

# A hypomorphic allele of *dab1* reveals regional differences in reelin-Dab1 signaling during brain development

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## SUMMARY

The disabled 1 (Dab1) p80 protein is essential for reelin signaling during brain development. p80 has an N-terminal domain for association with reelin receptors, followed by reelin-dependent tyrosine phosphorylation sites and about 310 C-terminal residues of unknown function. We have generated mutant mice that express only a natural splice form of Dab1, p45, that lacks the C-terminal region of p80. The normal development of these mice implies that the receptor-binding region and tyrosine phosphorylation sites of p80 are sufficient for reelin signaling. However, a single copy of the truncated gene does not support normal development of the neocortex and hippocampus. The CA1 region of the hippocampus is split into two well-organized layers, while the marginal zone of the neocortex is invaded by late-born cortical plate neurons. The haploinsufficiency

of the p45 allele of Dab1 implies that the C terminus of p80 affects the strength of reelin-Dab1 signaling, yet there is no apparent change in reelin-dependent tyrosine phosphorylation of p45 relative to p80. Therefore, we suggest that the C-terminal region of Dab1 p80 is involved in signaling to downstream effector molecules. Furthermore, the presence of late-born cortical plate neurons in the marginal zone reveals a requirement for reelin-Dab1 signaling in late-born cortical plate neurons, and helps distinguish models for the cortical inversion in the reeler mutant mouse.

Key words: reelin, Hippocampus, Marginal zone, Brain development, Tyrosine phosphorylation, Disabled 1, Mouse

## INTRODUCTION

During mammalian brain development, many neurons take complex paths between their places of birth and sites of eventual settling and differentiation (Caviness and Rakic, 1978; Hatten, 1993; Hatten and Heintz, 1995; Rakic and Caviness, 1995). These migration routes and settling points are defined by short-range and long-range cues from the surrounding environment, many of which later guide axonal growth cones to their targets. One such cue is the secreted protein reelin, which controls migrations of many neurons, including Purkinje cells in the cerebellum and most glutamatergic excitatory neurons of the neocortex and hippocampus (D'Arcangelo and Curran, 1998; Rice and Curran, 1999).

In the neocortex, reelin is made by the Cajal-Retzius (CR) neurons in the outer leaf of the preplate (D'Arcangelo et al., 1995; Ogawa et al., 1995; Schiffmann et al., 1997). When early cortical plate (CP) neurons are created in the ventricular zone below the preplate, they migrate outward on radial glia and between the cells of the inner leaf of the preplate. Upon reaching the CR cells, the early CP neurons detach from the radial glia and differentiate (Caviness, 1982; Caviness and Sidman, 1973; Marin-Padilla, 1971; Sheppard and Pearlman, 1997). The early CP neurons thus split the inner from the outer leaves of the preplate, driving the reelin-secreting cells ahead

of them and leaving the inner part of the preplate behind as the subplate. Later born CP neurons also migrate through the subplate and between the early CP neurons, settling only when they reach the CR cells (Luskin and Shatz, 1985; Marin-Padilla, 1992; Rakic, 1974). This age stratification within the CP is known as inside-out layering, because the first neurons are innermost, forming layers V-VI, and the last ones are outermost, forming layers II-IV. The CR neurons occupy the cell-poor marginal zone (MZ) or layer I.

In reeler (*Reln*) mutant animals, three defects result from the absence of the reelin signal (Caviness, 1982; Caviness and Sidman, 1973; Sheppard and Pearlman, 1997). First, a subplate is missing and all preplate cells are in layer I, indicating that reelin is needed for migrating CP neurons to invade between the subplate cells. Second, early-born CP neurons are found in outer layers (including layer I) and late-born CP neurons in inner layers (outside-in layering), indicating that reelin is needed for CP neurons to pass by their predecessors and stop migrating at the edge of layer I. Third, the dendrites and axons of CP neurons are disoriented, indicating that reelin is needed for proper orientation. In addition, there are defects in radial glia that may be primary or secondary to altered neuronal placement (Hunter-Schaedle, 1997; Pinto-Lord et al., 1982).

In vitro experiments are consistent with reelin stopping the radial migration of CP neurons and changing the adhesive properties of the neurons (Dulabon et al., 2000; Forster et al.,

1998; Hoffarth et al., 1995; Ogawa et al., 1995). The leading edges of migrating CP neurons have been observed to stop advancing when the cell nuclei have reached the edge of the reelin zone (Nadarajah et al., 2001; Tabata and Nakajima, 2001). Together, these results suggest that reelin is a 'stop signal' for migrating neurons of the CP (Frotscher, 1997; Ogawa et al., 1995; Pearlman and Sheppard, 1996; Schiffmann et al., 1997). However, this model does not provide a simple explanation for the accumulation of late-born CP neurons below early neurons in the *Reln* mutant cortex (outside-in layering). The late born neurons are thus accumulating in a region where reelin is not normally expressed. It has been proposed that outside-in layering is a secondary effect, caused by early CP neurons that remain attached to the radial glia and block outward movement of later CP neurons (Pinto-Lord et al., 1982). However, it is also possible that reelin normally signals to these neurons at a distance, attracting them to migrate to the edge of layer I.

Development of the CA1 region of the hippocampus also involves reelin. Reelin is made by the CR cells of the outer MZ (Nakajima et al., 1997) below which the pyramidal cells settle to form the hippocampal plate or stratum pyramidale (SP). As in the cortex, later arriving pyramidal cells pass between their predecessors and settle above them, creating an inside-out pattern (Caviness, 1973; Stanfield and Cowan, 1979). In the absence of reelin, the CA1 to CA3 regions of the hippocampus are disorganized, with pyramidal neurons broadly scattered in outside-in order (Caviness, 1973; Stanfield and Cowan, 1979). There is also a disruption of the dentate gyrus and aberrant innervation of the hippocampus from the entorhinal cortex (Del Rio et al., 1997).

Reelin-dependent cellular movements in the cerebellum are somewhat different. Purkinje cells are generated from a basal neuroepithelium, and move outwards along radial glia guides to form a monolayer (D'Arcangelo and Curran, 1998). Reelin is expressed by other neurons positioned below as well as above the migrating Purkinje cells (Miyata et al., 1996; Schiffmann et al., 1997). In the absence of reelin, Purkinje cells do not migrate outwards properly, suggesting that they may be repelled by the deep source of reelin. Later, they arrest movement when they reach the superficial layer of reelin-expressing cells.

All the reelin responses described above involve a common signal transduction pathway that requires one or two lipoprotein receptors [very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (apoER2 or Lrp8)] and an intracellular molecule, Dab1 (Rice and Curran, 1999). Combined mutations of *Vldr* and *Apoer2* or single mutation of *Dab1* phenocopies a *Reln* mutant (Howell et al., 1997b; Sheldon et al., 1997; Trommsdorff et al., 1999; Ware et al., 1997). The 80 kDa Dab1 protein (p80) contains an N-terminal PTB/PID domain that binds in vitro to phospholipids and to the cytoplasmic tails of the VLDLR and apoER2 (Howell et al., 1999b; Trommsdorff et al., 1998). C-terminal to the PTB domain are four potential tyrosine phosphorylation sites, of which at least two, Tyr198 and 220, become phosphorylated in reelin-stimulated cells and in developing brain (Howell et al., 1999a; Howell et al., 2000; Keshvara et al., 2001). These phosphorylations are essential for transduction of the reelin signal (Howell et al., 2000). This suggests a mechanism whereby reelin stimulates tyrosine phosphorylation of Dab1 by

binding to VLDLR, apoER2 and perhaps other receptors (D'Arcangelo et al., 1999; Dulabon et al., 2000; Hiesberger et al., 1999; Senzaki et al., 1999), generating a Dab1 phosphotyrosine-dependent signal to regulate cell movement. In addition, Dab1 protein levels decrease in response to reelin signaling (Howell et al., 1999a; Rice et al., 1998). This decrease is partly independent of Dab1 tyrosine phosphorylation (Howell et al., 2000), suggesting that some other change induced by activated VLDLR and apoER2, for example, possible serine phosphorylation or relocalization of Dab1, triggers changes in the rates of Dab1 synthesis or degradation. Decreased Dab1 protein levels may be part of a negative feedback loop to help cells adapt to the reelin signal.

C-terminal to its PTB domain and tyrosine phosphorylation sites, Dab1 p80 contains approximately 310 residues of more C-terminal sequence with unknown function (Fig. 1A). Within this region, two subdomains show sequence conservation with Dab2, a related protein that functions independently of tyrosine phosphorylation (Xu et al., 1995). The C terminus also includes predicted phosphorylation sites for Cdk5, a protein serine/threonine kinase known to phosphorylate Dab1 in vitro and to function in regulating reelin-dependent migrations (D'Arcangelo et al., 1999; Ohshima et al., 1996). The extreme C terminus also contains the sequence Asp-Pro-Phe (DPF), a potential binding site for clathrin adaptor protein 2 (AP2), which is tantalizing in view of the association of lipoprotein receptors with clathrin-coated pits and vesicles (Krieger and Herz, 1994; Owen et al., 1999). Dab2 also contains DPF sequences through which it co-localizes with AP2 and lipoprotein receptors (Morris and Cooper, 2001). However, the function of none of these C-terminal motifs is known. They may function to promote binding of Dab1 to receptors, to boost tyrosine phosphorylation in response to reelin, to assist interactions with downstream targets, or they may be an evolutionary vestige of a function predating the Dab1/Dab2 duplication in the vertebrate lineage. To address these possibilities, we have created mutant mice that express only the p45 splice variant of Dab1, which is truncated C-terminal to the tyrosine phosphorylation sites (Fig. 1A). p45 RNA is normally expressed in embryonic brain (I. Bar, C. Lambert de Rouvroit and A. Goffinet, personal communication) (Howell et al., 1997a), but the p45 protein has not been detected. We find that the Dab1 p45 protein supports normal brain development, indicating that the C terminus of p80 is not required for reelin-dependent neuronal migrations. However, certain neuronal migrations are disrupted when the truncated protein is expressed at single copy. Therefore, the p80 C terminus is needed for efficient signaling in specific neurons. Moreover, the phenotype of p45 hemizygotes lends new support for the 'stop signal' hypothesis for reelin function in the neocortex and hippocampus.

## MATERIALS AND METHODS

### Generation of mutant mice

Construction of the p45 knock-in vector was performed as previously described for the wild-type (WT) and 5F alleles of Dab1 p80 (Howell et al., 2000), except that the 271 codon cDNA was used instead of the 555 codon cDNA (Howell et al., 1997a). A *BglIII-PstI* fragment was first cloned from pBS271 Dab1 to pBS555 Dab1 (Howell et al.,

1997a) in order to change the orientation of the cDNA relative to the polylinker. The *Bgl*III site is in the open reading frame of *Dab1*, 5' to the p45-specific exon, and the *Pst*I site is in the 3' untranslated region. The knock-in vector contains the p45 cDNA (from the second coding exon), followed by a strong polyadenylation signal and preceded by a neomycin phosphotransferase selectable marker that is flanked by loxP sites (Howell et al., 2000). Electroporation of 129S4-derived AK7 embryonic stem cells and identification of homologous recombinants was performed as previously described (Howell et al., 2000). Chimeric animals were generated using standard techniques and mated to C57BL/6J animals expressing Cre recombinase in the germline (*Meox2<sup>Cre/+</sup>*) (Tallquist and Soriano, 2000). Agouti pups were genotyped for the presence of both the p45 knock-in cDNA and *Meox2<sup>Cre</sup>*. Double heterozygous animals were then mated to each other and the loss of *Meox2<sup>Cre</sup>* and the loxP-flanked neomycin phosphotransferase marker was confirmed. *Dab1<sup>p45</sup>* animals used in this study were bred for multiple generations on a mixed background 129S4×C57BL/6J. *Reln*, *Dab1<sup>-/-</sup>*, *Dab1<sup>WT</sup>* and *Dab1<sup>5F</sup>* have been described elsewhere (Howell et al., 1997b; Howell et al., 1999a; Howell et al., 2000).

### Histology

Brains were dissected from 20-day-old littermates and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for a minimum of 2 days at 4°C. The fixative was changed once after 24 hours. Brains were dehydrated in ethanol, cleared in HistoClear and embedded in paraffin. Sections (7 µm) were taken and Nissl (Cresyl Violet) or Hematoxylin and Eosin staining was performed using standard techniques. Coronal sections in the region of the septal hippocampus are shown in Fig. 2 and Fig. 3.

Immunohistochemistry for CSPG was carried out according to Rice et al. (Rice et al., 1998) except that, after deparaffinization and rehydration, antigen was retrieved by submerging the slides in boiling 10 mM citric acid (pH 6.0) twice for 5 minutes. Endogenous peroxidases were quenched for 30 minutes in 0.3% H<sub>2</sub>O<sub>2</sub> diluted in methanol. The sections were then washed in PBS with 0.25% Tween 20 (PBST), blocked in 1.5% horse serum for 2 hours and incubated overnight with anti-CSPG (Sigma) diluted 1:1,000 in PBST at 4°C. After washing three times for 10 minutes in PBST, sections were processed with the Vectastain Elite ABC kit (Vector Laboratories) using nickel enhancement for 45 seconds.

### Cell birthdating

*Dab1<sup>p45/+</sup>* and *Dab1<sup>p45/-</sup>* mice were mated, and timed-pregnant females were injected intraperitoneally with a single dose of 150 µg per g body weight of BrdU solution (10 mg/ml in 0.9% NaCl, 7 mM NaOH) at E12.5 or E16.5. Twenty days after birth, brains were fixed for 2 days in 4% paraformaldehyde, cryo-protected in 10%, 20% and 30% sucrose in PBS, and frozen in tissue freezing medium. Coronal sections (20 µm) were mounted on Fisher Superfrost/Plus slides, placed in 100% ethanol for 3 minutes at -20°C, and air-dried. Slides were incubated in 0.1% trypsin 2 mM EDTA at 37°C for 1 hour, washed in PBS, incubated in 2 M HCl for 1 hour at room temperature, and washed several times in PBS until the pH was neutral. Sections were blocked overnight in PBST (PBS + 0.01% Triton X-100) with 10% calf serum, then incubated sequentially with: anti-BrdU (Becton Dickinson; diluted 1:25 in PBST with 5% calf serum); sheep-anti mouse (Jackson Labs; diluted 1:100 in PBST with 10% calf serum); and donkey-anti sheep FITC conjugated (Jackson Labs; diluted 1:400 in PBST with 10% calf serum). Each incubation was for 1 hour, separated by three 5 minute washes in PBST. Sections were mounted using ProLong Antifade (Molecular Probes, Eugene, OR) and photographed by epifluorescence.

### Immunoprecipitations and western blotting

Briefly, E16.5 or P20 cerebral hemispheres were homogenized in neural RIPA (0.15 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl

sulfate (SDS), 1% sodium deoxycholate, 10 mM sodium phosphate (pH 7.4), 2 mM EDTA, 14 mM 2-mercaptoethanol, 50 mM NaF, 20 µg/ml aprotinin, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylarsine oxide and 0.1 mM PMSF) at 1 ml per 80 mg tissue weight. After lysis on ice for 10 minutes, the samples were clarified by centrifugation at 17,000 g for 30 minutes and normalized for protein content. 150-250 µg of protein were rotated overnight with 1 µl affinity-purified anti-Dab1(B3) antibody (Howell et al., 1997a), and 20 µl of 50% suspension protein A Sepharose at 4°C. The following day, beads were washed three times in neural RIPA. Immunoprecipitations and total lysates were separated by 10% SDS polyacrylamide gel electrophoresis followed by western blotting with either anti-Dab1(B3) or anti-phosphotyrosine (4G10) antibodies.

### Primary neuron cultures and reelin stimulation

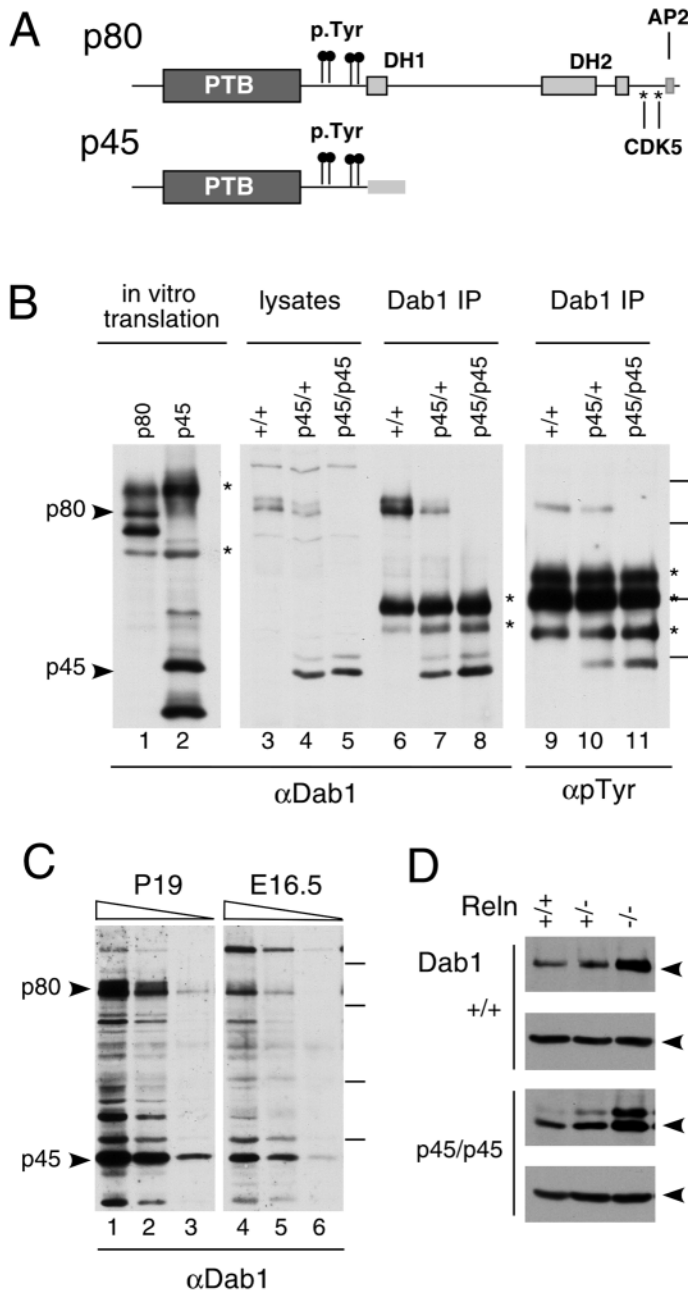
Reelin was collected as previously described (Howell et al., 1999a), with modifications. 293T cells were plated on 10 cm tissue culture dishes and grown until 70% confluent. They were then transfected with 2 µg of pVA-1 (Hiesberger et al., 1999) and 20 µg of either a reelin-encoding plasmid, pCRL (D'Arcangelo et al., 1997) or pCDNA3 (used as a control). Twenty-four hours later, the cells were re-plated in 3 ml of Opti-MEM (GibcoBRL) on 6 cm bacterial dishes. Reelin or control supernatant was collected once a day for up to 3 days. Fresh Opti-MEM was replaced with each collection. Reelin protein expression was confirmed by polyacrylamide gel electrophoresis and western blotting using a mixture of three reelin antibodies (G10, 74 and 742) (de Bergeyck et al., 1998). On occasion, reelin protein was purified by ammonium sulfate precipitation (Keshvara et al., 2001).

E16.5 embryos were collected from crosses between *Dab1<sup>p45/p45</sup>* and wild-type animals. Cerebral hemispheres were dissected and neurons were harvested and cultured essentially as previously described with the following modifications. Cerebral hemispheres from each embryo were homogenized by pipetting in 0.5 ml of 0.1% Trypsin diluted in Hanks Balanced Salt Solution (HBSS, GibcoBRL), and incubated at 37°C for 15 minutes. Samples were placed on ice and 0.2 ml of STI solution (0.4 mg/ml soybean trypsin inhibitor, 0.25 mg/ml DNaseI, 0.3% BSA and 12 mM MgSO<sub>4</sub> in HBSS) added. Following gentle centrifugation, the pellet was washed once in 0.7 ml of HBSS then resuspended in 0.4 ml of STI solution. Clumps were disrupted by passage through a flamed glass Pasteur pipette with a reduced opening. After a final centrifugation, cells were resuspended in 0.5 ml of neuron growth media (Neurobasal media (GibcoBRL) containing 2% B27 supplement (Collaborative Research), 500 µM glutamine, 25 µM glutamic acid and penicillin/streptomycin). Cells from individual embryos were pooled and plated in neuron growth media on dishes pre-coated for 1 hour at 37°C with poly-L-lysine and E-C-L cell attachment matrix (Upstate Biochemicals). The neurons were allowed to differentiate for 4-5 days, with one medium change after 2 days. Cells were treated with reelin-conditioned media or control supernatant for the times and doses indicated. Immunoprecipitations and western blotting were performed as described above.

## RESULTS

### Construction of *Dab1<sup>p45</sup>* allele and expression of *Dab1* p45

Previously, mice were created in which wild-type (WT) or phosphorylation site (5F) mutant forms of *Dab1* p80 were expressed from cDNAs inserted into the *Dab1* gene (Howell et al., 2000). To study the function of the p80 C terminus, the 271 codon cDNA for the p45 splice form of *Dab1* (Howell et al., 1997a) was similarly inserted into the *Dab1* gene (see Materials and Methods). This allele is called herein *Dab1<sup>p45</sup>*.



**Fig. 1.** Expression of Dab1 p80 and p45. (A) Schematic of p80 and p45 protein structures, showing PTB domain, tyrosine phosphorylation sites (p.Tyr), two regions of high sequence identity to Dab2 (DH1 and DH2), and predicted sites for Cdk5 phosphorylation and AP2 binding. (B) SDS PAGE analysis of samples from wild-type, *Dab1*<sup>p45/+</sup> heterozygous and *Dab1*<sup>p45/p45</sup> homozygous cerebral hemispheres sampled at E16.5. Brain lysates were analyzed directly (lanes 3-5) or after immunoprecipitation with anti-Dab1(B3) antibody (lanes 6-11). The 555-codon and 271-codon Dab1 mRNAs were translated in vitro and analyzed in parallel (lanes 1 and 2, respectively). Proteins were detected by western blotting with anti-Dab1(B3) antibody (lanes 1-8) or anti-phosphotyrosine (4G10) (lanes 9-11). Bands below p80 and p45 in lanes 1 and 2 represent internal initiation sites. Bands above p80 and p45 in lanes 3-11 may represent phosphorylated forms. Asterisks mark nonspecific bands. (C) Relative levels of expression of p80 and p45. *Dab1*<sup>p45/WT</sup> brains were harvested at P19 and E16.5. Serial threefold dilutions of each brain lysate were analyzed by SDS PAGE and western blotting with anti-Dab1(B3) antibody. (D) Effect of *Reln* mutation on expression of p80 and p45. Littermate embryos from a *Dab1*<sup>p45/+</sup> *Reln*<sup>-/+</sup> intercross were harvested at E16.5 and cerebral hemispheres were analyzed for Dab1 content. Tubulin was used as a loading control. Genotypes were *Dab1*<sup>+/+</sup> (top two panels) or *Dab1*<sup>p45/p45</sup> (bottom two panels), and wild type, heterozygous or mutant for *Reln*. Molecular mass standards were 95, 70, 54 and 47 kDa.

respectively). This suggests that the p45 protein is not normally expressed at a significant level, even though its mRNA has been detected in embryonic brain cDNA libraries (I. Bar, C. Lambert de Rouvroit and A. Goffinet, personal communication) (Howell et al., 1997a).

To determine whether p45 is tyrosine phosphorylated, immunoprecipitates were prepared from cerebral hemispheres using antibody to Dab1, and analyzed by western blotting with anti-Dab1 or anti-phosphotyrosine antibody (Fig. 1B, lanes 6-11). p80 and p45 were phosphorylated to similar stoichiometry.

Reelin signaling normally causes a reduction in Dab1 p80 levels, detected as a significant increase in Dab1 p80 in *Reln*<sup>-/-</sup> or *apoER2*<sup>-/-</sup> *Vldlr*<sup>-/-</sup> embryo brain samples (Howell et al., 1999a; Rice et al., 1998; Trommsdorff et al., 1999), with little, if any, change in Dab1 mRNA (Rice et al., 1998). We noticed that p45 is expressed at higher levels than p80 (Fig. 1B, lanes 3-5), suggesting that the C terminus of p80 may inhibit translation or reduce protein stability. To control for possible changes in mRNA levels resulting from the structure of the *Dab1*<sup>p45</sup> allele, the *Dab1*<sup>WT</sup> allele, which is identical but for the absence of the p45-specific 270 bp exon, was used for comparison (*Dab1*<sup>WT</sup> encodes p80) (Howell et al., 2000). *Dab1*<sup>p45/WT</sup> embryos and pups were generated, and cerebral hemisphere proteins were analyzed (Fig. 1C). As western blotting does not provide a linear response, different protein quantities were analyzed, and similar intensity bands compared. The values were adjusted for the efficiency of blotting and antibody detection, using as controls in vitro translated p80 and p45. The quantity of p45 was  $2.8 \pm 1.4$  ( $n=3$ ) times the level of p80 at E16.5 and  $2.5 \pm 0.07$  ( $n=3$ ) times the level of p80 at P19. Therefore, removal of the C terminus from p80 permits expression at higher level. However, when the *Dab1*<sup>p45</sup> allele was bred into a *Reln* mutant background, the levels of p45 protein increased (Fig. 1D, upper) to a similar

The p45 protein diverges from p80 at residue 241, replacing the C-terminal 314 residues of p80 with 30 unique residues from a p45-specific exon.

*Dab1*<sup>p45/+</sup> heterozygotes and *Dab1*<sup>p45/p45</sup> homozygotes were born at expected frequency. Unlike null mutants of *Reln* or *Dab1*, *Dab1*<sup>p45/p45</sup> homozygotes have normal life span, can breed and show no apparent neurological deficits. To confirm p45 protein expression, embryos from a heterozygous *Dab1*<sup>p45/+</sup> intercross were sacrificed at E16.5 and lysates were prepared from their cerebral hemispheres. Western blots showed that only p45 is expressed in the cerebral hemispheres of *Dab1*<sup>p45</sup> homozygotes, with no sign of read through or splicing to make p80 (Fig. 1B, lane 5). p45 co-migrates with the in vitro translated product of the 271-codon cDNA clone (lane 2). Heterozygous animals express both p45 and p80, while wild-type animals express only p80 (lanes 4 and 3,

extent as p80 (Fig. 1D, lower), indicating that the p45 protein is still subject to reelin-dependent down-regulation.

### Defective neocortex and hippocampus in *Dab1*<sup>p45</sup> hemizygous brain

Histological analysis of *Dab1*<sup>p45/p45</sup> brains revealed no defects (Fig. 2, Fig. 3 and data not shown). This indicates that the C terminus of p80 is dispensable for normal development. *Dab1*<sup>p45/p45</sup> or *Dab1*<sup>p45/+</sup> were bred to *Dab1*<sup>+/-</sup>, to generate *Dab1*<sup>p45/-</sup> hemizygotes. These animals are also overtly normal and there are no defects in the cerebellum (data not shown), which is grossly disorganized and underdeveloped in *Dab1*<sup>-/-</sup> (Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997). However, two reproducible defects were observed in *Dab1*<sup>p45/-</sup> hemizygotes but not in *Dab1*<sup>+/-</sup> hemizygotes that were reproducible in all individuals analyzed in detail (Fig. 2A, Fig. 3A). These dose-sensitive phenotypes mean that *Dab1*<sup>p45</sup> is a hypomorphic allele.

One phenotype is a distinct splitting in the stratum pyramidale (SP) in the CA1 region of the hippocampus (Fig. 2A,A'). A second pyramidal layer (arrow) lies closer to the hippocampal fissure (circle) and further from the alveus (square), which mark the former outer and inner edges of the structure. The ectopic pyramidal layer becomes the main layer in the CA2 region. The CA3 region and the dentate gyrus appear to develop properly. The hippocampus of *Dab1*<sup>p45/-</sup> animals is markedly different from *Dab1*<sup>p45/+</sup> heterozygotes (Fig. 2B,B'), *Dab1*<sup>p45/p45</sup> homozygotes (Fig. 2C) or *Dab1*<sup>+/-</sup> hemizygotes (Fig. 2E). It is also different from that of *Dab1*<sup>-/-</sup>,

*Reln*<sup>-/-</sup> or *Dab1*<sup>5F/5F</sup> animals, in which pyramidal cells are scattered over a broad region and the dentate is disrupted (Fig. 2D and data not shown).

The second defect is in the neocortical marginal zone. In *Dab1*<sup>p45/-</sup> hemizygotes, the marginal zone is thin and crowded with small cells (Fig. 3A, arrows), compared with *Dab1*<sup>p45/+</sup> heterozygotes (Fig. 3B), *Dab1*<sup>p45/p45</sup> homozygotes (Fig. 3C) or *Dab1*<sup>+/-</sup> hemizygotes (Fig. 3E). Ostensibly, the marginal zone resembled those of *Dab1*<sup>-/-</sup>, *Reln*<sup>-/-</sup> or *Dab1*<sup>5F/5F</sup> animals, which are also packed with polymorphic cells (Fig. 3D and data not shown).

To determine whether the effect of reducing *Dab1*<sup>p45</sup> gene dosage could be mimicked by limiting reelin signaling in a *Dab1*<sup>p45/p45</sup> homozygote, *Dab1*<sup>p45/p45</sup> *Reln*<sup>+/-</sup> animals were analyzed. However, the hippocampus and neocortex appeared normal (Fig. 2F, Fig. 3F).

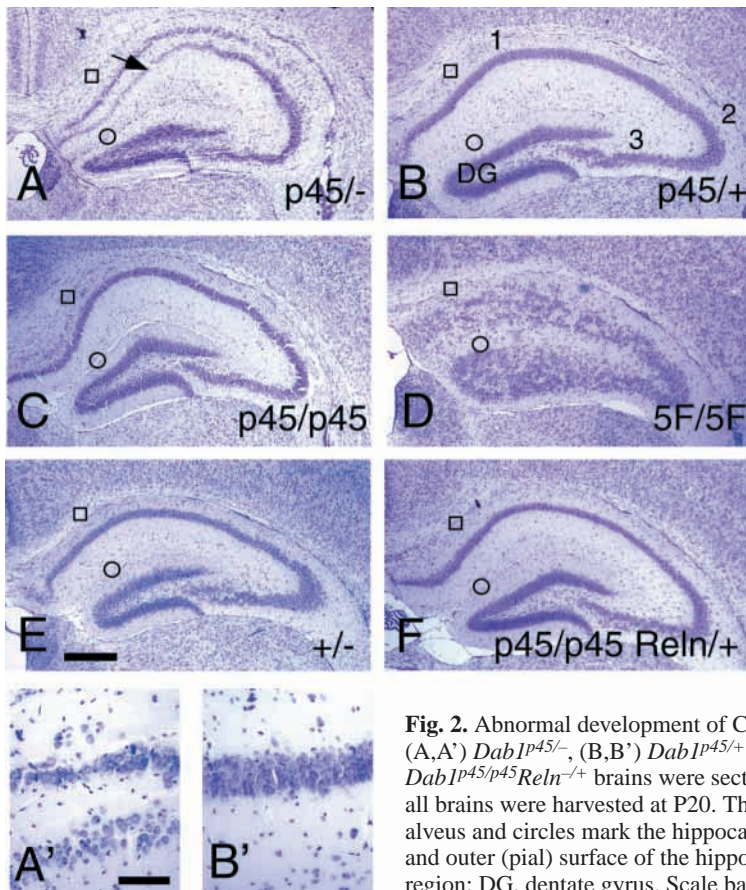
Despite the apparent similarities between the marginal zones of *Dab1*<sup>p45/-</sup> and *Dab1*<sup>-/-</sup> animals, further analysis indicated different underlying causes. First, we assessed whether the preplate split into two layers, using chondroitin sulphate proteoglycan (CSPG) as a marker for preplate derivatives (Sheppard and Pearlman, 1997). E16.5 embryos from a cross of *Dab1*<sup>p45/+</sup> and *Dab1*<sup>p45/-</sup> individuals were collected, sectioned and immunostained with CSPG antibody (Fig. 4). In animals of all genotypes tested, the preplate had split to subplate and marginal zone. This suggests that the C terminus of p80 is not needed to split the preplate.

Next, we performed birthdate studies to determine whether the cortical plate is assembled 'inside-out' or 'outside-in'.

Again, *Dab1*<sup>p45/+</sup> and *Dab1*<sup>p45/-</sup> animals were crossed, and pregnant females were injected with a single dose of 5-bromodeoxyuridine (BrdU) on either E12.5 or E16.5. The progeny were sacrificed on P20 and cryostat sections stained for BrdU (Fig. 5). The neuronal layering of *Dab1*<sup>p45/p45</sup> homozygotes appears to be normal (data not shown). Defects were apparent in *Dab1*<sup>p45/-</sup> hemizygotes. In these animals, early-born neurons (labeled at E12.5) migrated appropriately to deep regions of the cortical plate (Fig. 5A), as in the control (Fig. 5C). However, late born neurons (labeled on E16.5) migrated not only into the upper levels of the cortical plate but also into the marginal zone (Fig. 5B). By contrast, in the control, late neurons were only in the upper cortical plate (Fig. 5D). Thus, *Dab1*<sup>p45/-</sup> hemizygotes show normal 'inside-out' layering, and the excess marginal zone neurons are late-born CP neurons, unlike *Reln*<sup>-/-</sup>, *Dab1*<sup>-/-</sup> and *apoER2*<sup>-/-</sup>*Vldlr*<sup>-/-</sup>, where the marginal zone neurons are born early (Caviness, 1982; Caviness and Sidman, 1973; Howell et al., 1997b; Trommsdorff et al., 1999).

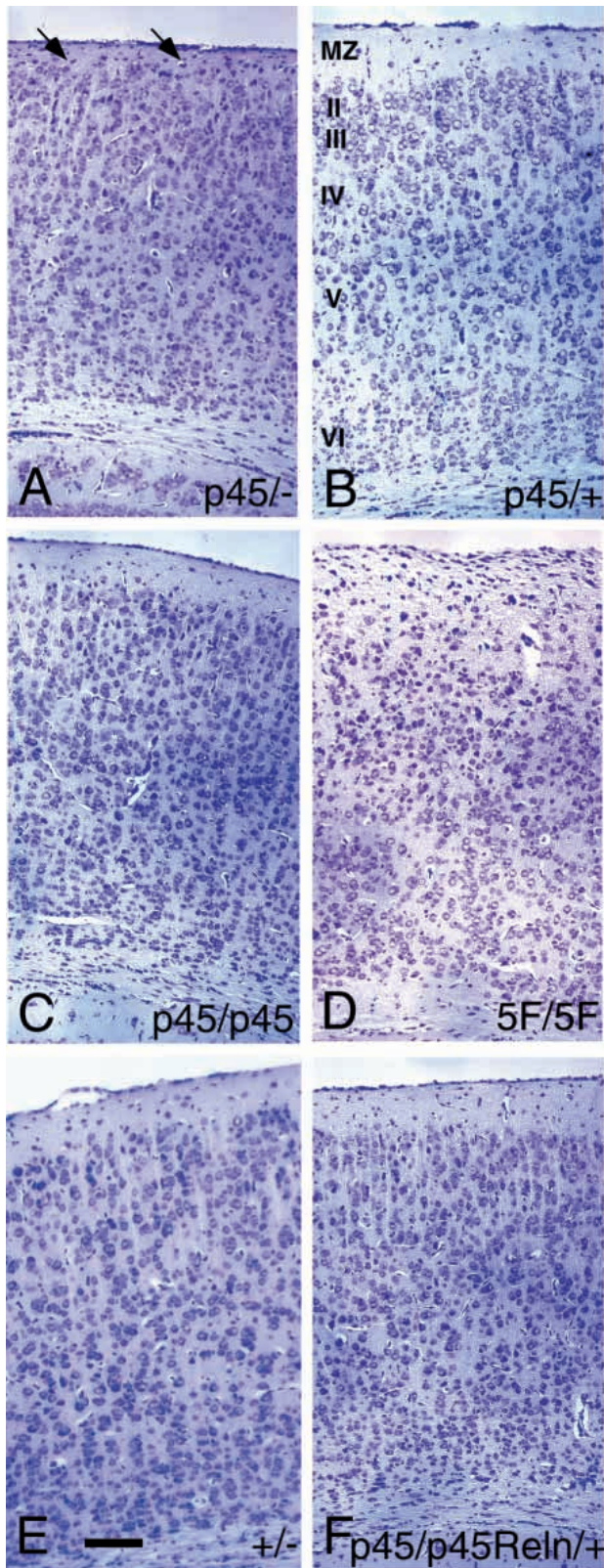
### Reelin signaling via *Dab1* p45

The defects in *Dab1*<sup>p45/-</sup> hemizygotes may result from

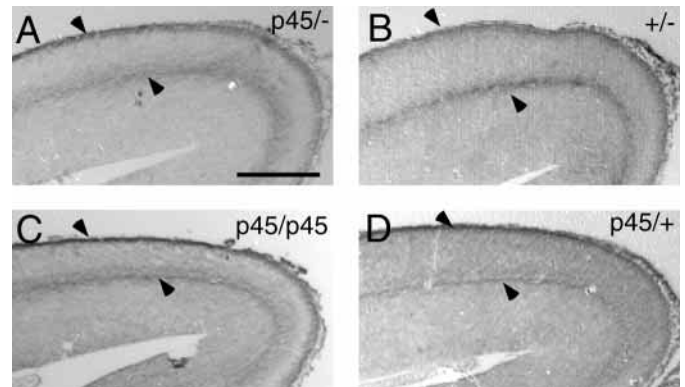


**Fig. 2.** Abnormal development of CA1 region of hippocampus in *Dab1*<sup>p45/-</sup> hemizygotes.

(A,A') *Dab1*<sup>p45/-</sup>, (B,B') *Dab1*<sup>p45/+</sup>, (C) *Dab1*<sup>p45/p45</sup>, (D) *Dab1*<sup>5F/5F</sup>, (E) *Dab1*<sup>+/-</sup> and (F) *Dab1*<sup>p45/p45</sup> *Reln*<sup>+/-</sup> brains were sectioned and stained with Nissl. Animals A-C were littermates, and all brains were harvested at P20. The arrow indicates ectopic pyramidal cell layer, squares mark the alveus and circles mark the hippocampal fissure. These provide landmarks for the inner (ventricular) and outer (pial) surface of the hippocampus. Abbreviations: 1, CA1 region; 2, CA2 region; 3, CA3 region; DG, dentate gyrus. Scale bar: 400  $\mu$ m in A-F; 25  $\mu$ m in A',B'.



**Fig. 3.** Abnormal development of neocortex of *Dab1*<sup>p45/-</sup> hemizygotes. (A) *Dab1*<sup>p45/-</sup>, (B) *Dab1*<sup>p45/+</sup>, (C) *Dab1*<sup>p45/p45</sup>, (D) *Dab1*<sup>5F/5F</sup>, (E) *Dab1*<sup>+/-</sup> and (F) *Dab1*<sup>p45/p45</sup> *Reln*<sup>-/+</sup> brains were sectioned and stained with Nissl. Arrows indicate ectopic neurons in the marginal zone. Abbreviations: MZ, marginal zone (layer I); II–VI, layers II–VI of the cortical plate. Scale bar: 100  $\mu$ m.



**Fig. 4.** Normal preplate splitting in *Dab1*<sup>p45/-</sup> hemizygotes. Immunohistochemical detection of chondroitin sulphate proteoglycan (CSPG) in sagittal sections of E16.5 brains. (A) *Dab1*<sup>p45/-</sup>, (B) *Dab1*<sup>+/-</sup>, (C) *Dab1*<sup>p45/p45</sup> and (D) *Dab1*<sup>p45/+</sup> littermate brains. Arrowheads indicate CSPG staining of marginal zone and subplate. Scale bar: 200  $\mu$ m.

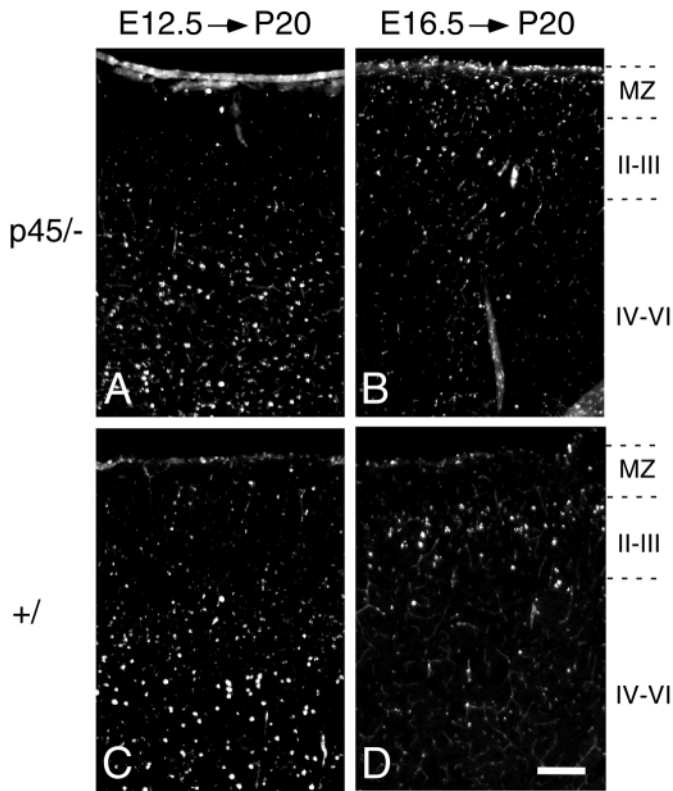
reduced reelin signaling. To test whether tyrosine phosphorylation of p45 requires higher reelin doses or longer times than p80, we examined the dose-response and kinetics of p45 phosphorylation in vitro. To provide an internal control, we made use of cerebral neurons from E16.5 *Dab1*<sup>p45/+</sup> embryos, which express both p80 and p45. Neuronal cultures were stimulated with reelin-containing or control media. Cell lysates were then immunoprecipitated with anti-Dab1 antibody and tyrosine phosphorylation was assayed by western blotting. The tyrosine phosphorylation of Dab1 p45 paralleled that of p80 at different doses of reelin (Fig. 6A) or at different times of treatment (Fig. 6B). This indicates that p45 and p80 are equivalently phosphorylated in response to reelin, and suggests that the phenotype of *Dab1*<sup>p45/-</sup> brains is due to decreased downstream signal transmission by p45.

## DISCUSSION

The 555-codon splice form of *Dab1*, encoding p80, has previously been shown to be sufficient for normal brain development (Howell et al., 2000). We now show that the 271-codon splice form, encoding p45, is also sufficient for known reelin-dependent cell migrations. Thus, just the PTB domain and tyrosine phosphorylation sites are adequate for normal Dab1 protein function, and the C terminal 314 residues of p80, including predicted Cdk5 phosphorylation sites, homology regions with Dab2, and motifs that interact with endocytosis machinery, are dispensable. However, p45 is quantitatively less effective at signaling than p80: when the gene dose of *Dab1*<sup>p45</sup> but not *Dab1*<sup>p80</sup> is reduced, the positions of specific neurons are abnormal. Therefore, the C terminus of Dab1 p80 that is absent from p45 plays a quantitative role in some but not all reelin-dependent neuronal migration events.

### Signaling by the Dab1 p80 C terminus

P45 expression was two- to threefold higher than p80, even when comparing *Dab1*<sup>p45</sup> and *Dab1*<sup>WT</sup> knock-in alleles with comparable gene structure. This higher expression may have partially obscured the reduced efficiency of Dab1 p45 function



**Fig. 5.** Late cortical plate neurons in marginal zone of *Dab1*<sup>p45/-</sup> hemizygotes. *Dab1*<sup>p45/-</sup> and *Dab1*<sup>p45/+</sup> mice were crossed and pregnant females injected with BrdU on E12.5 (A,C) or E16.5 (B,D). (A,B) *Dab1*<sup>p45/-</sup>, (C) *Dab1*<sup>+/+</sup> and (D) *Dab1*<sup>p45/+</sup> littermates were sacrificed on P20 and BrdU-labeled nuclei detected by immunofluorescence. Note that, in both *Dab1*<sup>p45/-</sup> and control, nuclei labeled at E12.5 are deep in layers IV-VI and nuclei labeled at E16.5 are shallow in layers II-III. Neurons labeled at E16.5 migrate into the marginal zone (MZ) in *Dab1*<sup>p45/-</sup> but not in controls. Scale bar: 100  $\mu$ m.

revealed in hemizygotes. The increased level of p45 may be due to increased translation or increased stability. However, the p45 protein is still subject to the post-transcriptional controls that normally downregulate p80 levels in response to reelin (Howell et al., 1999a; Rice et al., 1998).

Dab1 p45 and p80 appear to be phosphorylated to a similar stoichiometry in vivo. Whether comparing brains in which only p45 or only p80 is expressed, or brains in which neurons

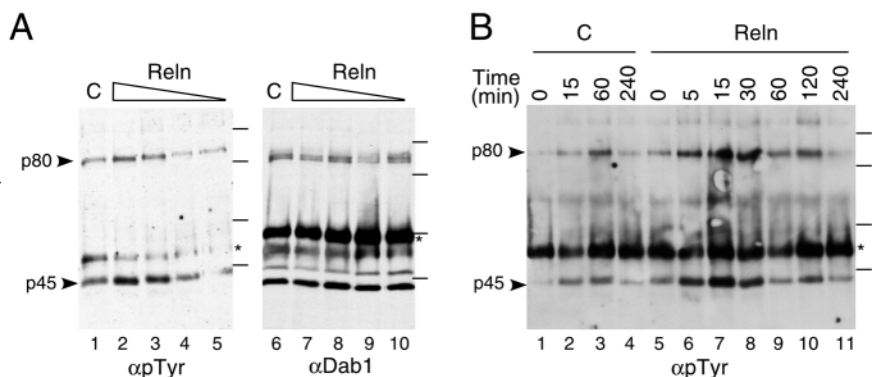
express both proteins (*Dab1*<sup>p45/+</sup>), the ratio of phosphotyrosine to Dab1 protein was similar. This implies that Dab1 tyrosine phosphorylation does not require the Dab1 p80 C terminus. This conclusion was substantiated in vitro, when neurons expressing both the p80 and p45 were stimulated with reelin. The time course and concentration dependence of reelin-induced tyrosine phosphorylation of p45 paralleled that of p80. Thus, the C terminus of p80 is unlikely to be important for promoting interaction between Dab1 and cell-surface reelin receptors or tyrosine kinases. Instead, the reduced efficiency of p45 in mediating reelin signaling in late-born CP neurons and a subset of CA1 pyramidal cells is possibly due to a decrease in signal relay downstream from tyrosine phosphorylated Dab1. One could speculate that the C terminus of p80 cooperates with the Dab1 tyrosine phosphorylation sites to assemble a signaling complex that controls cell movement. Test of this model will require identification of proteins that bind to the tyrosine phosphorylation sites and to the C terminus.

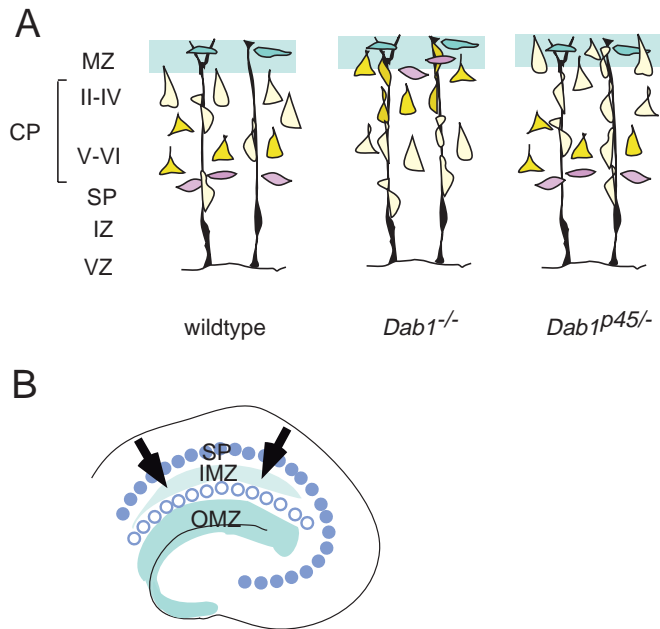
### A novel brain development phenotype

*Dab1*<sup>p45</sup> is the first hypomorphic allele of a gene in the reelin pathway, and the phenotype is novel. The appearance of a *Dab1*<sup>p45/-</sup> hemizygous brain is not simply intermediate between a *Dab1*<sup>-/-</sup> or *Reln*<sup>-/-</sup> and a wild-type brain. The phenotype has certain unexpected and striking features. First, layer I of the neocortex contains late-born CP neurons (Fig. 7A), while in *Dab1*<sup>-/-</sup>, *Dab1*<sup>5F/5F</sup>, *Vldlr*<sup>-/-</sup>*apoER2*<sup>-/-</sup> or *Reln*<sup>-/-</sup> brains layer I is populated by preplate and early-born CP neurons. Second, the stratum pyramidale (SP) of the CA1 region is split (Fig. 7B), while in *Dab1*<sup>-/-</sup>, *Dab1*<sup>5F/5F</sup>, *Reln*<sup>-/-</sup> or *apoER2*<sup>-/-</sup> brains, the SP neurons are scattered in one or more broad bands. The tight alignment of the pyramidal cells in each of the two SPs of the mutant brain is unique. However, *Dab1*<sup>p45/-</sup> brains show proper placement of CA3 pyramidal cells, dentate granule cells, Purkinje cells in the cerebellum, and subplate and early-born CP neurons in the neocortex. The current analysis has revealed defects only in the CA1 pyramidal cells and the late-born CP neurons.

We consider two explanations for why the C terminus of p80 may be quantitatively more important in some cell types but not others. First, some neurons, including Purkinje cells, dentate granule cells and early-born CP neurons, might express signaling molecules that compensate for the reduced signaling activity of p45. There are several precedents for cell-type specific signaling molecules that regulate neuronal migrations.

**Fig. 6.** Reelin response of cultured cerebral neurons. Neurons were cultured from *Dab1*<sup>p45/+</sup> embryos, which express both p80 and p45, and treated with reelin-containing (Reln) or control (C) supernatant from 293T cells. Samples were immunoprecipitated with anti-Dab1(B3) and analyzed by SDS PAGE and western blotting with anti-Dab1(B3) (part A, lanes 6-10) or anti-phosphotyrosine (part A, lanes 1-5 and part B). (A) Incubation with serial fourfold dilutions of reelin-containing supernatant for 15 minutes, or (B) with undiluted reelin-containing supernatant for various times. Asterisks to the right of each panel mark nonspecific bands. Molecular mass standards were 95, 70, 54 and 47 kDa.





**Fig. 7.** Working models to explain over-migration of late cortical plate neurons and split hippocampus in *Dab1*<sup>p45/-</sup> hemizygotes. (A) Positions of different classes of neocortical neurons at birth for wild type (left), *Dab1*<sup>-/-</sup> or *Reln*<sup>-/-</sup> (center) and *Dab1*<sup>p45/-</sup> (right). At this time, the latest cortical plate (CP) neurons (pale yellow) are migrating up radial glia (black) from the ventricular zone (VZ) towards the marginal zone (MZ). Cajal-Retzius neurons (dark green) in the MZ have previously secreted reelin (pale green), although reelin production in the MZ declines after birth. In wild type, reelin excludes CP neurons and stimulates their release from radial glia. Because the early CP neurons (dark yellow) have already been released from the glia, later CP neurons (light yellow) can migrate past them until they reach the MZ. In *Dab1*<sup>-/-</sup> or *Reln*<sup>-/-</sup>, early CP neurons enter the MZ and only release slowly from radial glia. The ability of late CP neurons to respond to reelin is unknown, because they are held up below the traffic jam caused by the early-born CP neurons. In addition, the subplate neurons (purple) are pushed into the MZ. In *Dab1*<sup>p45/-</sup>, early-born CP neurons do release from the radial glia, and the inability of the late CP neurons to respond to reelin is evident from their entry into the MZ. Release of the late CP neurons from radial glia may also be defective, and cause a traffic jam as shown. (B) Migration paths and settling points of pyramidal neurons in the hippocampus. Normally, pyramidal cells (dark blue) settle in the stratum pyramidale (SP), which is proximal to the IMZ in the CA1 region. At the time of arrival of these cells, the IMZ is expressing a low amount of reelin (pale green). In *Dab1*<sup>p45/-</sup>, a significant population of pyramidal cells passes through the SP and forms a second layer (open blue symbols). We suggest that this layer may form at the edge of the OMZ, where higher concentrations of reelin are expressed (dark green). As the hippocampus matures, the OMZ becomes the stratum lacunosum moleculare and the IMZ becomes the stratum radiatum.

For example, the Cdk5 regulatory subunit p35 is needed for proper inside-out lamination of the CP but not for a normal marginal zone, preplate splitting, hippocampus or cerebellar development (Chae et al., 1997; Kwon and Tsai, 1998). ApoER2 is more important than VLDLR in the neocortex and hippocampus, while the roles are reversed in the cerebellum (Trommsdorff et al., 1999). The *cdf* gene is required for the reelin-dependent migration of a subset of zebrin II-negative

Purkinje cells but not for migration of zebrin II-positive cells (Beierbach et al., 2001). It is not known whether any of these molecules interacts with p80. Recently, early- and late-born CP neurons have been shown to express different gene products (Tarabykin et al., 2001). The early CP neurons arise by divisions in the ventricular zone and express *Otx1*, while late CP neurons undergo further divisions in the subventricular zone (between the ventricular zone and subplate) and express *Svet1* before migrating into the CP. Gene expression differences such as these might explain the different requirements of early and late CP neurons for the C terminus of p80.

A second potential explanation for the distinctive phenotype of *Dab1*<sup>p45/-</sup> brains is quantitative: the strength of the reelin signal may differ in different parts of the brain and at different times during development. In the neocortex, reelin is highly expressed by CR neurons until birth, but expression then declines in layer I and increases in layers V-VI (Alcantara et al., 1998). Late-born CP neurons (E16.5 and later) take 3 days to reach the boundary of layer I (Caviness, 1982; Caviness and Sidman, 1973), so they arrive when reelin levels are declining in layer I but increasing in layers V-VI. If the absence of the p80 C terminus in *Dab1*<sup>p45/-</sup> neurons quantitatively impairs reelin signaling, then the late arrivals may be more affected than early ones.

Regional differences in reelin levels may also explain the *Dab1*<sup>p45/-</sup> phenotype in the CA1 region of the hippocampus (Fig. 7B). Although reelin is present at the highest level in the outer MZ (OMZ), it is also expressed in the layer below the OMZ, the inner MZ (IMZ), at E18 (Alcantara et al., 1998). The earliest pyramidal cells arrive at this time (Stanfield and Cowan, 1979), and settle below the IMZ. The last pyramidal cells arrive after birth when expression of reelin in all layers is declining (Alcantara et al., 1998; Stanfield and Cowan, 1979). Nonetheless, they also settle below the IMZ (which becomes the stratum radiatum) to form the upper part of the SP. In the *Dab1*<sup>p45/-</sup> mutant, a set of CA1 neurons moves beyond the normal position of the SP to a second tight zone, which may correspond to the edge of the OMZ. It is thus possible that these cells do not respect the low reelin signal from the IMZ but do sense the higher reelin signal from the OMZ. This interpretation is consistent with an increased reelin requirement of cells expressing *Dab1* p45, and a model in which the C terminus of *Dab1* p80 promotes interaction with downstream signaling molecules.

### The 'stop signal' model

The entry of late-born CP neurons into layer I in *Dab1*<sup>p45/-</sup> brains implies that these neurons normally require reelin signaling to prevent over-migration. This lends support to the 'stop signal' explanation for inside-out layering of the wild-type neocortex (Frotscher, 1997; Ogawa et al., 1995; Pearlman and Sheppard, 1996; Schiffmann et al., 1997). According to this model (Fig. 7A, left), both early-born and late-born CP neurons are subject to the same migratory controls, advancing until they reach the reelin-producing CR cells in layer I. Although this model explains one aspect of the *Dab1*<sup>-/-</sup> and *Reln*<sup>-/-</sup> mutant phenotypes – why layer I is crowded with early CP and preplate neurons – it does not explain why late CP neurons accumulate below the early CP neurons, causing 'outside-in' layering (Fig. 7A, center). One proposal is that



reelin signaling normally releases CP neurons from radial glia, and in *Reln*<sup>-/-</sup> mutants, earlier CP neurons remain attached and obstruct the movement of later neurons (Pinto-Lord et al., 1982). The positioning of late-born CP neurons in reelin signaling mutants may thus be secondary to defects in early neurons. An alternative explanation is a 'chemoattractant' model: as late born neurons enter the bottom of the CP, they detect reelin diffusing down from layer I and respond by migrating past earlier CP neurons (Sheppard and Pearlman, 1997). In the absence of such a chemoattractive signal, the later neurons accumulate below the earlier ones. Distinguishing these models requires study of brains in which early but not late neurons respond properly to reelin signaling.

The *Dab1*<sup>p45/-</sup> cortical phenotype is more simply interpreted in terms of the 'stop signal' model than the 'chemoattractant' model. The early-born *Dab1*<sup>p45/-</sup> neurons seem to respond appropriately to reelin, leaving late-born *Dab1*<sup>p45/-</sup> CP neurons an unimpeded migratory path to the edge of layer I. However, the late-born *Dab1*<sup>p45/-</sup> neurons do not sense the reelin 'stop signal' and they mistakenly enter layer I (Fig. 7A, right). Thus the *Dab1*<sup>p45/-</sup> phenotype implies that the late-born CP neurons would enter layer I in a normal brain but for the reelin 'stop signal'. The phenotype is more difficult to interpret if reelin is normally a 'chemoattractant' for late CP neurons entering the base of the CP. Based on this result, we predict that late-born CP neurons, which lack a cell-autonomous component of the reelin response pathway (such as Dab1), would over-migrate into layer I if transplanted into a normal brain. We also predict that conditional ablation of reelin or Dab1 during corticogenesis would allow late CP neurons into layer I. These predictions remain to be tested using chimeras and conditional gene ablation.

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