Reelin Promotes Neuronal Orientation and Dendritogenesis during Preplate Splitting

Anna J. Nichols1 and Eric C. Olson1,2

1Department of Neuroscience and Physiology, SUNY Upstate Medical University, Syracuse, NY 13210, USA and 2Department of Electrical Engineering and Computer Science, Syracuse University, Syracuse, NY 13210, USA

Address correspondence to Eric C. Olson, PhD, Department of Neuroscience and Physiology, SUNY Upstate Medical University, 3295 Weiskotten Hall, 750 East Adams Street, Syracuse, NY 13210, USA. Email: olsone@upstate.edu.

The secreted ligand Reelin is thought to regulate the translocation and positioning of prospective layer 6 (L6) neurons into the preplate, a plexus of neurons overlying the ventricular zone. We examined wild type and Reelin-deficient cortices and found that L6 neurons were equivalently positioned beneath the pia during the period of preplate splitting and initial cortical plate (CP) formation. The absence of detectable L6 ectopia in “reeler” cortices at this developmental time point indicates that Reelin-signaling might not regulate L6 neuron migration or gross positioning during preplate splitting. To explore the acute response of L6 neurons to Reelin, subpial injections of Reelin were made into Reelin-deficient explants. Reelin injection caused L6 neurons to orient their nuclei and polarize their Golgi toward the pia while initiating exuberant dendritic (MAP2+) outgrowth within 4 h. This rapid Reelin-dependent neuronal orientation and alignment created CP-like histology without any significant change in the mean position of the population of L6 neurons. Conversely, subpale cells and chondroitin sulfate proteoglycan immunoreactivity were found at significantly deeper positions from the pial surface after injection, suggesting that Reelin partially rescues preplate splitting within 4 h. Thus, Reelin has a direct role in promoting rapid morphological differentiation and orientation of L6 neurons during preplate splitting.

Keywords: cortex, dendrite, epilepsy, migration, neurite, orientation, preplate

Introduction

Preplate splitting, an early event in cortical development, involves the movement of prospective layer 6 (L6) neurons from the ventricular zone (VZ) into the preplate, a loose overlying plexus of differentiated neurons (Marin-Padilla 1978). Once assembled, these L6 neurons constitute the early cortical plate (CP). The formation of the CP within the preplate splits the preplate into a superficial layer of neurons, the marginal zone (MZ), and a deeper layer called the subplate (SP; Marin-Padilla 1978). This fundamental event, possibly unique to the development of the mammalian dorsal pallium, enables the assembly of upper cortical layers (Super et al. 1998; Aboitiz et al. 2005).

Preplate splitting is the first event during corticogenesis that requires the secreted ligand Reelin (Sheppard and Pearlman 1997). In “reeler” mice, which lack Reelin, L6 neurons fail to split the preplate (Sheppard and Pearlman 1997), do not form a clear CP, and are found in a disorganized layer of cells that lack consistent apical–basal orientation and normal dendrites (Goffinet and Lyon 1979). After preplate splitting fails in the reeler cortex, later born neurons destined for cortical layers 2–5 are found in abnormal, deeper positions creating an approximate inversion of cellular layering (Caviness and Sidman 1973).

Functional analyses and the complementary expression patterns of Reelin (D’Arcangelo et al. 1995) and its receptors (Trommsdorff et al. 1999) indicate that Reelin signals to migrating and differentiating neurons (Dulabon et al. 2000; Olson et al. 2006) as well as neural precursors (Hartfuss et al. 2003; Weiss et al. 2003). Reelin is secreted by Cajal–Retzius (CR) neurons in the preplate and MZ (D’Arcangelo et al. 1995; Hirotsume et al. 1995; Ogawa et al. 1995). Reelin binds to ApoER2 and the very low density lipoprotein receptor (D’Arcangelo et al. 1999; Hiesberger et al. 1999) leading to tyrosine phosphorylation of an essential adapter protein, Dab1 (Howell et al. 1997, 1999; Sheldon et al. 1997; Ware et al. 1997). Phospho-Dab1 interacts with proteins involved in both actin and microtubule dynamics (D’Arcangelo 2006).

Despite progress in understanding the biochemical cascade initiated by Reelin, how Reelin-signaling produces CP formation is unknown. It is generally agreed that Reelin positions neurons but the cellular mechanism is unclear and may involve stimulating (Super et al. 2000; Magdaleno et al. 2002), inhibiting (Dulabon et al. 2000), repelling (Yip et al. 2009), or detaching (Hack et al. 2002; Sanada et al. 2004) migrating neurons. Time-lapse studies of neuronal migration suggest that Reelin-signaling controls two key processes: the rapid, glial-independent movement that occurs immediately prior to migration arrest (Nadarajah et al. 2001). Similar movements are observed during the period of preplate splitting, suggesting that L6 neurons may translocate directly into the preplate (Nadarajah et al. 2001). Although there is evidence that neurons translocate “out” of the VZ, there is no definitive evidence that neurons translocate “into” the preplate to split it. Additionally, no studies have examined translocation in reeler mice. Thus, the cellular mechanisms of preplate splitting and the specific roles of Reelin in this critical process are unknown.

To explore Reelin’s role in preplate splitting, we used reporter mice crossed into the reeler background and examined the position of L6 neurons during the period of preplate splitting. Surprisingly, we did not detect ectopic L6 neurons in reeler cortices during this period despite the failure of preplate splitting. This finding argues against an important role for Reelin in gross cellular positioning of L6 neurons during preplate splitting. Therefore, to determine the acute response of L6 neurons to Reelin, we injected recombinant Reelin underneath the meninges of whole-hemisphere cortical explants from reeler embryos. Within 4 h of Reelin injection, L6 neurons in mutant explants elaborated dendrites and reoriented their somata toward the pial surface. This morphological differentiation occurred without significant L6 neuron movement and was accompanied by a partial rescue of preplate splitting. These findings argue that promotion of L6 neuron orientation and differentiation are primary functions of Reelin-signaling during preplate splitting.
Materials and Methods

Mice
All animal procedures were approved by the Institutional Animal Care and Use Committee of SUNY Upstate Medical University. Eomes::GFP mice (The Gene Expression Nervous System Atlas Project, NINDS Contract no. N01NS02531 to The Rockefeller University, New York, NY) were mated to reeler (B6C3F1 a/a-Reln+/+, Jackson Laboratories, Bar Harbor, ME) mice to produce compound heterozygotes that were then intercrossed to produce eomes::GFP/Reln+/− mutants. Both Reln+/+ and Reln−/− embryos exhibit normal preplate splitting (Sheppard and Pearlman 1997) are referred to as wild type in this study and are denoted Reln+/+. The day of plug discovery was designated embryonic day 0 (E0).

Explant Cultures
Whole hemispheres were cultured medial side down on collagen-coated polytetrafluoroethylene filters with a 3-μm pore size (Transwell-COL, Corning, NY) using an established protocol (Jossin et al. 2003, 2004). The explants were cultured in DMEM-F12 medium containing GlutaMAX and supplemented with 2% B27, 1% B5, 7.5 mM glucose, 1X penicillin-streptomycin, and 0.05 mg/mL gentamicin. Explants were maintained in a 37 °C, high oxygen environment (95% O2/5% CO2) throughout the experimental period. Explants were allowed a 4-h recovery period prior to experimental treatments. All cell culture reagents were from Invitrogen (Carlsbad, CA).

Production of Reelin
Supernatants containing recombinant Reelin were produced from a stable HEK293 cell line as described (Forster et al. 2002) with minor modifications. Conditioned media from the Reelin-secreting HEK293 cell line (Reelin medium [RM]) or a control HEK293 cell line (Control medium [CM]) was collected after 48 h of incubation in serum-free Opti-MEM media supplemented with 1X GlutaMAX and 1X Pen/Strep (all cell culture reagents were from Invitrogen). The conditioned media were concentrated approximately 10-fold using Amicon Ultra 100 000 molecular weight cut off filters (Millipore, Billerica, MA) and used for explant injection on the same day.

Reelin Injection
Wiretrol 10-μL pipettes (Drammond, Broomall, PA) were pulled to a fine point with an electrode puller and the tip snapped off with jeweler's forceps. Approximately 0.5-1 μL of recombinant Reelin was injected into multiple lateral and medial points in each explant. Injection periods were brief (<10 min) and were performed at room temperature. The explants were then cultured for an additional 4 h.

Histology/Immunohistochemistry
Explants and brains were drop fixed for 1-6 h in 4% paraformaldehyde/Pagano solution (250 mM sucrose, 25 mM MgCl2, 2.5 mM KCl, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 7.4). The tissue was then embedded in 10% calf gelatin (Sigma, St. Louis, MO), postfixed for <24 h in 4% paraformaldehyde/Pagano solution and then sectioned at 100 μm molecular weight cut off filters (Millipore, Billerica, MA) and used for explant injection on the same day.

Immunofluorescence Analysis
The mean pixel intensity of MAP2 and F-actin immunofluorescence was examined in both drop-fixed sections and explants. Optical sections were made in ImageJ v1.38 (Wayne Rasband, National Institutes of Health [NIH]) or using the LSM Image Browser (Zeiss, Thornwood, NY).

Cell Orientation
Golgı orientation was assessed from GM130 immunofluorescence, and the position of the GM130 signal relative to the corresponding cell soma was assigned to 1 of 4 quadrants Q1–Q4, Q1 was pia-directed and Q3 was ventricle-directed. When the Golgi extended between two quadrants orientation was assigned to the quadrant that contained the majority of the organelle. Nuclear orientation was categorized as either vertical or horizontal based on the orientation of the longest axis of the nucleus.

Statistical Analyses
Unpaired t test with Bonferroni correction was used to compare mean cell position in each zone between reeler and control sections (Fig. 1). For all experiments involving RM or CM injection, one-way analysis of variance (the single factor being the presence of Reelin) with a post hoc Tukey’s honestly significant difference tests were performed to test the hypotheses that measurements obtained from 1) RM-treated mutant explants were not different from CM-treated mutant explants, 2) CM-treated wild-type explants were not different from CM-treated mutant explants, and 3) CM-treated wild-type explants were not different from RM-treated mutant explants.

Results
To examine excitatory layer 6 (L6) neurons involved in preplate splitting, we crossed eomes::GFP transgenic mice (Gong et al. 2003) with the reeler (Reln) mouse line. Eomes::GFP transgene expression initially identifies Tbr1+ intermediate precursor cells that generate Tbr1+ excitatory neurons in the developing...
However, we find that GFP protein expression persists after Tbr2 and encompasses the period of L6 neuron migration and positioning (Supplementary Fig. 1), which is the presumed Reelin-responsive period.

L6 Cell Position in the reeler Mutant

To characterize neuronal position during preplate splitting, pregnant dams were injected with BrdU (50 mg/kg) on E12 to label the population of cells destined to split the preplate in the embryonic cortex (Takahashi et al. 1999). Embryos were harvested on E13, genotyped, and the brains sectioned and processed for either BrdU or Tbr1 immunoreactivity. Abnormal migration and positioning in \( \text{Reln}^{-/-} \) cortex would be revealed by ectopic groups of L6 neurons in the VZ or intermediate zone (if Reelin-signaling stimulated translocation) or in the superficial preplate (if Reelin-signaling arrested migration). To facilitate analysis, the medial-to-lateral extent of the neocortex was divided into 6 zones, each 150 \( \mu \text{m} \) in length along the pia. Zone 3 was defined as the zone of active preplate splitting, and zones were compared between matched sections of \( \text{eomes}::\text{GFP;Reln}^{-/-} \) and mutant \( \text{eomes}::\text{GFP;Reln}^{-/-} \) neocortex. Cell position was measured from the basal lamina of the pia. BrdU+/GFP+ cell positions were compared between 6 wild-type embryos and 6 mutant embryos, >160 cells were measured in each zone. Tbr1+/GFP+ cell positions were compared between 5 wild-type embryos and 5 mutant embryos, and >110 cells were measured in each zone. Unpaired \( t \)-tests with Bonferroni correction were performed to compare mean positions in each zone. Scale bars: 50 \( \mu \text{m} \) in \( d, e \).

Figure 1. Equivalent positioning of L6 neurons during preplate splitting in E13 wild-type cortex and reeler cortex. Mean cell position was compared between genotypes along the medial-to-lateral extent of the neocortex. Each zone is 150-\( \mu \text{m} \) wide, and Z3 is the zone of active preplate splitting, zones 1 and 2 are at the preplate stage, while zones 4-6 show CP. No significant differences in the mean L6 neuron position were identified when \( \text{a-c} \) BrdU+/GFP+ neurons (labeled one day prior on E12) or \( \text{d-f} \) Tbr1+/GFP+ neuron position was compared between wild-type neocortex (eomes::GFP;Reln+/?) and mutant (eomes::GFP;Reln+/C0/C0) neocortex. Cell position was measured from the basal lamina of the pia. BrdU+/GFP+ cell positions were compared between 6 wild-type embryos and 6 mutant embryos, >160 cells were measured in each zone. Tbr1+/GFP+ cell positions were compared between 5 wild-type embryos and 5 mutant embryos, and >110 cells were measured in each zone. Unpaired \( t \)-tests with Bonferroni correction were performed to compare mean positions in each zone. Scale bars: 50 \( \mu \text{m} \) in \( d, e \).

neocortex (Englund et al. 2005; Kowalczyk et al. 2009). However, we find that GFP protein expression persists after Tbr2 and encompasses the period of L6 neuron migration and positioning (Supplementary Fig. 1), which is the presumed Reelin-responsive period.

L6 Cell Position in the reeler Mutant

To characterize neuronal position during preplate splitting, pregnant dams were injected with BrdU (50 mg/kg) on E12 to label the population of cells destined to split the preplate in the embryonic cortex (Takahashi et al. 1999). Embryos were harvested on E13, genotyped, and the brains sectioned and processed for either BrdU or Tbr1 immunoreactivity. Abnormal migration and positioning in \( \text{Reln}^{-/-} \) cortex would be revealed by ectopic groups of L6 neurons in the VZ or intermediate zone (if Reelin-signaling stimulated translocation) or in the superficial preplate (if Reelin-signaling arrested migration). To facilitate analysis, the medial-to-lateral extent of the neocortex was divided into 6 zones, each 150 \( \mu \text{m} \) in length along the pia. Zone 3 was defined as the zone of active preplate splitting, and zones were compared between matched sections of \( \text{eomes}::\text{GFP;Reln}^{-/-} \) and wild-type littersmates. We found that the mean position of BrdU+/GFP+ neurons relative to the basal lamina differed at most by 10 \( \mu \text{m} \) (e.g., approximately the diameter of a nucleus) between genotypes in each zone along the lateral-to-medial extent of the neocortex (Fig. 1a-c) and in no zone was the difference significant.

To more precisely identify L6 neurons, we examined the position of Tbr1+/GFP+ cells in the same set of sections. While Tbr1 immunoreactivity identifies both L6 and preplate neurons (Hevner et al. 2001), we find that the combination of strong GFP expression and strong Tbr1 immunoreactivity is selective for those neurons within the forming CP (Fig. 1d,e, also Fig. 7b). We therefore measured the distance from the pial basal lamina, identified with anti-laminin immunoreactivity, to the Tbr1+/GFP+ cell somata. We found no significant differences between genotypes, in any zone, in the mean Tbr1+/GFP+ neuron position (\( P \geq 0.22 \) for all zones). Thus, no cytoarchitectural evidence was observed that would indicate that Reelin-signaling affects the translocation or initial positioning of L6 neurons during the period of preplate splitting.

Rapid Dendritogenesis 4 h after Reelin Injection

Prior studies showed that bath-applied Reelin rescued preplate splitting in cortical slice explants from \( \text{Reln}^{-/-} \) mutants after 2 days in vitro (Jossin et al. 2003, 2004). Because E12 BrdU+/GFP+ and Tbr1+/GFP+ cells were found equivalently positioned under the pial surface in E13 \( \text{Reln}^{-/-} \) cortex, we examined the acute response of these superficial cells to injected Reelin. To assess the response, Reelin conditioned medium (RM) or control conditioned medium (CM) was injected underneath the pia of E13 \( \text{eomes}::\text{GFP;Reln}^{-/-} \) whole-hemisphere explants using a positive displacement glass microelectrode. Multiple (3-6) 0.5-1 \( \mu \text{L} \) injections were performed along the medial/lateral and rostral/caudal axes of the whole-hemisphere explants. Four hours after injection, the explants were fixed and processed for histological analysis.
Figure 2. Pia-oriented dendritic growth 4 h after Reelin injection into whole-hemisphere eomes::GFP explants. (a) Schematic of a coronal section of E13 wild-type cortex showing the lateral area of analysis. (b) Reln−/− explants injected with conditioned medium (CM) showed few subpial neurites while (c) Reln−/− explants injected with Reelin conditioned medium (RM) showed extensive subpial neurites (within dashed lines). (b′, c′) Traced outlines of representative neuronal somata and proximal neurites in explants. (d−f′) MAP2 immunolabeling of explants revealed an increase in MAP2 content to wild-type levels after RM injection into Reln−/− mutants. MAP2 expression in (d, d′, d″) CM-injected wild type (Reln+/?), (e, e′, e″) CM-injected mutant (Reln−/−), and (f, f′, f″) RM-injected mutant (Reln−/−) explants. (d−f) Higher magnification images of MAP2 signal in neurites. (g−i) Alexa Fluor-phalloidin staining of explants showed an increase in F-actin after RM injection. (j−k) Quantification of mean fluorescence signal in arbitrary units (a.u.) in ROIs below the pia. J Map2 signal (k) F-actin signal. At least 3 explants were examined in each condition. Error bars denote standard error of the mean. One-way analysis of variance with post hoc Tukey tests were performed between treatment conditions in each depth region. *P ≤ 0.05; **P ≤ 0.01. Scale bars: 20 μm in (c), 25 μm in (f), 40 μm in (f″, i′). Abbreviations: Nctx, neocortex; PP, preplate; LGE, lateral ganglionic eminence; hem, cortical hem; ROI, region of interest.
Four hours after injection, GFP+ neurons in RM-injected Reln−/− explants displayed greater neurite elaboration into the region subjacent to the pial basal lamina (MZ) than corresponding CM-injected Reln−/− explants (Fig. 2h,c). Measurement of the mean fluorescence intensity of the canonical dendritic marker MAP2 (Caceres et al. 1984) revealed a significant increase in apical MAP2 content in the MZ of RM-injected Reln−/− explants compared with CM-injected Reln−/− explants (Fig. 2d-f,f, P < 0.0004). Notably, RM injection elevated MAP2 levels in the mutant explants to levels not significantly different from wild-type explants (P = 0.35). RM-rescued explants also showed a significant increase in F-actin in the first 20 µm below the pia (Fig. 2g-i,k; P = 0.02) measured by Alexa Fluor-phalloidin signal. These results are consistent with previous findings that show a role for Reelin-signaling in both actin dynamics (Pramatarova et al. 2003; Chai et al. 2009) and dendritic development (Niu et al. 2004; Olson et al. 2006; Matsuki et al. 2008).

Rapid Cellular Orientation 4 h after Reelin Injection

RM injection also significantly increased the fraction of neurons with the longest axis of their nuclei oriented perpendicular to the pial surface (termed here “pia-orientation”). Pia-orientation of the GFP+ nuclei increased from 64 ± 4.5% (CM-injected mutant) to 89 ± 1.1% (RM-injected mutant; P < 0.01; Fig. 3f), a value that was not significantly different from the pia-orientation observed with CM-injected wild-type explants (90 ± 1.1%; P = 0.09). This pia-orientation of the long axis of the nucleus may be due to directional elongation or nuclear rotation. To further assess pia-orientation, we examined the position of the cis-Golgi in the neuronal soma, identified by GM130 immunolabeling (Nakamura et al. 1995). Because the cis-Golgi is localized to the basal portion of the apical dendrite (Horton et al. 2005), Golgi position identifies cellular orientation in cortical neurons. The percentage of pia-oriented Golgi in GFP+ neurons is significantly different in Reln−/− explants (51 ± 1.1%) compared with wild type 92 ± 1.1% (P < 0.0001). Four hour after RM injection into Reln−/− explants, 91 ± 1.1% of Golgi were pia-oriented (Fig. 3b,c). The rescue value was not significantly different from the wild-type (CM-injected wild-type explant) value (Fig. 3g, P = 0.77). In addition, the rescued Golgi phenotype resembled the wild type with elongated morphology (Fig. 3a–c insets). These findings indicate a rapid, Reelin-dependent organization of Golgi positioning and nuclear orientation in developing cortical neurons.

Partial Rescue of Preplate Splitting 4 h after Reelin Injection

The rescue of cellular morphology and the apparent formation of CP after Reelin injection suggested a possible rescue of preplate splitting. Remarkably, Reln−/− hemispheres exhibited some evidence of preplate splitting 4 h after RM injection. CSPG immunoreactivity identifies the MZ and SP (Pearlman and Sheppard 1996; Sheppard and Pearlman 1997) and we observed a deep band of CSPG immunoreactivity in RM-injected explants that was similar in extent, yet less intense, than the CSPG immunoreactivity of wild-type explants (Fig. 4a–c).

We also examined the position of the unlabeled (GFP−) population of cells. Labeling in wild-type brain sections with the nuclear dye Hoechst revealed a population of GFP– cells that was intermingled with GFP+ cells in the unsplit preplate but that becomes significantly segregated from the GFP+ cells in areas of CP formation (Supplementary Fig. 2). This suggested that, at a minimum, a subset of preplate cells was included within the GFP– cell population. Measured from the pia basal lamina, the mean position of GFP– cells is 25-µm deeper in wild-type (CM-injected) explants compared with mutant (CM-injected) explants (P < 0.0001). In mutant explants that were RM-injected, the mean position of GFP– cells was 10.5-µm deeper from the pia compared with mutant explants that were

Figure 3. Pia-orientation of Golgi and the long axes of neuronal nuclei 4 h after Reelin injection. GM130 immunolabeling of Golgi and Hoechst labeling of nuclei in (a, a’) CM-injected wild type (Reln+/+), (b, b’) CM-injected mutant (Reln−/−), and (c, c’) RM-injected mutant (Reln−/−) explants. High magnification images of (d) CM-injected mutant (Reln−/−) and (e) RM-injected mutant (Reln−/−) explants showing oriented and elongated Golgi in the pia-directed process (arrows) subsequent to RM injection. (f) Quantification of vertical (V) and horizontal (H) nuclear orientation. Three explants and >90 cells were examined in each condition. (g) Quantification of Golgi orientation by quadrant (Q). Error bars denote standard error of the mean. One-way analysis of variance with post hoc Tukey tests were performed between conditions. **P ≤ 0.01; ***P ≤ 0.001; ****P = 0.0001. Scale bars: 40 µm in (c’) and 10 µm in (c’ inset, e).
CM-injected (Fig. 4d–h, P = 0.02) indicating a partial rescue of preplate splitting in these explants within 4 h. To more precisely identify preplate cells, we examined the mean position of calretinin+ cells in the explants (Fig. 4i–m). Calretinin immunolabelling identifies MZ cells, primarily CR cells, as well as a subset of SP cells in rodents (Fonseca et al. 1995). In RM-rescued mutant explants, calretinin+ cells were 8-μm deeper than calretinin+ cells in CM-injected mutant explants (P = 0.03). These findings indicate that significant preplate splitting occurs within 4 h of RM injection.
No Significant Change in Mean L6 Neuron Position 4 h after Reelin Rescue
The cellular mechanisms driving Reelin-dependent preplate splitting could involve short-range movement of L6 neurons past SP cells. Based on our analyses of cell position (Fig. 1), any difference in the mean position of L6 neurons should be small (i.e., <10 μm) but potentially detectable in the comparison of RM-rescued explants and CM-injected explants. Comparison of mean position of E12 BrdU+/GFP+ cells in mutant (CM-injected), control (CM-injected), and rescued mutant (RM-injected) explants did not reveal any significant differences (Fig. 5a–e, P > 0.94 for all comparisons). In contrast, a modest 4.3-μm difference in mean position was detected in Tbr1+/GFP+ neurons between wild-type explants (CM-injected) and mutant explants (CM-injected; P = 0.04) that was partially rescued by RM injection into the mutant explant. The mean position of Tbr1+/GFP+ neurons in the RM-injected explants was shifted 3.2 μm (e.g., approximately one-third the diameter of a nucleus) toward the pia compared with CM-injected mutant explants (Fig. 5f–j, P = 0.09). These analyses suggest that at best, only modest Reelin-dependent L6 neuron movement may accompany SP cell displacement.

Sequence of Cellular Events during Preplate Splitting
To order the sequence of cellular events that coincide with preplate splitting, we compared medially and laterally localized superficial Tbr1+/GFP+ neurons in explants. The neocortex exhibits a TNG (Miyama et al. 1997; Takahashi et al. 1999). This gradient is also manifest by preplate splitting; CP formation and preplate splitting are first apparent laterally on E13, then move medially by E14. Superficial Tbr1+/GFP+ neurons on E13 constitute the forming CP; therefore, comparison of the superficial Tbr1+/GFP+ cells along the medial-to-lateral extent provides an approximate 24-h developmental time-line for prospective L6 neurons. In the most immature (medial) regions of both wild-type explants and Reln−/− explants, Tbr1+/GFP+ neurons were predominantly horizontally oriented with few identifiable neurites (Fig. 6b). In more lateral regions of wild-type sections, in the area of active preplate splitting, Tbr1+/GFP+ neurons showed greater pia-orientation with modest pia-directed neurite outgrowth (Fig. 6c). In the most mature (lateral) areas of wild-type CP, Tbr1+/GFP+ neurons displayed uniform pia-orientation, with clear dendritic outgrowth (Fig. 6d). Thus, observable dendrite formation may coincide with, but does not significantly precede, pia-orientation. These findings suggest that Reelin-signaling stimulates dendrite initiation and cellular orientation in a close temporal sequence.

Reelin Does Not Stimulate Orientation or Dendritogenesis in Immature Cortex
Although RM injection rescued both dendritic growth and orientation within 4 h in mature (lateral) regions, neither endogenous nor exogenous Reelin induced robust dendritic growth or orientation in immature medial regions where preplate splitting had not yet occurred. In this region, prospective L6 neurons could be found within a cell diameter of the Reelin-positive CR cells, yet they showed predominant horizontal orientation and lacked identifiable neurites (Fig. 7b). While neurons in immature medial regions appeared relatively unresponsive to endogenous Reelin, this might be due to insufficient levels of secreted or bioactive Reelin in this area. To explore this, we examined cell orientation and Golgi position in immature medial regions of injected explants. We did find modest but significant differences in Golgi orientation between CM-injected wild type and CM-injected mutant explants (Fig. 7c–g) suggesting basal levels of Reelin-signaling in this region. However, this difference was not rescued by RM injection into mutant explants (Fig. 7f/g). This contrasts with the robust rescue of Golgi and nuclear orientation in lateral regions of the same explants. These findings indicate that Reelin itself is insufficient to trigger the orienting and neurite response associated with preplate splitting and suggest that a developmentally regulated cofactor may be required for the cellular responses that initiate preplate splitting.

Discussion
Cortical neurons are found in ectopic locations in reeler mouse cortex (Caviness and Sidman 1973) and thus the reeler phenotype has been categorized as a neuronal migration disorder. While Reelin-signaling has a critical role in regulating
migration and positioning during later cortical development, we find that Reelin’s first role in early cortical development appears to be largely restricted to orientation and neurite elaboration. During preplate splitting, prospective L6 neurons elongate and orient toward the pial surface and extend F-actin and MAP2-positive neurites, while presumptive SP neurons are passively displaced or actively move below the CP. We find that these events require Reelin-signaling but are probably not exclusively triggered by Reelin during normal development because postmitotic Tbr1+ neurons in immature areas of the preplate are relatively unresponsive to endogeneous Reelin provided by adjacent CR cells or exogenous recombinant Reelin provided by injection. This indicates that an unknown developmentally regulated cofactor for Reelin-signaling may trigger preplate splitting.

It is surprising that we did not detect a difference in L6 neuron position between genotypes as preplate splitting is identified by a forming CP within the preplate of normal embryos and an absence of a distinct CP in the reeler embryos. Several possibilities might account for this observation. First, preplate splitting is often experimentally defined by the clear displacement of SP cells beneath the forming CP rather than by the position of L6 neurons. Second, the CP forming within the preplate is visually identified by tightly aligned neuronal somata, thus the pia-directed alignment of cortical neurons during Reelin-signaling may be critical to the perception of CP. Finally, we note a large increase in the apparent deposition of CSPG in the MZ and SP during the process of preplate splitting, which may contribute the cell-sparse gaps that frame and highlight the developing CP during preplate splitting.

We envision two nonexclusive cellular mechanisms underlying SP cell displacement: increased adhesion between L6 neurons (leading to cell sorting) or possibly active SP cell movement. Decreased neuron–neuron binding is a hypothesized consequence of the reeler mutation (Goffinet and Lyon 1979; Goffinet 1984), and reaggregated clusters of embryonic neurons show a more laminated organization when Reelin-signaling is intact (DeLong and Sidman 1970; Ogawa et al. 1995). Selective cell adhesion has long been hypothesized as driving cell sorting in reaggregated embryos while forced expression of homotypic adhesion molecules in a subset of cells will drive cell sorting in otherwise homogenous populations of cultured cells (Nose et al. 1988). Thus, the partial preplate splitting we observed in RM-rescued explants may be driven by an increase in homotypic L6 neuron adhesion leading to exclusion (sorting) of SP neurons. Active SP neuron motility seems less likely to explain preplate splitting since SP neurons are not known to express components of the Reelin-signaling receptor complex, and they can have extensive neurites which are not typically associated with motile cells.

Although we observed only minor differences between genotypes in the mean position of Tbr1+/GFP+ neurons before and during preplate splitting, large differences in L6 neuron position emerge in later cortical development as the cortical inversion develops in reeler mutants. The specific cellular events leading to the inversion are not known in detail but may be caused by a traffic jam of migrating neurons (Pinto Lord et al. 1982) that accretes subsequent to the failure of L6 differentiation and preplate splitting. As such the cellular inversion may not be directly informative as to the primary cellular function of Reelin-signaling.

In immature areas of cortex, we found that Reelin alone is insufficient to elicit the orienting and dendritic responses. Although we cannot exclude L6 neuronal competence as the
Dendritic and cell orientation abnormalities are a well-characterized feature of neurons within the reeler cortex (Goffinet and Lyon 1979; Pinto Lord and Caviness 1979). Although some of these abnormalities in vivo may be secondary to the general histological disruption of the reeler cortex, more recent work has shown a direct role for Reelin-signaling in dendritic growth and branching both in vitro and in vivo (Niu et al. 2004; Olson et al. 2006; Jossin and Goffinet 2007; Matsuki et al. 2008; Niu et al. 2008). While Reelin application increased dendritic growth and branching of hippocampal neurons in vitro, the effect was relatively slow (discerned at 4 days after plating) and can be modest (MacLaurin et al. 2007). This seemingly contrasts with our findings of dramatically enhanced dendritic growth and branching of hippocampal neurons in vitro, the effect was relatively slow (discerned at 4 days after plating) and can be modest (MacLaurin et al. 2007). This finding argues that Reelin exposure by itself is insufficient to stimulate significant morphological differentiation in all neurons, thus other factors that work in concert with Reelin may be critical. If these cofactors were found at reduced levels in vitro and this would account for the apparent differences in potency of Reelin-signaling.

Although it is generally believed that dendritic growth occurs after the cessation of migration, some studies indicate that the nascent dendrite might emerge through direct transformation of the leading process of the migrating neuron (Pinto Lord et al. 1982; Olson et al. 2006). The genetics of neuronal migration disorders have suggested an interrelationship between dendritic growth and cell positioning. Cell autonomous interference with Reelin-signaling by Dab1 suppression caused layer 2/3 neurons to be malpositioned ~40-μm deep in the CP and later resulted in impaired dendritic growth and branching in the MZ (Olson et al. 2006). Similarly, cell autonomous interference with the serine-threonine kinase cdk5 caused migration and dendritic developmental errors (Oshshima et al. 2007). Conversely, mice deficient in MAP1b (Gonzalez-Billault et al. 2005) or doubly deficient for MAP1b and MAP2 showed neuronal positioning deficits in addition to dendritic growth abnormalities (Teng et al. 2001). The relationship between dendritogenesis and cell layer formation is underscored by the findings presented in this study as we observed L6 neurons rapidly extending dendrites into the MZ (future layer 1). Layer 1 is not the target layer for L6 dendrites; consequently, these neurites will be remodeled and withdrawn from layer 1 during later development. Thus, appropriate cell orientation and dendritogenesis may be required for appropriate cortical layer formation rather than vice versa.

**Figure 8.** Model of Reelin’s permissive role in neuronal orientation, differentiation, and preplate splitting. (a, d) Prior to preplate splitting, L6 neurons are equivalently positioned in mutant (–/–) and wild-type (+/+). L6 neurons are horizontally oriented and lack definitive neurites independent of the presence or absence of Reelin. (b, e) Developmentally regulated cofactors trigger pia-orientation and neurite initiation of L6 neurons but only in the presence of Reelin (+/+). (c, f) Immediately after preplate splitting has occurred, L6 neurons remain equivalently positioned in wild-type cortex and mutant cortex, but recognizable CP has formed only in the wild-type cortex. In contrast, L6 neurons remain intermingled with preplate cells in the mutant and are primarily horizontally oriented with stunted dendrites. Later born cohorts of migrating neurons (not shown) migrate past L6 neurons in wild-type cortex, but fail to do so in the mutant cortex, creating the inversion of cortical layering characteristic of the mutant phenotype.

**Funding**

Department of Neuroscience and Physiology, the Hendrick’s Foundation, and a grant from the NINDS (NS066071 to E.C.O.). A Departmental Graduate Fellowship supported A.J.N.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Critical prerequisite of the response, we found that many of the nonresponsive cells in the immature medial area are strongly immunopositive for Tbr1 and BrdU (E12-labeling) suggesting equal maturity with Reelin-responsive neurons in more mature (lateral) areas of rescued explants. Thus, a developmentally regulated cue rather than the maturity of L6 neurons may be the cofactor responsible for triggering orientation, dendritogenesis, and preplate splitting. In this model, Reelin functions permissively, consistently with prior studies in which ectopic expression of Reelin or bath-applied recombinant Reelin rescued preplate splitting (Magdaleno et al. 2002; Jossin et al. 2003). This model is further supported by the observation that in 2 of 3 RM-rescued explants, we were able to immunohistochemically detect the injected recombinant Reelin. Injected Reelin was detected relatively uniformly across the cerebral wall (Supplementary Fig. 3), yet rescued neurons showed a pia-directed orientation and neurite response. This finding argues that Reelin itself is not a directional cue for orientation and dendritogenesis. In aggregate, these observations outline a model in which Reelin is constitutively present and diffuse in wild-type cortex and in which preplate splitting is triggered by limiting and spatially localized cofactors for neuronal orientation and dendritogenesis (Fig. 8). This model predicts that exogenous Reelin will only stimulate rapid rescue in those areas where the hypothetical cofactors have exceeded a critical threshold.
Notes
We thank Rick Matthews, Anjen Chenn, Dave Cameron, Chris Turner, and Brian Howell for comments on the project and the manuscript. We thank Michael Frotscher (University of Freiburg) for the stable cell line expressing Reelin. Conflict of Interest: None declared.

References


