disability-1 Functions Cell Autonomously during Radial Migration and Cortical Layering of Pyramidal Neurons

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Genetic mosaics offer an excellent opportunity to analyze complex gene functions. Chimeras consisting of mutant and wild-type cells provide not only the avenue for lineage-specific gene rescue but can also distinguish cell-autonomous from non-cell-autonomous gene functions. Using an independent genetic marker for wild-type cells, we constructed Dab1+/+ ↔ Dab1−/− chimeras with the aim of discovering whether or not the function of Dab1 during neuronal migration and cortical layering is cell autonomous. Dab1+/+ cells were capable of radial migration and columnar formation in a Dab1−/− environment. Most Dab1+/+ cells segregated to the superficial part of the mutant cortex, forming a multilayered supercortex. Neuronal birth-dating studies indicate that supercortex neurons were correctly layered, although adjacent mutant cortex neurons were in reversed order. Immunocytochemistry using Emx1, a marker for pyramidal neurons, indicates that the vast majority of Dab1+/+ neurons in the supercortex were Emx1 immunoreactive. Confirmation of the pyramidal phenotype was demonstrated by the absence of GABA immunoreactivity among Dab1+/+ cells in the supercortex. Myelin staining using 2′,3′-cyclic nucleotide 3′-phosphodiesterase showed the supercortex was supported by a secondary white matter from which thick fiber tracts appear connected to the underlying mutant white matter. The presence of Dab1+/+ cells failed to rescue inversion of cortical layers and the abnormal infiltration of the marginal zone by Dab1−/− cells. Conversely, mutant cells did not impose a mutant phenotype on adjacent wild-type neurons. These results suggest that Dab1 functions cell autonomously with respect to radial migration and cortical layering of pyramidal neurons.

Key words: disabled-1; Reelin; development; cortex; Emx-1; radial migration

Neurons that assemble to form the cerebral neocortex migrate in multiple waves after their birth in the ventricular zone. Early-born neurons are the first to reach the outermost aspects of the cortical plate (CP), but these become displaced inward as later-born neurons migrate past them (Angevine and Sidman, 1961; Rakic, 1974). In this manner, cohorts of neurons born at different times become organized into precise cortical layers. The genetic mechanisms behind this inside-out order of cortical layering have been under intense scrutiny, stimulated in part by the recent discovery of reelin, the gene responsible for the classic mouse mutant reeler (D’Arcangelo et al., 1995). Mutations in reelin give rise to inversion of cortical layers (Caviness and Sidman, 1973), in addition to other abnormalities in the hippocampus and cerebellum that are associated with the typical reeling gait and motor defects (Goldowitz et al., 1997). Whereas the Reelin protein is secreted by Cajal-Reidtzius cells in the marginal zone (MZ) (Ogawa et al., 1995; D’Arcangelo et al., 1997), neurons arriving into the adjacent CP are devoid of Reelin (Rice et al., 1998). Instead, CP neurons express a number of other proteins, some of which function downstream of Reelin (for review, see Rice and Curran, 1999). Together, these proteins form the Reelin signaling pathway, and disruption of some of these components, either spontaneously or by gene-targeting, results in the reeler-like phenotype with behavioral ataxia and inversion of cortical layers (Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997; Trommsdorff et al., 1999).

A key player in this molecular tapestry is disabled-1 (Dab1), a mouse relative of Drosophila disabled, that encodes an intracellular protein with properties of an adapter molecule in protein kinase signaling (Howell et al., 1997a). Dab1 lacks catalytic activity, but is required for Reelin signal transduction. After extracellular binding of Reelin to lipoprotein receptors [very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2)], increased tyrosine phosphorylation of Dab1 is observed (D’Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999). In addition, there is accumulation of Dab1 in cortical neurons of either reelin−/− or ApoER2−/−/ VLDLR−/− mice, suggesting failure of Dab1 degradation in the absence of Reelin pathway activation (Rice et al., 1998; Trommsdorff et al., 1999). The role for Dab1 tyrosine phosphorylation in Reelin signaling is further supported by mice that express a mutant form of Dab1 that is not a tyrosine kinase substrate. These mutant mice have a phenotype reminiscent of the Reeler mice and Dab1 null mutants, suggesting that correct Dab1 phosphorylation is a crucial event for correct neuronal positioning (Howell et al., 2000).

Apart from ApoER2/VLDLR receptors, Reelin has also been shown to bind to a number of other cell surface molecules present in migrating neurons, including cadherin-related neuronal receptors and α3β1 integrin (Senzaki et al., 1999; Dulabon et al., 2000).
Mutations in a number of other genes also cause cortical laminar inversions (e.g., Cdk5 and p35) and abnormalities (e.g., α3 integrin), suggesting multiple pathway involvement in lamina formation and that components of the Reelin pathway, although necessary, may not be sufficient for layer specification. The question then arises whether the Reelin pathway, acting through Dab1, functions cell autonomously during two key morphogenetic processes involved in cortical development: cortical neuron migration and layer specification. If the answer is yes, then restoration of Dab1 gene function by introducing Dab1\(^{+/+}\) cells into a Dab1\(^{-/-}\) environment should produce wild-type phenotypes in Dab1\(^{-/-}\) cells. In addition, there would be failure of rescue of mutant phenotypes among adjacent Dab1\(^{-/-}\) neurons. Conversely, non-cell-autonomous function of Dab1 would result in extension of gene function beyond cells expressing the original mutant phenotype, resulting in some Dab1\(^{+/+}\) neurons assuming the mutant phenotype, although other Dab1\(^{-/-}\) neurons may be rescued.

To distinguish between these possibilities, we constructed Dab1\(^{+/+}\) ↔ Dab1\(^{-/-}\) chimeras with the aim of undertaking lineage-specific gene rescue (Rosant and Spence, 1998). Using an independent lacZ marker for Dab1\(^{+/+}\) cells, we found that Dab1\(^{+/+}\) cells formed a supernumerary “supercortex” above the Dab1\(^{-/-}\) cortex [not to be confused with the subplate (SP)] (Caviness, 1982), and there was no rescue of layer invasion among adjacent Dab1\(^{-/-}\) neurons. Despite comprising a minority of cortical cells, the Dab1\(^{+/+}\) neurons were able to form an inside-out cortical structure with respect to their birth dates. This structure bore some of the hallmarks of a “minicortex,” including staining for the pyramidal neuron marker Emx1 and extensive myelination of a secondary subcortical white matter. These results led us to conclude that Dab1 signaling in cortical neurons is cell autonomous with respect to the establishment of cortical layers among radially migrating neurons of the pyramidal class.

**MATERIALS AND METHODS**

**Chimeras.** To generate Dab1\(^{+/+}\) ↔ Dab1\(^{-/-}\) chimeras (Dab1\(^{-/-}\) chimeras), we introduced embryonic stem (ES) cells carrying the lacZ reporter gene into Dab1\(^{-/-}\) blastocysts. The latter were produced from Dab1\(^{+/+}\) × heterozygous crosses (maintained on C57BL/6J background), and Dab1\(^{-/-}\) offspring were identified by genotyping. The ES cells have normal Dab1 alleles (confirmed by PCR) and were derived from the H253 transgenic mouse line with the X lacZ/X lacZ genotype (Tan et al., 1993; Sturm et al., 1997). Previous experiments using this cell line have shown that the injection of one to two ES cells per blastocyst is sufficient to confer chimerism in the CNS, and the genetically marked cells migrated in essentially the same patterns as host cells (Tan et al., 1998; Reese et al., 1999). Injected blastocysts were implanted into pseudoconceptional foster females, and chimeric pups were reared for 14–16 d before genotype analysis and processing for X-gal histochemistry to visualize β-galactosidase (β-gal), and 14 μm coronal sections were prepared for immunocytochemistry. Sections for X-gal histochemistry were incubated overnight at 37°C in a solution of 0.1% X-gal containing 2 mM MgCl\(_2\), 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Nonidet P-40, 5 mM K\(_2\)Fe(CN)\(_6\), and 5 mM K\(_4\)Fe(CN)\(_6\) in 0.1 M Sorenson’s phosphate buffer. The X-gal was prepared as a 4% stock in dimethylformamide and was added to the mixture just before use.

For nuclear staining after X-gal histochemistry, sections were rinsed in PBS and then stained in 50 μg/ml Hoechst 33342 (Sigma, St. Louis, MO) for 2 min. Fluorescent cell nuclei were visualized using a UV filter.

**Histology.** After a lethal injection of Avertin, chimeras were perfused with 0.1 M Sorenson’s phosphate buffer, pH 7.4, with 2 mM MgCl\(_2\) and 5 mM EGTA, and the brains were removed for 20 min after fixation. After cryoprotection with 30% sucrose, brains were embedded in OCT (Tissue-Tek, Torrance, CA) and serial sections (50 μm) of the cortex were obtained for X-gal histochemistry to visualize β-galactosidase (β-gal), and 14 μm coronal sections were prepared for immunocytochemistry. Sections for X-gal histochemistry were incubated overnight at 37°C in a solution of 0.1% X-gal containing 2 mM MgCl\(_2\), 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Nonidet P-40, 5 mM K\(_2\)Fe(CN)\(_6\), and 5 mM K\(_4\)Fe(CN)\(_6\) in 0.1 M Sorenson’s phosphate buffer. The X-gal was prepared as a 4% stock in dimethylformamide and was added to the mixture just before use.

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**Immunocytochemistry.** For double-immunofluorescence studies, 14 μm coronal sections were mounted on slides and incubated with primary antibodies to NeuN (1:3000), calbindin (1:100 dilution; Swant); and a mouse monoclonal antibody to Dab1 (Howell et al., 1997a). Secondary antibodies were biotinylated anti-rabbit (1:200 dilution; Vector Laboratories, Burlingame, CA), donkey anti-mouse Cy3 (1:1000 dilution; Jackson ImmunoResearch, West Grove, PA), and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:500 dilution; Molecular Probes, Eugene, OR). With BrdU, NeuN, and CNPase antigens, simultaneous visualization of β-gal was achieved with fluorescein-avidin D (1:200 dilution; Vector Laboratories). Incubation with primary antibody overnight was followed by 1 h in secondary antibodies before cover-slipping with Fluormount-G (Southern Biotechnology, Birmingham, AL).

For simultaneous detection of β-gal and Emx1 or β-gal and GABA, we used a method that allows discrimination of primary antibodies raised in the same species, in this case the rabbit (Shindler and Roth, 1996). This method works on the rationale that primary antisera against one antigen (Emx1 or GABA) is used at such dilute levels that only tyramide signal amplification (TSA) allows for its detection. The second primary anti-serum against β-gal is used at normal concentration and is detected by conventional immunocytochemistry. The secondary antibody used to detect rabbit IgG against β-gal is incapable of detecting the rabbit IgG against Emx1 (or GABA) by virtue of the extreme dilution of the latter.

Sections were blocked in 10% normal horse serum (CSL Ltd., Melbourne, Australia) in PBS for 1 h and then in 0.5% blocking reagent (Renaissance TSA Indirect; NEN, Boston, MA) in buffer containing 0.1

5'CGCAAGCGACCTTTGATAAAGA-3' and reverse, 5'T-ACTCATGTGTCATCCCGAGCT-3'Neo forward, 5'ATCAGGATGTATCGGAGCAAGA-3' and reverse, 5'CCACAGTGATGATCCGAGA-3'.
Figure 1. Genotyping to distinguish chimeric from nonchimeric animals and wild-type from mutant animals. A, Top band represents the mutant allele (Neo 170bp), and the bottom band the endogenous allele (Dab1 81bp). B, Top band represents lacZ from introduced ES cells (LacZ 154bp), the bottom band represents Dab1 (81 bp). Lane 1, pUChPurII markers; lane 2, homozygous mutant (Dab1−−); lane 3, heterozygous mutant (Dab1+−); lane 4, wild type (Dab1++); lane 5, weak Dab1−− chimeras; lanes 6–9, strong Dab1+/− chimeras; lane 10, injected ES cells; lane 11, no DNA. As expected, a weak Dab1−− chimera gave a strong Neo band and a weak Dab1 band (lane 5); conversely, strong Dab1−− chimeras (lanes 6–9) gave intense Dab1 signals from the ES cell allele. Confirmation of the presence of the injected ES cells was obtained by assaying for the presence of the lacZ gene. As expected, this band was present in the ES cells (lane 10) and in chimeras (lanes 5–9).

RESULTS

Previous studies using stem cell chimeras have shown that descendants of a single injected ES cell are capable of normal participation in CNS development and provide good resolution on cell migration pathways and cell fates (Tan et al., 1998; Hawkes et al., 1999; Reese et al., 1999). Using this model, we introduced one to two wild-type ES cells into blastocysts from Dab1−−−− intercrosses. Under these conditions, ES cells with normal Dab1 genes can populate blastocyst hosts with Dab1−−, Dab1−−−−, or Dab1−−−−−− genotypes.

Chimeras (from Dab1−−, Dab1−−−−, or Dab1−−−−−− hosts) were identifiable by their patchy coat colors, and Dab1−−−−−− chimeras (subsequently confirmed by genotyping) displayed ataxia, trembling, dragging of hind limbs, and frequent flipping onto their backs. In all Dab1−−−−−− chimeras, regardless of the contribution of wild-type cells to their brains, this behavioral phenotype was not rescued, and these mice did not survive beyond 17 d after birth. In all cases, chimeric cerebella were smaller and poorly foliated.

Genotyping results were used to distinguish Dab1−−−−−− chimeras from littermates. Nonchimeric mice did not provide a PCR product for lacZ (Fig. 1B, lanes 2–4). The presence or absence of Dab1 or Neo fragments allowed wild-type (Dab1++−−) hosts (Fig. 1A, lane 4) to be distinguished from heterozygous (Dab1−−−−) (lane 3) or null (Dab1−−−−−−) (lane 2) mutants. Weak Dab1−−−−−− chimeras (lane 5) tended to provide a weaker Dab1 band (from the smaller contribution of ES cells) but a strong band for the Neo gene present in the targeting construct (Howell et al., 1997b). Strong Dab1−−−−−− chimeras (lanes 6–9) gave bands of approximately equal intensity for Dab1 and Neo, reflecting a greater contribution of Dab1−−−−−− ES cells. Lane 10 shows the presence of Dab1 in ES cells used for making the chimeras.

Wild-type cells in Dab1−−−− chimeras form a supercortex

Chimeric littermates with Dab1++−− or Dab1−−−− genotypes were used as controls for studying cortical cell dispersion. In these mice, βgal-expressing cells in the neocortex exhibited cell dispersion patterns seen in normal chimeras (Tan et al., 1998). Radial columns of cells extending across the full cortical depth were seen in Dab1−−−− chimeras, suggesting that wild-type neurons migrate normally, despite an environment of cells heterozygous for Dab1 (Fig. 2A). In contrast, the distribution of wild-type cells in Dab1−−−− chimeras was unexpected, with ectopic segregation of βgal-positive cells mainly in the superficial area and occupying a cortical rind beneath the pial surface (Fig. 2B, arrows). Deeper into the cortex, a minor but still substantial fraction of βgal-positive cells was diffusely intermingled with Dab1−−−− cells, making up the bulk of the cortical tissue underneath the densely packed wild-type cells (Fig. 2B, D). Despite the uneven distribution, the βgal-positive cells in both deep and superficial areas appeared to share a radial dispersion pattern with parallel registration of lateral boundaries (Fig. 2B, inset). In strong chimeras, radial columns were not visible, probably because of the merging of neighboring columns (Fig. 2C, D). All Dab1−−−− chimeras displayed the intense packing of βgal-positive Dab1++−− cells into a dense superficial band, resembling a supernumerary cortex that we termed a supercortex (Fig. 2C, D). Dab1−−−− cells appeared to fill the remaining cortical thickness with a non-uniform infiltration of Dab1−−−− cells, some of which coalesced into cellular nodules in the middle layers (Fig. 2C, white arrows). Interestingly, the supercortex did not extend beyond the entorhinal areas (Fig. 2C, arrowheads). The normally cell-poor MZ, situated above the supercortex, was invaded by a large number of cell bodies, similar to that reported for mice lacking Reelin, Dab1, or ApoER2-VLDLR. Cells of both genotypes were found in the MZ (Fig. 2D–F), although staining of cell nuclei with Hoescht 33342 (Fig. 3F) revealed that the vast majority of the infiltrate were Dab1−−−− cells. Thus, despite the presence of adjacent wild-type cells, a large number of mutant cells continued to invade the MZ as reported previously (Howell et al., 1997b).

Dab1++−− cells in the supercortex are Emx1-positive pyramidal neurons

To ascertain the identity of the cells making up the supercortex, we performed double-immunofluorescent experiments using antibodies against βgal and NeuN, a marker for neurons. The results
indicate widespread immunoreactivity for NeuN (Fig. 3A, red and double-labeled, yellow, or orange), with only a few cells expressing βgal (green) without NeuN (Fig. 3A). Double-immunofluorescence was also performed using antibodies against βgal and Emx1, a homeoprotein transcription factor homologous to the product of the Drosophila gene empty spiracles (Cecchi and Boncinelli, 2000). Emx1 has been shown to label cortical neurons (Gulisano et al., 1996) and, more specifically, to be a marker for pyramidal neurons (Chan et al., 2001). Widespread staining for Emx1 was confirmed across the entire cortex (Fig. 3B, green and double-labeled, yellow, or orange), but in the supercortex, most of the cells were double-labeled with βgal and Emx1 (Fig. 3B–D). At the bottom layer of the supercortex and parallel to the pia (Fig. 3B,C, dotted lines), a string of weakly positive Emx1 cells were found (Fig. 3D, arrows). The identity of these remain unclear; however, their large cell somas and immunoreactivity for NeuN suggests they are neurons. Within the supercortex, a small number of Emx1-positive but βgal-negative (i.e., Dab1<sup>+/+</sup>) cells were also found, indicating contribution by host mutant cells. In the MZ overlying the supercortex, wild-type βgal-positive cells were also found, although very few of these were weakly stained for Emx1 (Fig. 3B).

To confirm that Emx1 is a marker for radially migrating neurons, we performed double-immunofluorescent labeling on radial columns of wild-type chimeras. Staining with βgal-antibody revealed the columnar arrangement of lacZ-expressing cells (Fig. 3E, red); however, staining with anti-Emx1 showed that, without exception, all of the cells comprising the column also stained for Emx1 (Fig. 3F, yellow or orange). In light of previous studies that have showed that radially migrating cells are predominantly glutamatergic (Tan et al., 1998), coupled to electron microscopic evidence that Emx1 only marks pyramidal neurons (Chan et al., 2001), these results suggest that the Emx1-positive cells in the supercortex are pyramidal. Additional supporting evidence was obtained using antisera directed against GABA, a marker for
nonpyramidal interneurons. In the supercortex (Fig. 3G), βgal-positive cells (red) rarely stained for GABA (green), but in the mutant cortex, double-positive cells (green with yellow center) were found (Fig. 3G, arrows). Although GABA-positive neurons were seen in the supercortex (Fig. 3G, arrowheads), they were invariably Dab1−/− (green). Thus, it would appear that wild-type GABA interneurons, which normally have a tangential mode of cell dispersion and the bulk of which originate from the underlying ganglionic eminence (Anderson et al., 1997, 2001), did not contribute to the formation of the supercortex.

**Supercortex is linked to mutant white matter by myelinated fibers**

The myelin-associated enzyme CNPase is normally found in the plasma membrane of oligodendrocytes (Sprinkle, 1989; Thompson, 1992). In the normal cortex, CNPase immunoreactivity is associated with myelin-producing cells found in deeper layers and also in the subcortical white matter (Vogel et al., 1988). This pattern of CNPase staining was confirmed in wild-type and Dab1−/− cortices (Fig. 4A,B, arrows), with occasional and faint fibrillar staining within the lower cortical layers (arrowheads). In addition, oblique fiber bundles were sporadically observed within the mutant cortex. The superficial cortical layers clearly lacked significant immunoreactivity, with the exception of slight increased staining where the MZ normally resides in the Dab1−/− cortex (asterisk).

In contrast, the cortex of Dab1−/− chimeras displayed a striking picture of CNPase immunoreactivity (Fig. 4C). Intense labeling, suggestive of widespread myelination, was observed beneath the supercortex in a horizontal fiber tract running parallel to the supercortex (Fig. 4C, arrow). Many of the fibers had coalesced to form thick bundles. Intense immunoreactivity was also observed in the white matter and also in vertical fiber bundles traversing the Dab1−/− cortex. These vertical fiber bundles appeared to link the subcortical fiber tracts beneath the supercortex with mutant white matter (Fig. 4C, arrowheads). The supercortex per se was remarkably free from myelin staining, except for a thin margin of occasional immunoreactivity in the superficial pial region and also in the deeper layers of the supercortex. Because CNPase expression and activity is dependent on the presence of nerve axons (Goto et al., 1990), it is reasonable to assume that CNPase activity is associated with nerve fiber tracts occupying a “white matter” region beneath the supercortex. In this scenario, the vertical bundles of myelinated tracts may represent nerve fibers attempting to connect the supercortex with other subcortical structures. Indeed, the intensity of staining in white matter of Dab1−/− chimeras greatly exceeds that of either wild-type or Dab1−/− mutants, suggesting an increased number of myelinated fibers (Fig. 4C). However, it remains unclear whether fiber tracts from the supercortex are functional as corticofugal fibers emanating from supercortical neurons or corticopetal pathways innervating the supercortex.

So far, neurons in the supercortex have been shown to be exclusively pyramidal in composition by virtue of positive staining for Emx1 and negative for GABA. Pyramidal neurons in the supragranular layers have been shown to be differentially stained for calbindin, a calcium binding protein (Demeulemeester et al., 1989; van Brederode et al., 1991). Antibodies revealed calbindin immunoreactivity only among neurons present in the upper half of the supercortex (Fig. 4D, yellow and arrows), whereas the lower half supercortex neurons stained only for βgal (Fig. 4D, red). Other calbindin-positive neurons with extensive processes but belonging to the host Dab1−/− population were also seen beneath the supercortex, and their morphology suggests them to be mutant interneurons (Fig. 4D, green).

**A subplate is formed beneath the supercortical plate**

The calcium-binding protein calretinin labels the marginal zone and subplate, derivatives of the preplate, at specific developmental stages (Vogt-Weisenhorn et al., 1994; Fonseca et al., 1995). Using an antibody to calretinin, the cortices of E16.5 (wild-type, Dab1−/− mice, and Dab1−/− chimeras) were immunostained to examine for the presence and localization of the subplate. In wild-type cortices, both the MZ and the SP were labeled (Fig. 5A, arrows), whereas in Dab1−/− brains, the unsplit preplate known as the subplate was labeled for calretinin (Fig. 5B, arrow). Dab1−/− chimeras displayed an interesting pattern of staining: a strong band of calretinin staining was seen in the presumptive MZ (Fig. 5D, arrow), but in addition, there was clearly a string of calretinin-positive subplate cells underneath the “supercortical” plate (Fig. 5D, arrow). The location of the supercortical plate in these sections was identified by examining neighboring sections immunostained for βgal (Fig. 5C, asterisk). These results suggest that the supercortical plate has been positioned between the MZ and SP. The genotype of the SP in Dab1−/− chimeras revealed a mixture of both mutant and wild-type cells (our unpublished observations).

**Dab1 is expressed in βgal-positive cells**

In Dab1−/− chimeras, wild-type cells of ES cell origin are distinguished by expression of the lacZ marker. To confirm that wild-type cells express Dab1, we performed immunostaining using antibodies to Dab1 and βgal on adjacent sections of E16.5 neocortices. At this stage, the “cortical plate” of the future supercortex can be identified as a βgal-positive structure underneath the pial surface (Fig. 5E, asterisk). Staining of the adjacent section revealed the presence of Dab1 protein in the supercortex (Fig. 5F, asterisk), but as expected, not in the underlying Dab1−/− host.

**Neurons of the supercortex are born in the correct inside-out order**

A key hallmark of defective Reelin signaling is the inversion of cortical neurons born at different times of the neuronegenetic period (Caviness, 1982). The thymidine analog BrdU was used to label neurons undergoing the last round of mitotic cell division, before their migration to the CP. Neuronal birth dating was performed for wild-type, mutant Dab1−/− (nonchimeric), and Dab1−/−/Dab1−/− chimeras to compare the positions of neurons labeled with BrdU. In the nonchimeric Dab1−/− cortex, BrdU labeling at E12.5 and E13.5 confirmed aberrant displacement of early-born cohorts to superficial layers (Howell et al., 1997b) (Fig. 6B, arrows). As expected in the wild type, superficial layer labeling was observed after BrdU injection at E17.5 (Fig. 6A). In Dab1−/− chimeras, BrdU injection at E16.5 showed labeling of Dab1−/− cells (yellow) in the upper layers of the supercortex (Fig. 6C, arrows), which is compatible with the expected labeling position of later-born neurons. The host BrdU-labeled Dab1−/− neurons were mostly distributed in the deeper half of the mutant cortex (data not shown), as reported by others (Howell et al., 1997b), but in the supercortex, a very small number of Dab1−/− cells could also be found close to the pial surface (Fig. 6C, red). Additional confirmation that layers are inverted in the mutant cortex is provided by the relative absence of E16.5 BrdU-labeled cells in the area directly beneath the supercortex (Fig. 6C). Dab1−/− chimeras were also injected with BrdU at E12.5, and early born
Figure 3 legend.
Dab1\(^{+/+}\) neurons (yellow) were found in the deeper layers of the supercortex (Fig. 6D, arrows). In these brains, the early-born mutant neurons (red) were mostly found in the upper part of the mutant cortex, situated just beneath the supercortex (Fig. 6D, red). A low-power view showed that BrdU cells of Dab1\(^{-/-}\) genotype (Fig. 6E, arrows), labeled at E12.5, had migrated to the upper one-third of the mutant cortex, suggesting persistent layer inversion of the mutant cells, despite the presence of the Dab1\(^{+/+}\) supercortex above. Thus, Dab1\(^{+/+}\) neurons born at different times in the chimeric cortex appear to migrate to the appropriate deep and superficial layer positions within the supercortex. Together with the calbindin-staining data (Fig. 4D), neuronal positioning of Dab1\(^{+/+}\) cells in the supercortex appears to be independently driven and correlated with their Dab1 genotype.

**DISCUSSION**

Classically, the first step toward elucidation of gene function is by targeted gene deletion. In the case of Dab1, the spontaneous
mutant scrambler and Dab1 knock-out mice have been very useful in revealing the central role for Dab1 in neuronal migration and cortical layering (Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997). Using chimeras, our results shed additional light on Dab1 gene function in cortical development. By considering the phenotype of Dab1−/− cells in the context of a Dab1−/− environment, another facet of Dab1 gene function in the Reelin signaling pathway has been exposed. We asked whether the function of Dab1 during cortical cell migration and layering is cell autonomous or whether Dab1 activation produces a non-cell-autonomous effect, for example, by regulating the production of cell surface or extracellular molecules that may affect the behavior of other cells. Answers to these questions will assist in identifying the primary role of Dab1 gene function.

The assembly of multiple neuronal layers as a function of development is a complex process, possibly requiring dynamic feedback of positional information between early- and late-born neurons. Various models have been proposed for the molecular control of this process, and they invariably include the Reelin signaling pathway, but other cell surface and cell matrix molecules may also be involved. Confirmation of multiple pathway involvement is evidenced by layering abnormalities in mutant mice for a number of genes, in addition to those described in the Reelin pathway, e.g., p35, Cdk5, and α3 integrin (Gilmore et al., 1998; Kwon and Tsai, 1998; Anton et al., 1999). To further understand this complex process, an important step would be to demonstrate whether or not the key players have interdependent relationships. Whereas the activity of Reelin is clearly non-cell autonomous (Terashima et al., 1986; Yoshiki and Kusakabe, 1998), the results presented here suggest that Dab1 function in the neocortex is cell autonomous. This conclusion is supported by several lines of evidence. First, the mutant environment did not impose a mutant phenotype on the wild-type cells, and, inversely, there was no rescue of the mutant phenotype by wild-type cells. The capacity for radial cell dispersion by Dab1−/− cells in the mutant environment appeared to be unaffected, but columns were abnormal, with the majority of cells dispersing to the superficial part of the column. A minority of Dab1−/− neurons was found in the middle and deeper layers of the composite cortex, but the vast majority of wild-type neurons were segregated to the superficial region. Here, they formed a supercortex beneath the pial surface. Second, there was persistence of the mutant pheno-

Figure 5. Immunocytochemistry for calretinin shows that the preplate is split in wild-type (A) and Dab1−/− chimeras (C, D) but not in Dab1−/− (B). All tissues depicted were obtained from E16.5 neocortices. A, Staining for calretinin, a marker for preplate derivatives, shows normal splitting of E16.5 preplate into MZ and SP. B, In mutant Dab1−/− embryos, the preplate fails to split, as indicated by the calretinin-immunoreactive SPP. C, D, In Dab1−/− chimeras, the βgal-positive supercortex (asterisk) is bounded by calretinin-positive SP cells and the MZ. E, F, In adjacent sections (separately stained for βgal and Dab1), βgal-positive cells in Dab1−/− chimeras express Dab1 in the supercortex (asterisk). Scale bar, 50 μm.
type in the MZ, i.e., infiltration by a large number of mutant cell bodies despite the close proximity of the wild-type supercortex, again implying lack of rescue. Although the MZ was densely populated by mutant cells, few wild-type cell bodies were seen here, consistent with the notion that the wild-type preplate has been properly split and most of the wild-type cells have withdrawn from the MZ. This interpretation of cell-autonomous behavior is substantiated by the presence of calretinin-positive subplate cells underneath the chimeric supercortical plate. Evidently, despite the presence of wild-type cells, the mutant preplate had failed to split, otherwise a second calretinin-positive subplate would be observed below the mutant cortical plate. On the other hand, the observable subplate contains a mixture of both mutant and wild-type cells, suggesting that some mutant cells may have responded to the subplate-splitting cues and have assembled in the correct location with respect to the wild-type, but not mutant, cortical plate. Most likely, mutant cell bodies seen in the composite MZ (Fig. 2F) represent the bulk of the ectopically displaced subplate cells from the unsplit mutant preplate (Rice et al., 1998).

Within the supercortex, the dense packing of cells made it difficult to distinguish between cortical layers on the basis of cellular morphology or size, but birth-dating experiments using BrdU and staining with calbindin, a marker of upper layer neurons (Demeulemeester et al., 1989; van Brederode et al., 1991), indicate that they have formed in the “correct” inside-out order. Furthermore, the presence of an appropriately localized subplate in the embryo, and later in the adult, the extensive myelination in the secondary “white matter,” suggest that restoration of Dab1 function has promoted assembly of a separate cortex, despite it being sandwiched between mutant cortical tissue. However, there were a number of unusual features in the supercortex compared with the normal cortex. First, the supercortex was overwhelmingly pyramidal in character as evidenced by double-staining for Emx1. On the other hand, staining for GABA, a marker of inhibitory neurons, revealed hardly any Dab1+/− GABAergic cells inside the supercortex. The few GABAergic cells present in the supercortex were invariably Dab1+/− in genotype (Fig. 3G, arrowheads). Gauging from a similar density of mutant GABAergic cells in the underlying mutant cortex, their presence in the supercortex may simply reflect sharing of a common cortical space. Although Dab1+/− GABAergic neurons were hardly seen in the supercortex, they were freely present beneath (Fig. 3G, arrows), mixed in with other cells of the mutant cortex. This finding raises the possibility that the supercortical environment, bounded between subplate and MZ, may be especially conducive to the pyramidal phenotype (Gilmore et al., 1998). However, this fails to account for the under-representation of wild-type GABAergic cells in the supercortex. Perhaps interneuron migration, which is primarily tangential and mostly from underlying striatum (Anderson et al., 1997, 2001; Tan et al., 1998), may be unresponsive to Reelin signaling and their positions mediated by other mechanisms that are non-cell autonomous with respect to Dab1. It would be interesting for future studies to determine whether the function of Dab1 extends equally to both pyramidal and nonpyramidal cells.

Another unusual feature of the supercortex was the extraordinary compression of the layers at the expense of neuropil space. Furthermore, the supercortex was invariably positioned in the upper part of the composite cortex with a minor but still substantial population of straggling βgal-positive cells in the middle and deeper layers. The superficial location of the supercortex may be attributable to space constraints imposed by Dab1−/− cells in an overpopulated cortex, resulting in upward pressure exerted on the undersurface of the supercortex, preventing their downward expansion into the deeper layers. Therefore, straggling cells, many of which are pyramidal neurons, may also represent incomplete attempts to fill the depth of cortical space.

Alternatively, the asymmetric placement of the supercortex may simply reflect increased affinity of wild-type neurons for a source of Reelin near the pial surface. This observation invites speculation for a mechanistic role of Dab1 in neuronal positioning. One model suggests temporary increases in cell–cell adhesion at the Reelin–cortical plate interface as incoming neurons...
expressing high levels of Dab1 are activated by Reelin (Fig. 7A). Under normal circumstances, a momentary increase in cell adhesion would draw the most recent arrivals past preexisting neurons (displacing them in the process) to the MZ (Fig. 7B). After arrival near the MZ, Reelin binds to other surface molecules (e.g., α3β1 integrin) to initiate neuronal de-adhesion and detachment from radial glia (Dulabon et al., 2000). Reiteration of this process, involving successive waves of Dab1-expressing cells, may explain how incoming cohorts of neurons can leapfrog over older and less adhesive neurons whose Dab1 may be downregulated or degraded (D’Arcangelo et al., 1999) (Fig. 7C). What may be the mechanism for increased cell adhesion after Dab1 activation? One lead is available from recent work with p35-activating proteins. The p35 protein is a regulator of Cdk5 kinase, and mutations in both proteins result in abnormalities of cortical layering. It has been demonstrated that increased kinase activity leads to decreased cell adhesion because of disruption of N-cadherin–β-catenin complexes (Kwon et al., 2000). Although a direct link between Dab1 and p35–Cdk5 kinase remains elusive, it has been suggested that one effect of Reelin signaling is to inhibit p35–Cdk5 kinase activity in newly arriving neurons, leading to increased N-cadherin-mediated adhesion (Homayouni and Curran, 2000).

Finally, the distribution of the straggling βgal-positive cells at midcortical levels deserves additional comment. These Dab1+/+ cells may have failed to make it to the supercortex, suggesting that there are defects in the Dab1−/− environment that may not be fully overcome by a Dab1+/+ genotype. This is suggestive of non-cell-autonomous behavior mediated by structures that normally facilitate neuronal migration. One possibility is that radial glia in the mutant cortex may be defective, as has been reported in reeler mice (Pinto-Lord et al., 1982; Hunter-Schaedle, 1997), causing obstruction of neuronal migration affecting both mutant and wild-type neurons. The other possibility is that failure of Dab1−/− neurons to detach from radial glia may have caused a structural blockade imposed by malpositioned Dab1−/− neurons (Dulabon et al., 2000). However, the fact that the bulk of wild-type neurons have somehow been able to migrate past mutant neurons would argue against a log-jam hypothesis. Nonetheless, straggling wild-type neurons may have behaved like mutant neurons and been blocked in their migratory journey, perhaps because of sharing of glial fibers with mutant neurons that failed to detach. Thus, although the main conclusion of the present study points to a cell-autonomous role for Dab1, it needs to be tempered by the possibility of non-cell-autonomous behavior for neurons that do not insert into the supercortex.

REFERENCES


Figure 7. A. Proposed model to account for supercortex (SC) formation from increased cell adhesion among Dab1 cells in a Dab1−/− environment. In this example, layer VI neurons generated at E12 are attracted to a Reelin source, leading to increasing cell adhesion mediated by Dab1 as neurons approach the marginal zone. Dab1 activation in layer VI cells is followed by Dab1 degradation, leading to decreased cell–cell adhesion among layer VI neurons. At the same time, arriving layer V neurons (generated at E14) show increasing Dab1 activity as they respond to Reelin in the marginal zone, causing increased cell–cell adhesion and enabling them to migrate past layer VI cells.


