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Massive loss of Cajal-Retzius cells does not disrupt neocortical layer order

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Cajal-Retzius (CR) cells, the predominant source of reelin in developing neocortex, are thought to be essential for the inside out formation of neocortical layers. Fate mapping revealed that a large population of neocortical CR cells arises from the cortical hem. To investigate the function of CR cells, we therefore genetically ablated the hem. Neocortical CR cells were distributed beneath the pial surface in control mice, but were virtually absent in hem-ablated mice from embryonic day (E) 10.5 until birth. CR cells derived from other sources did not invade the neocortical primordium to compensate for hem loss. We predicted that neocortical layers would be inverted in hem-ablated animals, as in *reeler* mice, deficient in reelin signaling. Against expectation, layers showed the standard order. Low levels of reelin in the cortical primordium, or diffusion of reelin from other sites, may have allowed lamination to proceed. Our findings indicate, however, that the sheet of reelin-rich CR cells that covers the neocortical primordium is not required to direct layer order.

KEY WORDS: Mouse, Cajal-Retzius cells, Neocortex

INTRODUCTION

The normal inside-out formation of neocortical layers (Angevine and Sidman, 1961; Rakic, 1974) requires the secreted glycoprotein reelin (D'Arcangelo et al., 1995; Ogawa et al., 1995; Rice and Curran, 2001). In *reeler* mice, which lack functional reelin, neocortical layers are inverted and cells are abnormally dispersed (Caviness, 1982; Caviness and Sidman, 1973; D'Arcangelo et al., 1995; Goffinet, 1984). Similar defects are seen in mice lacking the reelin receptors very-low-density lipoprotein receptor (Vldlr) and apolipoprotein E receptor 2 (Apoer2; Lrp8 – Mouse Genome Informatics) (Hiesberger et al., 1999; Trommsdorff et al., 1999), or the downstream intracellular adaptor protein Dab1 (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997). Targets of reelin signaling, expressing both receptors and Dab1, include migrating neurons themselves (Perez-Garcia et al., 2004) and the radial glial cells (Hartfuss et al., 2003; Luque et al., 2003), which serve both as guides for migration and as neuronal progenitor cells (Noctor et al., 2001).

A core question remains unanswered: how does reelin influence migrating neocortical neurons so that they settle in distinct layers from deep to superficial? The functions proposed for reelin include: instructing migrating neurons to leave their glial guides and stop migration, forming a graded signal from the MZ that positions neuronal cell bodies in the cortical plate (CP) (Luque et al., 2003; Rice and Curran, 2001); or simply allowing neurons to pass other cells that have settled along their migratory route (Caviness, 1982; Pinto-Lord et al., 1982). One way to test these and other diverse ideas is to determine where the supply of reelin needs to be with respect to migrating neocortical neurons. If reelin is a guidance or positional signal, for example, then sources are likely to be required at specific sites. If reelin is a permissive or enabling signal, the precise source may not matter.

Since the identification of reelin, and the observation that it is robustly produced by Cajal-Retzius (CR) cells in the marginal zone (MZ) of the cortical primordium (D'Arcangelo et al., 1995; Meyer et al., 1999; Ogawa et al., 1995), CR neurons have been thought to be essential for laminar organization (Bielas et al., 2004; Rice and Curran, 2001). CR cells are strategically located, close to the pial end-feet of radial glia, at the end of the radial migratory pathway of neocortical pyramidal cells (Alcantara et al., 1998; Hartfuss et al., 2003; Luque et al., 2003). Reelin can signal to both radial glial cells and their daughter neurons (Luque et al., 2003; Magdaleno et al., 2002), potentially providing guidance for neurons throughout migration.

Much indirect evidence further supports a central role for CR cells in establishing neocortical layer pattern. For example, the neurotrophin BDNF regulates reelin in CR cells. BDNF overexpression reduces reelin in CR cells, and neocortical abnormalities include laminar defects that resemble those in the *reeler* mouse (Ringstedt et al., 1998). However, low levels of reelin mRNA (*Reln*) are expressed elsewhere in the cortical primordium (Alcantara et al., 1998; Meyer et al., 2002), and because most manipulations of CR cells are also general manipulations of reelin signaling, there is little direct evidence that CR cells themselves are absolutely required. Local application of a toxic agent to newborn mouse cortex ablates CR cells and disrupts cell migration to layers II/III (Super et al., 2000), but this approach might affect other cell types that regulate migration. In particular, meningeal cells maintain trophic interactions with CR cells, and themselves contain neuronal guidance molecules (Halfter et al., 2002; Hartmann et al., 1992; Lu et al., 2002; Super et al., 1997).

New evidence indicates that some steps in cortical histogenesis can take place without a reelin signal from CR cells in the MZ. In wild type, but not *reeler* mice, neurons form the CP by invading the preplate, separating its two main components, the MZ and subplate (Caviness, 1982; Luskin and Shatz, 1985; Stewart and Pearlman, 1987). In vitro, a specific fragment of reelin, applied in culture to slices from *reeler* telencephalon, provides a partial rescue of preplate

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partition, permitting the formation of a near-normal CP (Jossin et al., 2004). Furthermore, in *reeler* mice, forced reelin expression under the control of the nestin promotor (*nestin*-reelin), fully rescues this component of the *reeler* phenotype (Magdaleno et al., 2002).

Driven by the nestin promotor, reelin is expressed in the ventricular zone (VZ), but presumably also throughout the radial glial progenitor cells that express nestin and span the cortical primordium. Radial glial expression of reelin is not sufficient, however, to rescue normal layer patterning of the CP. Higher levels of reelin, delivered from the endogenous CR cell source, would seem to be required (Magdaleno et al., 2002).

Further studies, however, question even this requirement for CR cells (Meyer et al., 2004; Meyer et al., 2002; Yang et al., 2000). CR cells express p73 (Trp73 – Mouse Genome Informatics), a protein with isoforms that differentially affect apoptosis (Meyer et al., 2004; Pozniak et al., 2002; Yang et al., 2000). In p73-deficient mice, CR cells die, but neocortical layering is not inverted (Meyer et al., 2004; Yang et al., 2000). Perhaps, however, CR cells initiate normal lamination before their early death by apoptosis. CR cells are missing in *p73* mutants at E12.5 (Meyer et al., 2004), but are already present in the MZ by E10.5 (Alcantara et al., 1998). In the present study, we determined directly if CR cells in the neocortical MZ are the crucial source of reelin for neocortical layer patterning. To do so, we took a genetic approach to delete the progenitors of neocortical CR cells.

MATERIALS AND METHODS

Animals

Mice used were R26R reporter mice, *reeler* mutant mice (both from The Jackson Laboratory), an *Emx1-IRES-Cre* mouse line (Gorski et al., 2002), an *Otx1-Cre* mouse line (gift of Philippe Soriano, Fred Hutchinson Cancer Research Center, and Flora Vaccarino, Yale), and the two mouse lines generated for this study: *Wnt3a-IRES-Cre* and *Wnt3a-IRES-xneox-dt-a*. All mice were bred and maintained in compliance with National Institutes of Health guidelines. The University of Chicago IACUC approved the animal protocols used in this study.

Generation of mice to fate map the hem

In the telencephalon, the *Wingless-Int* gene *Wnt3a* is expressed only in the cortical hem, and is not expressed after birth in other cell types (Lee et al., 2000b; Shimogori et al., 2004). Consequently, the *Wnt3a* locus is ideal for genetic fate mapping and ablation of the hem. To fate map the hem *Cre recombinase* was inserted into the 3′ end of the *Wnt3a* locus, using standard homologous recombination in ES cells. An IRES preceding *Cre* permitted expression of both Wnt3a and Cre proteins from the same mRNA, and no disruption of Wnt3a activity was detected. That is, mice heterozygous or homozygous for the *Wnt3a-IRES-Cre* allele showed none of the striking phenotypic features of Wnt3a loss of function (Takada et al., 1994). We did not even detect a sensitive indicator of decreased canonical Wnt signaling from the hem, namely, a shrunken hippocampal dentate gyrus (Galceran et al., 2000; Zhou et al., 2004).

Cell fate mapping

Wnt3a-IRES-Cre mice were crossed with R26R mice. Offspring were genotyped and mice heterozygous or homozygous for both the *R26R* and *Wnt3a-IRES-Cre* alleles were processed further. An overview of cell types generated from the hem was obtained by staining whole-mount brains and sections for β-gal, using immunohistochemistry or X-gal histochemistry. Cell type was ascertained with antibodies against GFAP, Olig2, NeuN, reelin, GABA and p73. Mice were assessed at a range of ages from E10 to P30.

Genetic ablation of the hem

To ablate the cortical hem, a cassette (gift of Kevin Lee and Tom Jessell, Columbia) carrying an IRES, followed by stop codons flanked by loxP sites, followed by a cDNA encoding the diptheria toxin subunit A (dt-a) (Lee et al., 2000a), was inserted into the 3′ end of the *Wnt3a* locus, again using standard ES cell technology (Fig. 3A). Mice carrying one such allele were indistinguishable from wild-type mice, given that activation of the toxin was prevented by the stop codons preceding *dt-a* (Lee et al., 2000a). Dt-a was activated in the cortical hem by crossing mice carrying the *Wnt3a-IRES-xneox-dt-a* allele with an *Emx1-IRES-Cre* mouse line (Gorski et al., 2002). In mice heterozygous for both alleles, Cre-mediated recombination occurred selectively in cells that had once expressed both *Emx1* and *Wnt3a*. In the telencephalon, this was restricted to the cortical hem. Because the dt-a subunit is not transferred from cell to cell (Lee et al., 2000a), only the hem was directly ablated. Control brains, which were of the $Wnt3a^{xneoxdt\text{-}a/+}$; $Emx1^{+/+}$ genotype, showed an entirely normal hem. Recombination did occur elsewhere in the body of double heterozygotes, however, leading to a short lower jaw and an inability of the mouse pups to suckle. This recombination appeared to be due to an overlap of *Emx1* and *Wnt3a* expression in neural crest cells headed for the jaw. Hem-ablated mice consequently died shortly after birth. Unfortunately, this precluded a systematic birth-dating study of neocortical layers, given that many neurons will reach their final layer after birth.

Assessing hem loss and its effects

In situ hybridization (Grove et al., 1998) and immunohistochemistry were used to detect a panel of molecular markers of the hem, CR cells and other tissues. The hem expresses both *Wnt3a* and *Wnt2b* selectively (Grove et al., 1998). The transcription factor gene *Lmx1a* is expressed along the dorsal midline of most of the neuraxis, including the hem, and *Wnt8b* is expressed in the hippocampal primordium, hem and choroid plexus (Failli et al., 2002; Lee et al., 2000b). Hem-ablated mice were assessed with in situ hybridization and immunohistochemistry for the presence of reelin- and p73-positive cells (E10.5, E11.5, E12.5, E13.5, E15.5 and E18.5), preplate splitting (E15.5), and laminar organization (E18.5). At least six mutant mice were analyzed at each timepoint. The preplate, MZ and subplate were identified with immunoreactivity for calretinin and chondroitin sulfate proteoglycans (CSPGs). Developing layers in neocortex were identified by the laminar-specific expression patterns of a panel of genes as detailed below.

Testing the meninges covering the neocortical primordium

CR cells are highly sensitive to defects in the overlying meninges, and each provides trophic support for the other (Super et al., 2000). Loss of the meninges covering the neocortical primordium could be a plausible additional cause of loss of CR cells. The meninges were therefore compared between hem-ablated and control mice at a range of ages, using morphology and gene expression of IGF2 and MF-1, a forkhead/winged helix transcription factor, both expressed in meninges (Kume et al., 1998). Using these methods, the meninges in the hem-ablated mice were found to be similar to controls.

Antibodies

To analyze cell and tissue type, the following antibodies were used: rabbit anti-β-galactosidase, ICN (Cappel); mouse anti-NeuN (Chemicon, clone MAB377); mouse anti-reelin (Calbiochem, clone G10); mouse anti-p73 (Neomarker, clone ER-15); rabbit anti-calretinin (Chemicon); mouse anti-CSPG (Sigma, clone CS-56); rabbit anti-GABA (Sigma); and rabbit anti-Olig-2 (gift of David Rowitch, Harvard). Secondary antibodies were from Molecular Probes.

RESULTS

Fate mapping the cortical hem

Previous gene expression or dye-labeling studies indicate the hem as a source of CR cells (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004). To confirm and extend these findings with a direct cell lineage approach, we used the Cre-loxP recombination system, generating a *Wnt3a-IRES-Cre* mouse line in which *Cre recombinase* (*Cre*) was inserted into the *Wnt3a* locus (see Materials and methods; Fig. 1A). In these mice, *Cre* expression in the hem mimicked expression of endogenous *Wnt3a* (Fig. 1B,C). To label *Wnt3a*-expressing hem cells and their progeny permanently, the *Wnt3a-IRES-Cre* line was

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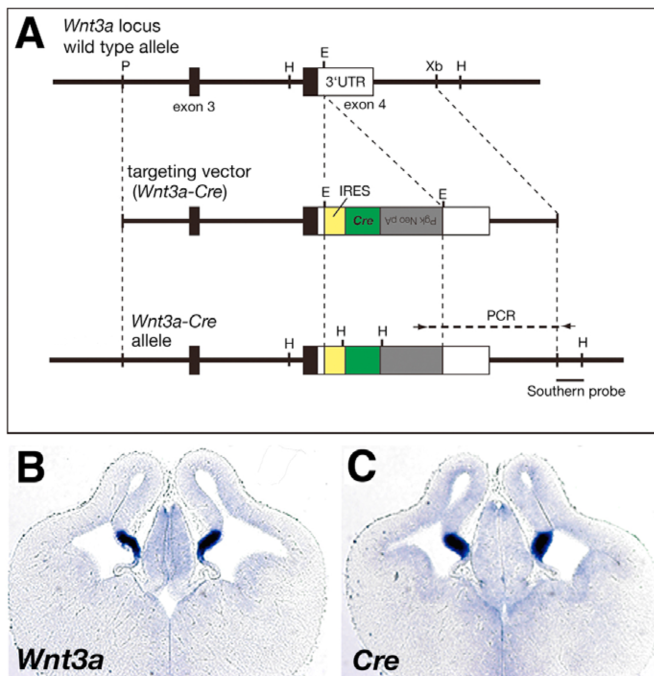

Fig. 1. Constructing *Wnt3a-IRES-Cre* mouse lines to fate map the hem. (**A**, top to bottom) Wild-type allele of *Wnt3a*; the targeting vector containing an IRES-Cre cassette; the targeted *Wnt3a-IRES-Cre* allele with Cre inserted into the 3′ end of the *Wnt3a* locus. (**B**,**C**) Coronal sections through E12.5 forebrains show *Cre* expression in the hem of *Wnt3a-IRES-Cre* mice (C), indistinguishable from expression of endogenous *Wnt3a* (B).

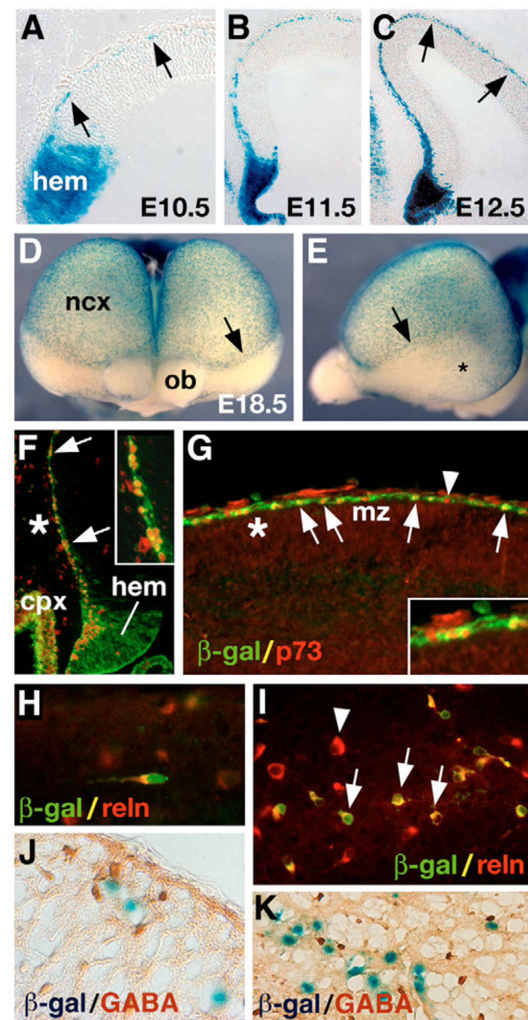

Fig. 2. Cells derived from the hem fit the criteria of Cajal-Retzius cells. (**A-C**) From E10.5-12.5, hem-derived β-gal-labeled cells (arrows) migrate into the MZ. (**D**,**E**) E18.5 cortical hemispheres in rostral (D) and lateral (E) views. β-Gal-expressing cells cover the neocortex with rough boundaries at the olfactory bulbs and future rhinal sulcus (arrows). Caudally, some cells migrate below the latter boundary (asterisk). (**F**,**G**) p73 immunofluorescence in cells of the hem, the cpx and the MZ of the hippocampal (F) and neocortical primordia (G). Asterisks in F,G indicate regions also shown at higher magnification (insets). Many cells are double-labeled with β-gal, indicating their origin in the hem (arrows, and higher magnification insets). A few were positive only for p73 (for example, see arrowhead in G). (**H**,**I**) Hem-derived, β-gal+ cells in the neocortex (H) and hippocampus (I) were reelin-immunoreactive (arrows), although some reelin-positive cells were single-labeled (arrowhead in I). (**J**,**K**) Hem-derived cells were not immunoreactive for GABA. cpx, choroid plexus; ncx, neocortex; ob, olfactory bulb; reln, reelin.

crossed with R26R reporter mice (Soriano, 1999), in which cells express β-galactosidase (β-gal) following Cre-mediated recombination. Because recombination in the telencephalon was confined to the *Wnt3a*-expressing hem, both spatially and temporally (see Materials and methods), cells labeled with β-gal could be taken as derivatives of the hem.

In R26R mice homozygous for the *Wnt3a*-Cre allele, recombination in the hem began at about E10. Between E10.5 and E12.5, β-gal-expressing cells filled the hem (Fig. 2A), and migrated laterally to form an increasingly dense layer close to the pial surface of the cortical primordium, where CR cells are located (Fig. 2B,C). At E18.5, MZ cells derived from the hem covered the neocortex (Fig. 2D,E). There were distinct but permeable boundaries of migration (Fig. 2D,E, arrows); these did not reflect a complete repulsion of hem-derived cells (Fig. 2E, asterisk). Rather, β-gal-expressing cells predominated in neocortex and hippocampus, tending not to migrate into the olfactory bulb or below a ventrolateral line, which is likely to mark the future rhinal sulcus: the border of olfactory cortex. These observations fit well with a recently reported complementary migration of CR cells to olfactory and rostromedial cortex from separate sites near the septum and the dorsal/ventral telencephalic boundary (Bielle et al., 2005).

The cortical hem generates CR cells in the neocortical MZ

The MZ contains a variety of cell types (Jimenez et al., 2003). However, CR cells are distinguished by their expression of reelin, calretinin and glutamate (Alcantara et al., 1998; del Rio et al., 1995; Meyer et al., 1999; Ogawa et al., 1995; Soda et al., 2003). A lack of GABA discriminates between CR cells and late-arriving interneurons that express reelin (Alcantara et al., 1998; Hevner et al., 2003b; Meyer et al., 1999; Soda et al., 2003). Finally, CR cells, but not interneurons, express p73, which may be the most specific single marker of CR cells in the MZ (Meyer et al., 2004; Meyer et al., 2002). In R26R mice carrying a *Wnt3a*-Cre allele, almost all β-gal-positive cells in the embryonic MZ or in layer 1

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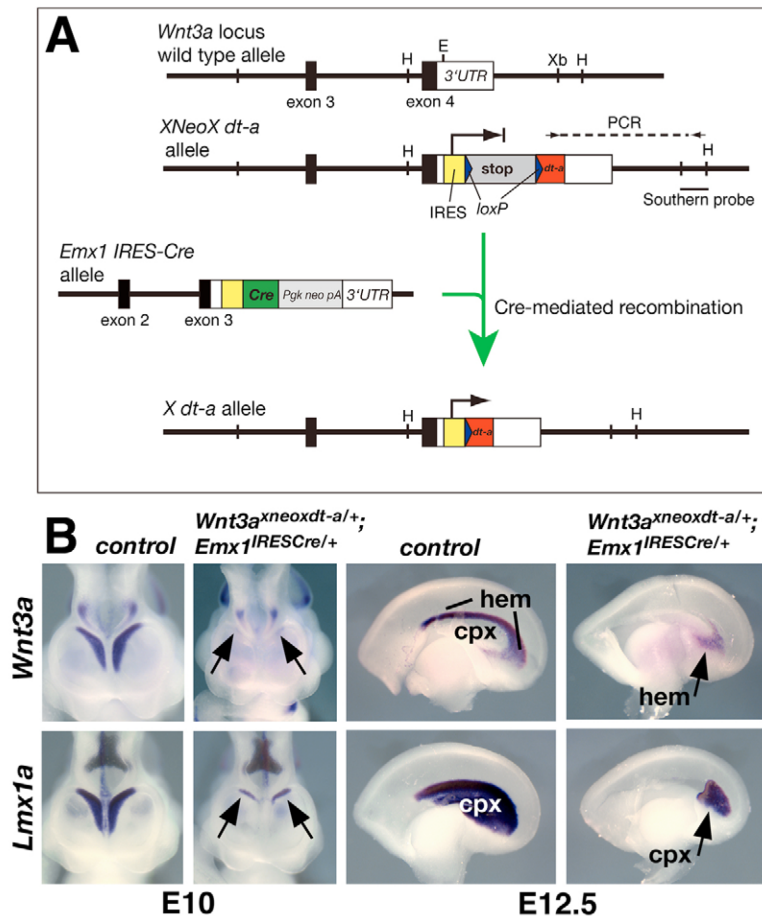

Fig. 3. Constructing a *Wnt3a-IRES-xneox-dt-a* mouse line to ablate the hem. (**A**, top to bottom) Wild-type *Wnt3a* allele; *Wnt3a-IRES-xneox-dt-a* allele; activation of dt-a toxin by Cre-recombination, using an *Emx1-IRES-Cre* mouse line (Gorski et al., 2002). (**B**) Recombination in the telencephalon was confined to the hem, which was almost completely ablated by E10 in Wnt3a$^{IRESxneoxdt\text{-}a/+}$;Emx1$^{IRESCre/+}$ mice (dorsal views). *Wnt3a* expression marks the hem at E12.5 (medial view) but not in a hem-ablated brain (arrow indicates caudal hem remnant). Expression of *Lmx1a* marks the hem and cpx; a part of the cpx (arrow) remains in the hem-ablated mouse.

of postnatal cerebral cortex expressed p73 and reelin, but not GABA (Fig. 2F-K), indicating that hem-derived MZ cells are CR cells.

We hoped to determine the fraction of neocortical CR cells that derive from the hem as the percentage of reelin- or p73-positive cells that co-express β-gal. The proportion of double-labeled cells will vary, however, according to levels of Cre recombinase and the time of effective recombination. Thus, in R26R mice, homozygous or heterozygous for *Wnt3a-IRES-Cre*, recombination in the hem began, respectively, at E10 or E11.

In mice of both genotypes, we determined the percentage of reelin- or p73-positive cells that co-express β-gal in medial cortex, adjacent to the hem (including the hippocampal primordium), and in dorsolateral neocortex. At E12.5, in heterozygotes, about 60% and 30% of p73-positive cells in the medial and dorsolateral cortex, respectively, were also β-gal-positive. In homozygotes, in which Cre recombinase is expressed earlier, and presumably at higher levels, these percentages were 90% and 60% (Fig. 2F,G). In both sets of brains, the proportion of hem-derived CR cells was higher in medial cortex, closer to the source. Moreover, the proportion rose at both medial and lateral sites as development proceeded (counts made at E12.5, E15.5 and E18.5), suggesting an ongoing lateral migration from the hem.

Direct cell lineage tracing therefore validates previous suggestions that the hem is a source of CR cells (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004). Most neocortical CR cells are hem derived. Nonetheless, we found that some reelin-expressing cells in the MZ of both dorsolateral neocortex and hippocampus were not β-gal positive. These might represent CR cells born before Cre recombination, or cells generated outside the hem (Bielle et al., 2005). To determine more accurately the proportion of CR cells derived from the hem, and to explore the function of this cell population, we ablated the hem genetically.

Genetic ablation of the hem

To ablate the hem, cDNA encoding the diptheria toxin, subunit A (dt-a) was inserted into the *Wnt3a* locus (see Materials and methods and Fig. 3A). Stop codons flanked by loxP sites preceded *dt-a* (Fig. 3A) (Lee et al., 2000a), preventing activation of the toxin in mice carrying the *Wnt3a-xneox-dt-a* allele. To remove the stop codons and activate the toxin, *Wnt3a-xneox-dt-a* mice were crossed with an *Emx1-IRES-Cre* line (Gorski et al., 2002) that directs Cre recombination in the dorsal telencephalon. In mice carrying both alleles, dt-a was activated in cells that had co-expressed *Emx1* and *Wnt3a*, i.e. in the telencephalon, only in the cortical hem.

Cre-mediated recombination directed by the *Emx1-IRES-Cre* mouse begins at E9.5. At E10, almost complete hem ablation was evident by the loss of characteristic hem expression of *Wnt3a* as well as by tissue loss (Fig. 3B, left; data not shown). *Wnt8b* and *Lmx1a* are expressed in the hem as well as adjacent tissues (Failli et al., 2002; Lee et al., 2000b); their expression was reduced appropriately (Fig. 3B, left; data not shown). At E12.5, *Wnt3a* and *2b* expression were missing, except in a small caudal remnant of the hem (Fig. 3B, right; data not shown), correlating with a region of comparatively weak Cre recombination (Gorski et al., 2002). By E12.5, other derivatives of the hem, such as the choroid plexus (cpx) were also largely missing, although a region of the cpx, probably derived from the caudalmost hem, was still evident by *Lmx1a* expression (Fig. 3B, right).

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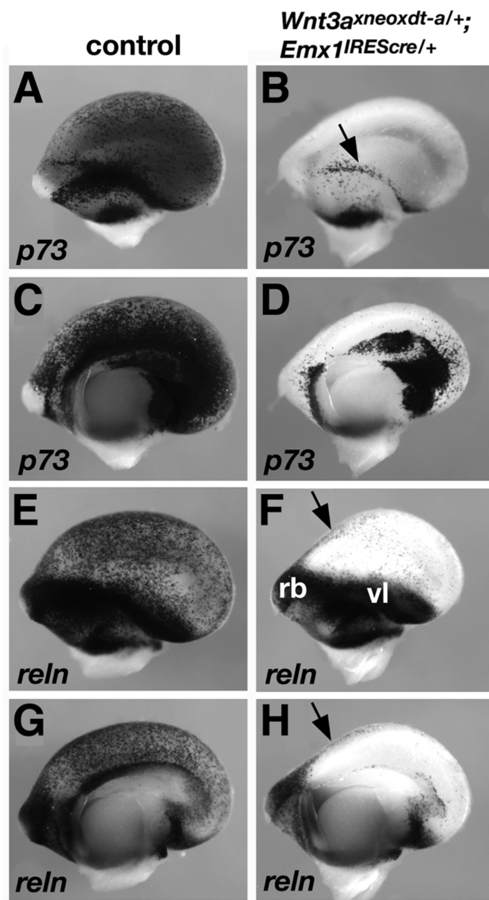

Fig. 4. Ablation of the hem leads to loss of CR cells. (**A-H**) E12.5 forebrains processed with in situ hybridization to show expression of reelin (*Reln*) or *p73* in control (A,C,E,G) or hem-ablated (B,D,F,H) mice. Hemispheres are viewed from the lateral (A,B,E,F) or medial face (C,D,G,H). *p73*- and *Reln*-expressing cells cover the cerebral cortex of controls, but are almost absent from medial cortex and lateral neocortex in hem-ablated animals. Ventrolateral (vl) and retrobulbar (rb) expression of *Reln* is more striking in the absence of neocortical *Reln*. Cells appear to stray from the rb into dorsomedial cortex (arrows, F,H). A lateral line of *p73* expression in hem-ablated cortex (arrow, B) roughly marks the boundary of major CR cell migration from the hem.

Loss of CR cells in the neocortical MZ

At E12.5, in control mice, the entire cerebral cortex was covered with *Reln*-expressing cells, with *p73* expressed in all but the olfactory bulb (Fig. 4A,C,E,G). By contrast, in double heterozygotes (*Wnt3a*$^{\text{xneoxdt-a/+}}$;*Emx1*$^{\text{IREScre/+}}$), *p73*- and *Reln*-expressing cells were virtually absent from the neocortical MZ (Fig. 4B,D,F,H). In sections through control E13.5 and E15.5 forebrains, *Reln*-expressing CR cells formed a dense band in the MZ (Fig. 5G,H; Fig. 6A). In hem-ablated mice this band of CR cells was missing, as assessed by *Reln* and *p73* expression (Fig. 5J,K; Fig. 6B), or immunoreactivity for reelin and p73 protein. At E18.5, few *Reln*-positive cells of any kind were seen in the neocortical MZ in the mutants (Fig. 6D,E), indicating that interneurons that express *Reln* had not yet migrated in bulk into the cortex (Alcantara et al., 1998; Hevner et al., 2003b; Soda et al., 2003). CR cells also show gene expression of stromal-derived factor (SDF1) and its receptor, CXCR4 (Lu et al., 2002; Tissir et al., 2004). Gene expression of both was lost in the MZ of hem-ablated mice. These observations strongly support the hem as being the origin of the great majority of neocortical CR cells.

Titration of CR cell number with partial hem ablation

If the hem provides the majority of neocortical CR cells, then shrinking the hem should lead to a significant loss of CR cells. To test this, *Wnt3a-IRES-xneox-dt-a* mice were crossed with an *Otx1-Cre* mouse line. When the latter was crossed with R26R mice, recombination occurred in a pepper-and-salt fashion in the hem. Accordingly, in mice heterozygous for *Wnt3a-IRES-xneox-dt-a* and *Otx1-Cre*, the hem was present, but smaller than normal. Correlating with the shrunken hem, many fewer CR cells appeared in the neocortical MZ, and these were dispersed, rather than forming a closely packed cell layer (data not shown).

Prominence of other sources of migratory reelin-positive cells

As noted above, additional sources of migratory, *Reln*-expressing cells have been reported in the ventral telencephalon, and a 'retrobulbar' zone near the olfactory bulb (Gong et al., 2003; Lavdas et al., 1999; Meyer et al., 1998; Meyer and Wahle, 1999). Genetic techniques have recently clarified the location and contribution of these sources. CR cells that migrate into olfactory and rostromedial cortex arise from *Dbx1*-expressing progenitor cells in or near the septum – perhaps equivalent to the retrobulbar zone – and at the boundary between dorsal and ventral telencephalon (Bielle et al., 2005).

Both zones stood out in the absence of hem-derived CR cells in the neocortex. *Reln* was densely expressed ventral to neocortex, and surrounding the developing olfactory bulb (Fig. 4F,H). From both regions, at E12.5, some *Reln*-expressing cells (Fig. 4F,H) appeared to move into the CR cell-depleted neocortex. A possibility, therefore, is that other sources supplied CR cells to the hem-ablated neocortex, at least during some periods of embryonic development.

To test this possibility, we examined hem-ablated embryos harvested on E10, E11.5, E12.5, E13.5, E15.5 and E18.5, the day before birth. *Reln*-expressing cells, probably derived from the septal/retrobulbar region, or the ventral/dorsal telencephalic boundary, were detected in rostromedial and caudomedial cortex at E13.5 (Fig. 5B,I,J), but not at E15.5 or later (Fig. 5K). Moreover, no *p73* was expressed in medial cortex (Fig. 5B,C), suggesting that these transient cells were not typical CR cells. A few *Reln*-positive cells also appeared to scatter into the lateral neocortical MZ from the dorsal-ventral telencephalic boundary (Fig. 5D), but these remained very sparse compared with *Reln*-expressing CR cells in the same region of control neocortex (Fig. 5E). In brains sectioned at E12.5 to E15.5, no more than one or two *Reln*-expressing cells per 40 μm section appeared in lateral neocortex (Figs 5, 6; see Fig. S1 in the supplementary material). The neocortical cells originating early from the septal/retrobulbar area or the ventrodorsal telencephalic boundary may be transient in wild-type neocortex too; alternatively, hem-ablated neocortex may be an inhospitable environment for the migration or survival of these cells. We concluded that in mice lacking a cortical hem, the neocortical MZ, from the onset of corticogenesis to birth, contains few or no cells, either defined CR cells or other cell types, that strongly express *Reln*.

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Low levels of reelin expression in the cortical primordium

In both hem-ablated and control mice, a faint band of *Reln* expression could be detected deep in the cortical primordium from about E12.5 (Fig. 5G) to at least E18.5. At E13.5, *Reln* expression appeared just beneath the pial surface. At E15.5 *Reln* expression was distinguishable in the lower intermediate zone (IZ), and/or subventricular zone (SVZ) (Fig. 6C). By E18.5 individual *Reln*-expressing cells could be resolved (Fig. 6D-F), and comparison with layer-specific gene expression indicated that these cells mostly overlapped expression of *Rorb* (the gene encoding RORβ) (Fig. 6D-F), which encodes a transcription factor found in cells of developing layers IV and V (Hevner et al., 2003a). Perinatal expression of *Reln* has been reported previously in layer V of the mouse (Alcantara et al., 1998), as has faint expression at earlier embryonic ages (Meyer et al., 2004; Meyer et al., 2002). We could not detect reelin protein with immunohistochemistry in the CP or IZ/SVZ, probably because of low protein levels, or reelin diffusion. Indeed, the levels of *Reln* mRNA were so low at earlier embryonic ages that they were not detected in every section (compare Fig. 5G with 5H,J,K).

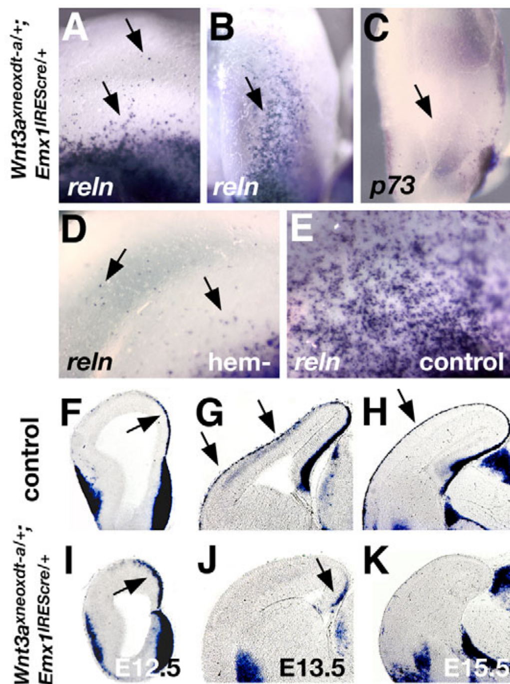

Fig. 5. Other sources provide only transient or sparse *Reln*+ cells to the hem-ablated neocortex. (**A-E**) E13.5 cerebral hemispheres from control (E) or hem-ablated (A-D) mice, processed with in situ hybridization to show *Reln* or *p73* expression. (A,D,E) Lateral views of hemispheres, rostral to the left; (B,C) dorsal views, rostral is downwards. (A-E) In hem-ablated cortex, ventral (A) and rostral (B) sources appear to provide *Reln*+ cells (arrows in A,B) to the neocortex. Dorsal cells that may originate from the rostral source do not express *p73* (arrow in C), and thus do not fit current criteria for CR cells. In a control (E), *Reln*+ CR cells cover lateral neocortex; in a hem-ablated mouse, the ventral source provides few *Reln*+ cells (arrows in D). (**F-K**) Coronal sections through E12.5, E13.5 and E15.5 hemispheres, medial towards the right, processed to show *Reln* expression (arrows). Evidence for a rostromedial source of *Reln*+ neurons (arrows) in sections at E12.5 in both control (F) and hem-ablated brains (I). In the latter, a small domain of *Reln*+ cells remains in medial cortex at E13.5 (arrow, J), but not at E15.5 (K).

Partition of the preplate in hem-ablated mice

In the absence of almost all intensely *Reln*-positive CR cells in the MZ, we expected a neocortical phenotype similar to that seen in the *reeler* mutant mouse. In *reeler* mice, neurons do not invade the preplate, splitting it into the MZ and subplate. Instead, the cortical plate forms beneath the subplate, which becomes a ‘superplate’ (Caviness, 1982). As the cortical plate expands, it develops an inverted layer pattern (Caviness, 1976; Caviness, 1982; Goffinet, 1984; Pinto-Lord et al., 1982).

In both hem-ablated brains and controls, at E13.5, the preplate was immunoreactive for the Ca^{2+}-binding protein, calretinin, and chondroitin sulfate proteoglycans (CSPGs) (Magdaleno et al., 2002; Sheppard and Pearlman, 1997) (Fig. 7A,C). By E15.5 in control mice, the preplate had split. An upper band of calretinin or CSPG immunoreactivity marked the CR cells of the MZ and a second lower band marked the subplate (Fig. 7B,D). In hem-ablated animals, as in controls, a cortical plate formed above a subplate. The lower band of immunoreactivity, the subplate, was therefore visible. As would be expected, the upper band, normally containing CR cells, was not (Fig. 7B,D).

Preplate partition can be rescued in the *reeler* mutant by forced expression of reelin in the VZ (Magdaleno et al., 2002). We speculated that the preplate splits in hem-ablated mice because of low levels of reelin in the IZ/SVZ. Forced expression of reelin in the VZ does not, however, rescue the normal layer pattern of the neocortex (Magdaleno et al., 2002). We therefore predicted that hem-ablated mice would show an inverted layer pattern, as in the *reeler* mouse, and/or extensive cell dispersion, as in the *reeler*+nestin-reelin mouse.

Neocortical lamination in hem-ablated mice

Laminar ordering was assessed in hem-ablated mice with gene expression markers that discriminate among developing neocortical layers (Hermans-Borgmeyer et al., 1998; Rubenstein

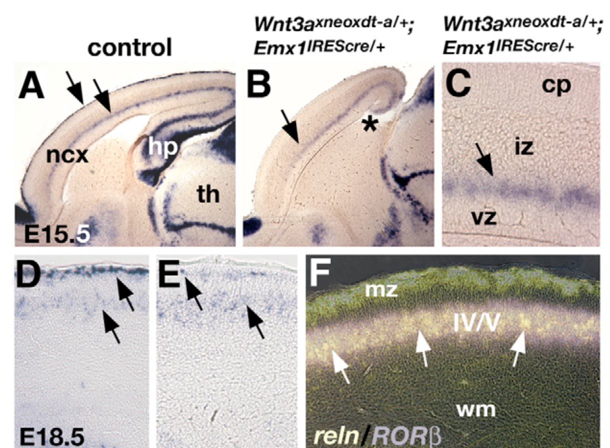

Fig. 6. Diffuse expression of *Reln* in control and hem-ablated mice. (**A-C**) Sagittal sections through E15.5 brains; (**D-F**) coronal sections through E18.5 neocortex. At E15.5 and E18.5 in control mice (A,D), but not hem-ablated mice (B,E), a dense layer of *Reln*+ CR cells fills the MZ (asterisk in B indicates the region of hem loss and consequent failure of hippocampal development). Below this is more diffuse *Reln* expression. At E15.5, a band of low *Reln*+ expression appears in the IZ/SVZ in both mutants and controls (A-C). By E18.5, scattered *Reln*+ cells appear in layers IV/V (D-F). (F) Layers defined by expression of *Rorb*: *Reln*+ cells are yellow, *Rorb* expression is light purple. Arrows in each panel indicate *Reln*+ cells.

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et al., 1999; Sugitani et al., 2002). A similar panel of molecular markers was used previously to show layer inversion in *reeler*, superimposed on abnormal cell dispersion (Hevner et al., 2003a). In control neocortex at E18.5, gene expression of the transcription factors SCIP (Pou3f1 – Mouse Genome Informatics), SorLA (Sorl1 – Mouse Genome Informatics), RORβ, ER81 (Etv1 – Mouse Genome Informatics), Fezl and Tbr1, distinguished emerging layers II/III, IV, V, VI and the subplate (Fig. 7E). To our surprise, hem-ablated mice also showed defined, correctly ordered layers (Fig. 7F). Expression of each protein in the panel appeared in the same pial-to-ventricular order as in control neocortex (Fig. 7E,F). Gene expression was in some cases more diffuse than in controls, or slightly out of position, suggesting modest migrational defects (Fig. 7E,F, *SorLA*/*Sorl1* and *Tbr1*). Thus, precise layer organization may require a larger complement of CR cells. Nonetheless, neocortical layers formed in a near normal pattern in the virtual absence of CR cells and, more specifically, without a strong reelin signal in the MZ.

Defects in the caudal cerebral cortex after hem ablation

A near normal laminar pattern predominated at rostral levels of the neocortex. Caudomedial cortex was highly disorganized and shrunken compared with more rostral regions (see Fig. S1 in the supplementary material). However, although *Reln*-expressing cells were barely detectable in caudal cortex, the disorganization was not a *reeler*-type defect. Laminar expression patterns of *SCIP*/*Pou3f1* and *Tbr1*, for example, were not inverted; instead *SCIP*/*Pou3f1* and *Tbr1* expression intermingled, forming jagged, patchy configurations (see Fig. S1 in the supplementary material), that we did not see in *reeler* cortex (data not shown). Plausibly, this defect arises from the reduction in hem-ablated mice of p73, part of a survival pathway in cerebral cortex. In mice with reduced levels of functional p73, caudal cortex is initially disrupted in a strikingly similar way (Meyer et al., 2004; Pozniak et al., 2002; Yang et al., 2000). After birth, *p73* mutants show progressive degeneration of the entire cerebral cortex, which becomes a thin ribbon of tissue (Pozniak et al., 2002; Yang et al., 2000).

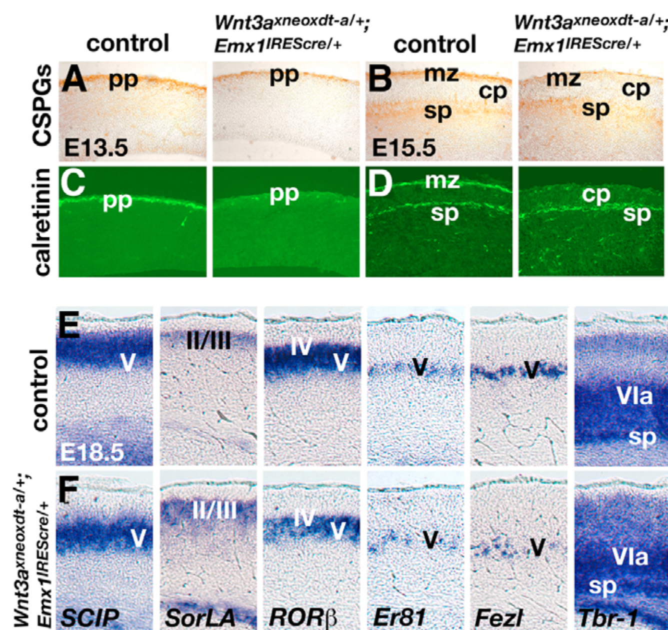

Fig. 7. In the absence of CR cells the preplate splits and neocortical layers are ordered correctly. (A-F) Coronal sections through control or hem-ablated neocortex. At E13.5, calretinin and CSPG-immunoreactivity (IR) distinguished the preplate (**A**,**C**). By E15.5, in control mice, two bands of calretinin- and CSPG-IR marked the MZ and subplate (**B**,**D**). Only the lower, subplate band appeared in hem-ablated animals; the upper band, which labeled CR cells in controls, was not detected (B,D). (**E**,**F**) In control neocortex at E18.5, expression patterns of *SCIP* (*Pou3f1*) *SorLA* (*Sorl1*), *Rorb*, *ER81* (*Etv1*), *Fezl* and *Tbr1* distinguished layers II/III, IV, V, VI and the subplate, along the pial-to-ventricular axis (E). In hem-ablated mice, expression of each gene appeared in a highly similar pial to ventricular order (F). Expression of some genes, notably *SorLA,* showed abnormal diffusion, indicating modest migration defects.

DISCUSSION

Remarkably, in the near absence of CR cells, neocortical layers assemble in the correct anatomical order. This observation is startling, given the body of evidence that reelin signaling is required for the layering of the neocortex (Rice and Curran, 2001).

A plausible explanation is that layer order does not depend, as has been thought, on an abundant, localized source of reelin in the MZ. The reelin-positive CR cells left in the neocortical MZ of hem-ablated or *p73*-null mice (Meyer et al., 2004) may be sufficient to guide layer position. A more likely mechanism, given that CR cells are few and far apart in both mutants, is that compensation occurs by reelin diffusing from subcortical structures or from other sources inside the cortical primordium.

Indeed, we identified low level reelin mRNA expression in the IZ before the preplate is split, continuing in the IZ while cells for deeper layers of neocortex are settling in the CP (Bayer et al., 1991; Caviness, 1982). Close to birth, *Reln* expression in layers IV/V coincides with the migration of layer IV and II/III neurons (Bayer et al., 1991; Caviness, 1982). Recent evidence suggests that the *Reln*-expressing layer V cells can regulate the migration of later-born neurons (Alcantara et al., 2006).

In both hem-ablated and wild-type mice, low level *Reln* is therefore expressed below the target layer of migrating neurons, as is the case for ectopic reelin in the VZ of *reeler*-nestin-reelin mice. In each context, neurons must pass through regions of reelin expression to reach their target layers. Thus, reelin seems not to be acting as a stop, glial-release or directional signal. Instead, present and recent findings (Magdaleno et al., 2002; Meyer et al., 2004) are in accordance with some of the earliest studies of the *reeler* mouse (Caviness, 1982; Pinto-Lord et al., 1982), which suggested that neocortical neurons in *reeler* lost the ability to push past settled cells that lay in their way. New and classic observations thus converge on a model in which reelin is a permissive or enabling cue for migration that is required for the mechanics of movement through cell-dense tissue.

Birth-dating studies in rats and mice have long shown that although upper and lower layer cells are largely born at different times, there is considerable overlap (Hevner et al., 2003a; Takahashi et al., 1999). That is, the assembly of layers by timed migration in the rodent is comparatively rough, and must presumably be refined by other mechanisms. Interestingly, disruption of reelin signaling also appears to disrupt layer refinement. In the *reeler* mutant, inverted layers are diffuse (Hevner et al., 2003a). Furthermore, in mice with mutations in other components of the reelin pathway, neurons for given layers are dispersed but layers are not always clearly inverted (Howell et al., 1997; Rice et al., 1998; Sheldon et al., 1997). We analyzed *reeler* neocortex at E18.5, using the panel of molecular

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markers cited above, and found that cell dispersion was more striking than layer inversion (data not shown). In brief, available evidence suggests that reelin signaling enables migration, and activates other (more precise) layering mechanisms (Magdaleno et al., 2002).

Previous studies have implicated CR cells and reelin in layer formation and connectivity in the hippocampus (Borrell et al., 1999; Del Rio et al., 1997). Hem-ablated animals lack a Wnt3a signal from the hem, and consequently the hippocampus does not develop (Lee et al., 2000b). Mice lacking functional p73, however, retain the hippocampus. As noted, *p73*-null mice lose almost all CR cells, which depend on p73 for their survival, but, like hem-ablated mice, show a standard neocortical layer pattern. By contrast, the hippocampus is highly abnormal. This finding could be taken to support a requirement for CR cell-derived reelin in hippocampal morphogenesis. However, at least some hippocampal abnormalities in the p73 mutant are not seen in *reeler*. Problems in the *p73* mutant hippocampus, particularly in the dentate gyrus, can also be ascribed to the loss of p73 itself, to defects in the meninges and to the absence of chemokine signaling mediated by CR cells (Meyer et al., 2004).

Observations of the *p73* mutant hippocampus and neocortex are not, therefore, in conflict. Moreover, these findings emphasize the complexity of CR cell signaling. CR cells express a variety of molecules implicated in cell motility, including *Sdf1* (*Cxcl12* – Mouse Genome Informatics) and *Cxcr4* (Bagri et al., 2002; Lu et al., 2002; Tissir et al., 2004), and as yet unidentified factors (Soriano et al., 1997). It seems likely that CR cells regulate the movement of other cell types, a conspicuous candidate being the interneurons that migrate into the cortex from subcortical sites (Hevner et al., 2004; Stumm et al., 2003). Because the MZ becomes layer I of neocortex, CR cells are also well placed to influence dendritic morphogenesis, axon growth and connectivity within layer I.

Finally, the proposal that layer pattern is rescued in the hem-ablated mouse by residual reelin is not completely satisfying. Forced reelin expression in the *reeler* cortical primordium, for example, does not rescue layer patterning (Magdaleno et al., 2002). The complexity of CR cell signaling suggests a new possibility that would square our findings and those of Meyer and colleagues (Meyer et al., 2004) with the mass of evidence that reelin is required for neocortical layering (Rice and Curran, 2001). Guidance of neocortical neurons to appropriate layers is likely to involve several signaling pathways that interact and counterbalance one another. A hypothesis to be tested, therefore, is that in the absence of reelin, other migratory cues from CR cells can act unopposed, disrupting a balance in mechanisms controlling migration and leading to the *reeler* phenotype. If CR cells are removed, it is predicted, migration cues remain in balance and generate a near normal layer pattern.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/3/537/DC1

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