Fig. 2D. The overall weaker and heterogeneous effect of ApoER2-CΔCD when compared with that of ApoER2-full-overexpression supports that plasma membrane ApoER2 overloading underneath by itself the migratory reeler cortical phenotype. Conceivably, our truncated ApoER2 form competes with endogenous receptors for reelin but does not relay the intracellular signal, thus way behaving like a dominant negative molecule (Jossin and Cooper, 2011). Then again, our full ApoER2 form might equally compete with endogenous receptors for reelin while efficiently relaying the intracellular signal. Thus, whether migration is halted in a given cell would depend on the particular reelin/ApoER2 stoichiometry of its plasma membrane; assuming that reelin levels must be limiting, the more full receptors remain without access to reelin and hence not being downregulated, the higher the probability of cell migration inhibition. This might also encompass a mechanism as to why NPCs do not migrate out of the VZ/SVZ. Interestingly, by longer harvesting times (P3) many of cells misexpressing ApoER2-CΔCD-GFP showed more branched and thicker processes (supplementary material Fig. S2). In agreement, neutralization of reelin at early postnatal stages increases dendritic complexity of cortical pyramidal neurons involving a signal transduction pathway independent of the canonical reelin receptors (Chameau et al., 2009).

ApoER2 knock-down rescues neuronal migration and Dab1 levels in reeler mice

As ApoER2 overloading indeed inhibits neuronal migration in wild type cortices, ApoER2 downregulation could somehow enhance migration. However, this can hardly be tested in wild types (i.e., in the presence of endogenous reelin) when reelin induces ApoER2 downregulation. In fact, knocking-down of either ApoER2 or Vldlr in wild type cortices was shown to have modest or no effect at all on neuronal positioning (Kubo et al., 2006). IPCs expressed high levels of functional reelin receptors (Fig. II–K). A short BromodeoxyUridine (BrDU, 50 mg/kg) pulse followed by BrDU immunolabeling was used to detect S-phase cycling progenitors. BrDU+ profiles in the basal most part of the VZ, the VZ/SVZ interface and the SVZ also expressed functional reelin receptors (Fig. 1L–N). By E17 AP-RR36 staining was still observed in NPCs expressing the generic NPCs marker nestin. Quite often a weaker band of receptors expression was observed within 20–30 μm from the ventricular lumen (supplementary material Fig. S1). In agreement with previous detections of antigenic determinants or mRNAs (Hartfuss et al., 2003; Kawaguchi et al., 2008; Keilani and Sugaya, 2008; Luque et al., 2003; Magdaleno et al., 2002), the expression of functional reelin receptors in both radial glia and IPCs indicates that NPCs are competent to receive reelin during the neurogenic phase of neocortical development. Consistent with this notion a previous study has demonstrated that reelin signaling regulates the temporal specification of NPCs (Lakomá et al., 2011). Most of the AP-RR36 signal was abolished in the ApoER2-deficient cortex while it remained virtually the same in the Vldlr-deficient one (Uchida et al., 2009; data not shown). Indeed, severe abnormalities in cortical neuronal positioning (layers II to VI) are present in the absence of ApoER2 but not of Vldlr (Benhayon et al., 2003; Hack et al., 2007). Thus, during mid/late development neocortical NPCs express mainly, if not exclusively, ApoER2.

Fig. 2. ApoER2 loading determines neuronal migratory behavior. Overexpression of ApoER2 inhibits neuronal migration (A–D). P0 brains that had been transfected at E14 with an EGFP plasmid (control) (A) or with truncated (B) or full-length (C) ApoER2 plasmids are depicted. Note the alignment of a layer of neurons beneath the marginal zone in control cortex (A). Fewer neurons are aligned and many of them appear halted in their radial migratory trajectories in the brains transfected with ApoER2 plasmids (B,C). ApoER2 knock-down rescues neuronal migration and Dab1 levels in reeler (E–O). E18 cortices that had been transfected at E14 either with an EGFP plasmid (control) or with an EGFP plasmid plus an RNAi plasmid for ApoER2 (siApoER2) are depicted. A band of aligned neurons is visible beneath the marginal zone in EGFP-electroporated heterozygous cortices (E). In EGFP-electroporated reeler cortices neurons do not form an apparent band and appears confined at the bottom of the cortical wall (F). Partial rescue of neuronal migration is apparent in EGFP+siApoER2-electroporated reeler cortices (G). Dab1 levels are reduced in reeler cortical neurons after EGFP+siApoER2 electroporation (I–O). Scale bar: 150 μm in A–C,E–G; 15 μm in E–K,L–N.
Admittedly counterintuitive, we decide instead to knock-down ApoER2 in reeler cortices. Three reelin heterozygous and 2 reeler cortices were electroporated at E14 with an EGFP plasmid. Three additional reeler cortices were electroporated with EGFP plasmid and an RNAi plasmid for ApoER2 (siApoER2). All brains were examined at E18. Again, like in WT cortices, in the EGFP-electroporated heterozygous it was apparent the alignment of a layer of cells beneath the marginal zone (Fig. 2E). In contrast, in the EGFP-electroporated reeler cortices cells did not form a discernible layer and appeared confined in the lower half of the cortical wall (Fig. 2F). However, in the EGFP+siApoER2-electroporated reeler cortices cells appeared more dispersed and positioned in a roughly inverted pattern as compared with those of EGFP-electroporated reeler cortices. A percentage of them appeared beneath the marginal zone at comparable positions to those of EGFP-electroporated heterozygous cortices (Fig. 2G).

A quantification of these effects is shown in Fig. 2H. Disruption of reelin signaling by genetic ablation of reelin, ApoER2, VLDLR, or Fyn and Src lead to the accumulation of Dab1 protein (Arnaud et al., 2003; Bock and Herz, 2003; Kuo et al., 2005; Rice et al., 1998; Sheldon et al., 1997; Trommsdorff et al., 1999), indicating that reelin limits its action in responsive cells by reducing Dab1 levels. We found that in reeler cortices the EGFP+siApoER2-electroporated neurons showed lower levels of Dab1 than the EGFP-electroporated neurons (Fig. 2I–O), raising the possibility that ApoER2 knock-down recapitulates reelin function in the absence of endogenous reelin. Furthermore, the reduction of ApoER2 genetic dosage also ameliorates neuronal positioning in reeler cortex (supplementary material Fig. S3). It implies that newborn neurons that have not activated the canonic reelin pathway retain their migratory potential suggesting that reelin signaling could influence migration by regulating the cell competence to respond to a second pathway. ApoER2 are indeed quite promiscuous receptors, with trombospondin-1, f-spondin, coagulation factor XI or protein C (Blake et al., 2008; Hoe et al., 2005; May et al., 2005; White-Adams et al., 2009; Yang et al., 2009) among its known ligands, making conceivable the existence of an inhibitory signal on cell migration that might be precluded upon reelin-induced ApoER2 downregulation. A facilitated activation of ephrin B signaling could equally be involved. In fact, ephrin Bs have been recently identified as reelin co-receptors as they bind directly to reelin and ApoER2/Vldlr, resulting in Dab1 phosphorylation. Significantly, the reeler cortical migratory phenotype was shown to be rescued by simple activation of ephrin B signaling, i.e., in the absence of endogenous reelin (Sentürk et al., 2011).

Partial rescue of lamination along with ApoER2 and Dab1 levels by expression of reelin in NPCs of reeler mice

The enriched expression of functional ApoER2 in NPCs and the evidence that ApoER2 loading determines the cellular migratory behavior also predict an action of reelin in the VZ/SVZ resulting in neuronal ApoER2 downregulation and positioning. To test this prediction we took advantage of the ne-reelin mice that ectopically express reelin in NPCs under the control of a nestin driver (Magdaleno et al., 2002). We performed immunolabeling at P0 combining the use of the subplate marker VGlu1 (Ina et al., 2007) and the cortical layer markers Tbr1 – for layer VI and preplate derivatives – (Bulfone et al., 1995) and Cux1 – for layers II/III/IV (Nieto et al., 2004). We confirmed that in the WT background, ectopic reelin did not alter cortical layering (Fig. 3A,B,E). However, against a previous notion, in the reeler background ectopic reelin did partly rescue cortical lamination, specifically in the cell population positioned above, but not that one below, an ectopic subplate (Fig. 3C,D,F). Properly positioned upper and lower layer cells contributed to the rescued CP region (i.e. above the subplate) with an approximate 15% and 70% of the total Cux1+ and Tbr1+ cell population, respectively. Consistent with previous observations (Feng and Cooper, 2009; Nieto et al., 2004), Cux1+ cells were virtually absent in the upper third of the conventional (unrescued) reeler CP (not shown). To corroborate these results we sequentially injected pregnant dams at E13 and E14 with the nucleotide analogues CdU and IdU, respectively, and harvested 4 pup brains at P0 for immunolabeling. We found that the inside-out pattern of cell positioning was partially rescued in reeler ne-reelin cortices. Indeed, later born cells (IdU+) pass earlier born cells (CdU+) over, but not beneath, the ectopic subplate (Fig. 3G,H). Thus, while the role of reelin in promoting splitting of the preplate does not seems entirely distinct from its effect on positioning, the failure of some cells to respond to reelin appears related to their migratory distance and hence to their birthdates. Interestingly, the neocortical (and cerebellar) phenotype of reeler ne-reelin is reminiscent of dab1<sup>cd/cd</sup> mutants, lacking one out of two pair of Dab1 tyrosine phosphorylons sites (Feng and Cooper, 2009; Morimura and Ogawa, 2009). This suggests that ne-reelin might support the kinase switch function of Dab1, but perhaps not sufficiently its scaffold function required for the migration of upper layers neurons. By means of in situ AP-RR36 staining we then evaluated the expression of functional ApoER2 in ne-reelin cortices. We confirmed that at E17 ApoER2 was much more abundant in the reeler than in the

![Fig. 3. Partial rescue of inside-out patterning in reeler by ectopic reelin in NPCs. Immunohistochemical detection at P0 of Tbr1, Cux1 and VGlu1 on adjacent sections of ne-reelin (A,B) and reeler-ne-reelin (C,D) cortices. In the WT background, the expression of the ne-reelin transgene does not alter cortical lamination. In the reeler background it does rescue cortical layering in the cell population above, but not that below, the ectopic subplate. Use of Fab blocking fragments confirmed these results allowing the combination of antibodies on the same section (E,F). Sequential injection of the nucleotide analogues CdU and IdU by E13 and E14, respectively, further demonstrate at P0 a rescue of the inside-out lamination pattern above the ectopic ne-reelin reeler subplate (G,H). Scale bar: 200 μm.](http://bio.biologists.org/Downloaded from http://bio.biologists.org/)
WT CP (Fig. 4A,B). In the presence of the endogenous protein (WT or heterozygous backgrounds) ectopic reelin did not alter ApoER2 expression. In contrast, in the reeler background, ectopic reelin induced a dramatic decrease of ApoER2 above the ectopic subplate (Fig. 4C,D). Comparable results were obtained at P0 (Fig. 4A–D’). A semi-quantification of these effects is shown in Fig. 4E,F. Moreover, normalized Dab1 levels were observed at P0 in the rescued CP region of reeler ne-reelin mice (supplementary material Fig. S4), further supporting that at least in part, the function of reelin in the proliferative compartment underlies its post-proliferative requirement for neuronal migration and positioning. The recent claim that deletion of Dab1 in migrating neurons alone phenocopies reeler lamination defects (Franco et al., 2011) seems apparently incompatible with our proposal. However, since the NEX promoter element used by Franco et al. (Franco et al., 2011) can drive expression in IPCs (Goebbels et al., 2006), it cannot be ruled out that Dab1 deletion in IPCs might have contributed to effects on the migratory properties of the neurons generated. Together, our results provide foundation for the highly orchestrated clearance of neocortical projection neurons from their birth site, indicating that a reelin-dependent ApoER2 downregulation mechanism uncouples newborn neurons from NPCs, thereby enabling neurons to migrate (Fig. 4G). Further elucidation of the underlying molecular mechanisms of ApoER2 regulation and mediated signaling (including reelin and ligands other than reelin as well as putative co-receptors) should reveal novel concepts and patterns that provide a clear link between NPCs proliferation/neuronal fate and neuronal migration. It might become increasingly evident that neurogenesis and neuronal positioning are less separately encoded than it has hitherto been thought.

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Competing Interests

The authors have no competing interests to declare.

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