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Migration Defects of *cdk5*$^{-/-}$ Neurons in the Developing Cerebellum is Cell Autonomous

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Cyclin-dependent kinase 5 (Cdk5) is a member of the family of cell cycle-related kinases. Previous neuropathological analysis of *cdk5*$^{-/-}$ mice showed significant changes in CNS development in regions from cerebral cortex to brainstem. Among the defects in these animals, a disruption of the normal pattern of cell migrations in cerebellum was particularly apparent, including a pronounced abnormality in the location of cerebellar Purkinje cells. Complete analysis of this brain region is hampered in the mutant because most of cerebellar morphogenesis occurs after birth and the *cdk5*$^{-/-}$ mice die in the perinatal period. To overcome this disadvantage, we have generated chimeric mice by injection of *cdk5*$^{-/-}$ embryonic stem cells into host blastocysts. Analysis of the cerebellum from the resulting *cdk5*$^{-/-}$ ↔ *cdk5*$^{+/+}$ chimeric mice shows that the abnormal location of the mutant Purkinje cells is a cell-autonomous defect. In addition, significant numbers of granule cells remain located in the molecular layer, suggesting a failure to complete migration from the external to the internal granule cell layer. In contrast to the Purkinje and granule cell populations, all three of the deep cerebellar nuclear cell groupings form correctly and are composed of cells of both mutant and wild-type genotypes. Despite similarities of the *cdk5*$^{-/-}$ phenotype to that reported in *reeler* and *mdab-1*$^{-/-}$ (*scrambler/yotari*) mutant brains, *reelin* and *disabled-1* mRNA were found to be normal in *cdk5*$^{-/-}$ brain. Together, the data further support the hypothesis that Cdk5 activity is required for specific components of neuronal migration that are differentially required by different neuronal cell types and by even a single neuronal cell type at different developmental stages.

Key words: neuronal migration; cdk5*; cerebellar development; Purkinje cell; granule cell; cell autonomous*

The cell cycle in all eukaryotes is controlled by a large family of serine/threonine protein kinases, the cyclin-dependent kinases (Cdks), that are activated by binding to regulatory subunits known as cyclins (Meyerson et al., 1992; Sherr, 1993). One unique member of the Cdk family is Cdk5. Unlike other Cdks, Cdk5 expression and kinase activity are not high during cell division. Furthermore, despite the wide distribution of its expression in the organism (Hellmich et al., 1992; Meyerson et al., 1992; Tsai et al., 1993), Cdk5 kinase activity is detected only in the nervous system (Tsai et al., 1993). These unique characteristics are because of the fact that, although Cdk5 has the ability to bind certain cyclins, no kinase activity results from this interaction (Xiong et al., 1992; Kanaoka et al., 1997). Two regulatory subunits that do activate Cdk5 are known as p35 and p39, and their expression is found only in neuronal tissue (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995). Curiously, neither p35 nor p39 has significant homology with the traditional cyclins (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995; Ohshima et al., 1996a). The appearance of active Cdk5 is correlated with the cessation of neurogenesis and the beginning of differentiation of neuronal cells in the developing brain (Tsai et al., 1993). Its endogenous substrates are unknown, but Cdk5 purified from nervous tissue is capable of phosphorylating neuronal cytoskeletal components, including neurofilament proteins (Lew et al., 1992; Shetty et al., 1993) and the microtubule-associated protein tau (Kobayashi et al., 1993; Paudel et al., 1993) *in vitro*. These findings indicate that Cdk5 may have unique functions in the development and differentiation of the brain, possibly through regulating the phosphorylation of neuronal cytoskeletal molecules.

The cerebellum is a powerful system for studying neuronal development. In the adult, it is a relatively simple structure consisting of a cell sparse molecular layer, a single layer of Purkinje cells, the internal granule cell layer (IGL), and deep white matter tracts that include the axons of Purkinje cells en route to their target, the deep cerebellar nuclei (DCN). Both Purkinje cells and neurons of the DCN are born in the germinal layer of the fourth ventricle before migrating to their final location. The granule cell precursors, originally derived from the rhombic lip, migrate as a dividing population to cover the external surface of the developing cerebellum forming the external granule cell layer (EGL). The neurons of EGL cease division postnatally and then migrate radially along the Bergmann glial fibers

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to the internal granule cell layer. As granule cells migrate into IGL, they form parallel fiber axons that synapse with the Purkinje cell dendrites in the molecular layer.

We have reported previously on the generation and initial characterization of *cdk5*$^{-/-}$ mice (Ohshima et al., 1996b). Mutant *cdk5*$^{-/-}$ mice exhibit perinatal mortality that is associated with a unique CNS pathology (Ohshima et al., 1996b). Disruptions of lamination are observed in the olfactory bulb, as well as the cerebral and cerebellar cortices (Ohshima et al., 1996b, 1997). Neuronal birthdate labeling by bromodeoxyuridine injections reveals an abnormal neuronal migration pattern in the cerebral cortex of *cdk5*$^{-/-}$ mice (Gilmore et al., 1998). Unfortunately, *cdk5*$^{-/-}$ mice die around birth. Thus, the abnormal brain organization cannot be characterized in brain areas, such as cerebellum in which much of development occurs postnatally.

To delineate the precise role of Cdk5 during postnatal cerebellar development, we have generated a series of *cdk5*$^{-/-}$ ↔ *cdk5*$^{+/+}$ chimeric mice (*cdk5* chimeras). The chimeras have an abnormal localization of a subpopulation of Purkinje cells, indicating a cell-autonomous defect in neuronal migration in these large *cdk5*$^{-/-}$ neurons. A substantial granule cell migration deficit is also apparent, a defect that would have been undetectable in the nonchimeric mutant. Finally, comparison of the cerebellar phenotype of the chimera with the previously described *reeler* and *scrambler/yotari* mutants (Ogawa et al., 1995; Sweet et al., 1996; Gilmore and Herrup, 1997; Goldowitz et al., 1997; Gonzalez et al., 1997; Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997; Yoneshima et al., 1997) reveals similarities but also significant differences. The levels and distributions of the mRNAs for *reelin* (D'Arcangelo et al., 1995) and *disabled-1* (Howell et al., 1997a) are normal in *cdk5*$^{-/-}$ mice, consistent with the suggestion that the Cdk5 deficiency disrupts a distinct migration process.

MATERIALS AND METHODS

Cdk5 mutant mice. The *cdk5*$^{+/-}$ mouse line is maintained on a C57BL/6 × 129/SvJ hybrid background. Genotyping of the offspring was performed by Southern blot analysis as described previously (Ohshima et al., 1996b). All mice were housed in the standard mouse facility (Association for Assessment and Accreditation of Laboratory Animal Care accredited) and were fed autoclaved diet and water.

Chimeric mice derived from cdk5$^{-/-}$ *embryonic stem cells. cdk5*$^{-/-}$ embryonic stem (ES) cell clones were obtained using high G418 culture method (Mortensen et al., 1992). *cdk5*$^{+/-}$ ES cells, derived from clone-57 (Ohshima et al., 1996b), were cultured in 1 mg/ml G418 (Life Technologies, Bethesda, MD) for 6–7 d, and the resistant colonies were picked and genotyped individually as described previously (Ohshima et al., 1996b). To generate chimeric mice, two independent clones of *cdk5*$^{-/-}$ ES cells (57G23 and 57G25) were injected into C57BL/6 blastocysts. The cerebella from three chimeric mice of each line were examined. Each had a high percentage of agouti (*cdk5*$^{-/-}$) coat color. Age-matched C57BL/6 (*cdk5*$^{+/+}$) mice were used as controls.

Histopathological and immunohistochemical analysis. For *in situ* hybridization, embryonic brains [embryonic day 16.5 (E16.5)] were immersed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. The tissue sections were cut at 6–8 μm and stained with hematoxylin. For immunocytochemistry of embryos, E18.5 brains were immersed in 4% paraformaldehyde overnight, equilibrated in PBS–20% sucrose–0.8% paraformaldehyde at 4° C, embedded in OCT (Tissue-Tek), frozen in dry ice, and cryostat sectioned at 10 μm. Adult brains from 8- to 12-week-old chimeras and controls were obtained after transcardial perfusion with PBS, followed by 10% buffered formalin (Fisher Scientific, Pittsburgh, PA). After a 1 hr post-fix in the same fixative, brains were kept in 20% sucrose–PBS for 2 d, and sections were cut on a cryostat at 20 μm. All cryostat sections were kept at −70°C before use. For immunohistochemistry, the following antibodies were used at the indicated dilutions: mouse anti-calbindin IgG D-28K, monoclonal (Sigma) at 1:1000 for double staining; rabbit anti-calbindin D-28K, polyclonal (gift from Sylvia Christakos) at 1:1000 for single staining; rabbit anti-Cdk5, polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100; and mouse anti-β2/β3 $GABA_A$ receptor bd17 (Boehringer Mannheim, Mannheim, Germany) at 1:100. For bright-field immunohistochemistry, HRP-conjugated secondary antibody was visualized using diaminobenzidene (DAB) reaction product as specified by Vectastain Elite protocol (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. For immunofluorescence, mouse IgG primary antibodies were visualized with fluorescein-conjugated secondary (Jackson ImmunoResearch, West Grove, PA) at 1:200. Rabbit primary antibodies were detected with Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch) at 1:400 for rabbit primary antibodies. For confocal microscopy of study granule cells, the Cdk5 antibody was detected with Oregon Green-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR), and the bd17 monoclonal was detected with Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch) at 1:400. All sections were examined by standard light, fluorescent, or confocal microscopic techniques.

reelin *and* disabled-1 *cDNA fragments.* Two cDNA fragments of *reelin*, rl-1, corresponding to nucleotides 10,228–10,665, and rl-2, corresponding to nucleotides 10,860–11,459 (GenBank accession number U24703), were amplified by PCR methods using a mouse brain cDNA pool (Marathon-Ready cDNA, catalog #7450–1; Clontech, Palo Alto, CA). The mouse *disabled-1* cDNA fragment, corresponding to nucleotides 1626–2116 (GenBank accession number Y08379), was amplified by the same PCR method. After subcloning these fragments into T-vector (Promega, Madison, WI), nucleotide sequencing was performed to confirm the identity of the insert with the reported sequences.

RNA isolation, Northern blot analysis, and in situ *hybridization.* RNA was isolated from individual brains at E16.5 by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1978). Twenty micrograms of total brain RNA was heat-denatured size-fractionated by electrophoresis through a 1.0% formaldehyde–agarose gel and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH) by capillary blotting as described previously (Sambrook et al., 1989). cDNA fragments were ^{32}P-radiolabeled by random priming. Hybridization was performed using the *reelin* (rl-1) and *disabled-1* cDNA probes in 6× SSC–50% formamide at 42°C overnight. The filter was washed twice in 0.1× SSC–0.1% SDS at 50°C for 30 min. After striping the probe, the same filter was used for hybridization with the mouse GAPDH probe.

For *in situ* hybridization, E16.5 brains were immersed fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 6–8 μm. ^{35}S-Labeled riboprobes for *reelin* (rl-1 and rl-2) were generated by *in vitro* synthesis. Hybridization was performed as described previously (Fox and Cottler-Fox, 1993; Yoshida et al., 1996). Briefly, after deparaffinization and rehydration, the sections were incubated with 0.2 N HCl, rinsed with DEPC-treated water, and then digested with proteinase K solution at 37°C. The sections were incubated successively in 0.1 M triethanolamine buffer, pH 8.0, succinic anhydride solution (1% solution in 0.1 triethanolamine buffer), and 0.1 M triethanolamine buffer. The prehybridization and hybridization were performed in a solution containing 50% formamide and 10% dextran sulfate at 45°C. After overnight hybridization, the slides were washed sequentially with 2× SSC, formamide wash solution, Triton X-100 wash solution, and 0.1× SSC. Then slides were exposed to RNase A at 37°C, washed in 2× SSC, and dehydrated in 0.3 M ammonium acetate and graded alcohols. The slides were exposed in the dark at 4°C for 3 weeks after being coated with autoradiography emulsion (NTB-3; Eastman Kodak, Rochester, NY).

RESULTS

Cerebellar formation in *cdk5*$^{-/-}$ mice

At birth, the cortex of the normal mouse cerebellum consists of two layers: a layer of Purkinje cells, several cells thick, and a superficial external granule cell layer. In the E18.5 cerebellum of the *cdk5*$^{-/-}$ mouse, this layered arrangement is disrupted. Although the EGL forms normally and is normal in appearance (Ohshima et al., 1997), the Purkinje cell layer cannot be readily distinguished. To identify the location of the different cerebellar cell types, immunocytochemical studies were performed on E18.5 brains. In *cdk5*$^{+/-}$ mice at E18.5, calbindin-positive Purkinje cells are located, as expected, as a broad layer of medium- to large-sized cell bodies in the developing cerebellar plate (Fig. 1*A*). In

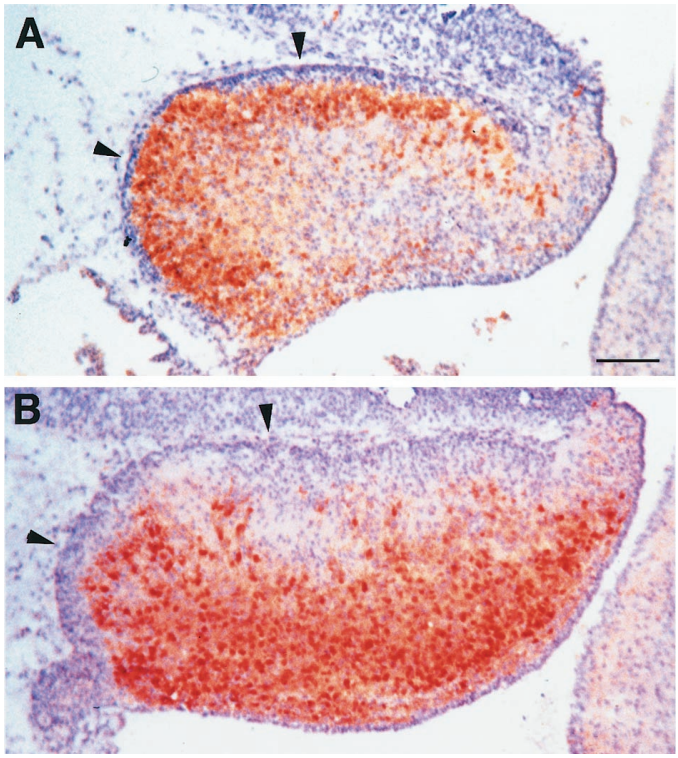

Figure 1. Calbindin staining of sagittal sections from E18.5 cerebellum reveals migration deficiencies of Purkinje cells in *cdk5*$^{-/-}$ mutants. Anti-calbindin antibody is visualized through the red-brown DAB reaction product, seen beneath the external granule cell layer (*arrowheads*) in normal (*cdk5*$^{+/-}$) cerebellum (*A*). Note that the Purkinje cells in this mouse are found in their proper cortical location. In *cdk5*$^{-/-}$ mice (*B*), calbindin-positive Purkinje cells are ectopically located deep in the cerebellum, near the ventricle at the bottom the cerebellum. Note the presence of a normal appearing external granule cell layer (*arrowheads*) in the *cdk5*$^{-/-}$ cerebellum. Scale bar, 100 μm.

contrast, in *cdk5*$^{-/-}$ mice, virtually no Purkinje cells are found in this cortical position. Instead, nearly all calbindin-positive cells are located deep in the cerebellar parenchyma (Fig. 1*B*). These observations imply that there is a disturbance of the prenatal migration pattern of Purkinje cells in the *cdk5*$^{-/-}$ cerebellum with little or no change in the initial migration of the granule cells to form the EGL.

The *cdk5*$^{-/-}$ ↔ *cdk5*$^{+/+}$ chimeric cerebellum

Because much of the process of cerebellar maturation occurs in the postnatal period, ES cell chimeric mice were generated with both *cdk5*$^{-/-}$ and *cdk5*$^{+/+}$ cells. Two clones of *cdk5*$^{-/-}$ ES cells were obtained by selection in high G418 medium (see Materials and Methods). Injection of the homozygous mutant ES cells into wild-type blastocyst was used to generate six *cdk5* chimeric mice with highly variegated coats. All chimeras were analyzed at 2 to 3 months of age. Chimeric mice appeared to have normal viability without noticeable ataxia. The cerebella of all six chimeras examined are either equal to or smaller in size than a typical wild-type mouse (Fig. 2*A*,*B*). Although smaller in size, the typical trilaminar pattern of the cerebellar cortex is always readily apparent, and the cerebellar lobules are well formed. The IGL is normal in position and cell composition, and the Purkinje cell layer is well formed, consisting of a row of calbindin-positive cells (Figs. 2*B*, 3*B*).

In wild-type animals, the molecular layer contains only a few well separated basket and stellate cells. The bulk of this layer consists mainly of parallel fibers (granule cell axons) and their targets, the dendrites of Purkinje cells. The molecular layer of the *cdk5* chimeras, however, has an abundance of granule-like cells (Fig. 2*D*,*E*). In all chimeras examined, the density of these granule-like cells varies from almost complete saturation of the molecular layer (Fig. 2*D*) to almost wild-type sparseness (Fig. 2*E*). Within the same animal, the density varies from region to region in both the mediolateral and anteroposterior dimension. The anatomical location of the cell-dense and cell-sparse regions between different chimeric cerebella does not appear to follow a reproducible pattern. Because granule cells should have completed migration to the IGL by the time these animals were examined, it appears that some aspect of the granule cell migratory process must be deficient. The migratory defect of the granule cells is not entirely unexpected because, in mice lacking p35 (one of the Cdk5 activators), an excess of granule cells is found in the molecular layer (Chae et al., 1997). Interestingly, the defect in *cdk5* chimeras appears to be much more severe.

In addition to the normally positioned Purkinje cells of the Purkinje cell layer of the chimeras, an unusually large number of neuronal-like cells are located near the white matter (Fig. 2*E*) and in regions occupied by the DCN. These neurons are found in positions ranging from near the fourth ventricle to just below the IGL at the bottom of a sulcus (Fig. 2*E*). The identification of these cells as mutant Purkinje cells was confirmed by immunohistochemical techniques, using antibodies against calbindin. In wild-type mice, staining with the Purkinje cell-specific calbindin antibody stains large cells exclusively in the Purkinje cell layer (Fig. 3*A*). In the chimeric brains, all of the Purkinje cells in the cortical Purkinje cell layer are immunopositive for calbindin, as expected. However, many of the unidentified large neurons in the deep cerebellar core region are also calbindin-positive. This finding demonstrates that a subset of the Purkinje cells in the chimera are in an abnormal location (Fig. 3*B*).

The presence or absence of Cdk5 protein can be used to determine the genotypes of the Purkinje cells in the chimera. As expected, all calbindin-positive neurons in wild-type brains are Cdk5-positive (Fig. 3*C*,*D*). In the chimera, all of the large calbindin-positive neurons in the Purkinje cell layer are also Cdk5-positive, confirming their *cdk5*$^{+/+}$ genotype (i.e., they were Purkinje cells derived from the wild-type C57BL/6 blastocyst) (Fig. 3*E*,*F*). It is highly significant that none of the calbindin-positive cells seen within the Purkinje cell layer are *cdk5*$^{-/-}$. This means that no mutant Purkinje cell is able to achieve its correct location in cerebellar cortex. In contrast, nearly all ectopic Purkinje cells are found to be *cdk5*$^{-/-}$ (Fig. 3*G*,*H*). Because no *cdk5*$^{-/-}$ Purkinje cells are found within the cerebellar cortex and virtually all Cdk5-positive Purkinje cells are located in the cerebellar cortex, it appears that the migration defect of Cdk5-deficient Purkinje cells is cell autonomous and fully penetrant.

The status of the DCN neurons was also investigated. There are no specific immunohistochemical markers for DCN neurons comparable with calbindin for Purkinje cells. Therefore, neurons of the DCN were identified by their size, their location within the cerebellum, and by the presence of a "halo" of calbindin staining surrounding their cell bodies (resulting from presynapic terminals of calbindin-positive Purkinje cells). In wild-type mice, all neurons in the DCN are Cdk5-positive, as expected (Fig. 4*A*,*B*). In the chimera, many of the identifiable DCN neurons are also Cdk5-positive (Fig. 4*C*,*D*). However there are also many clear

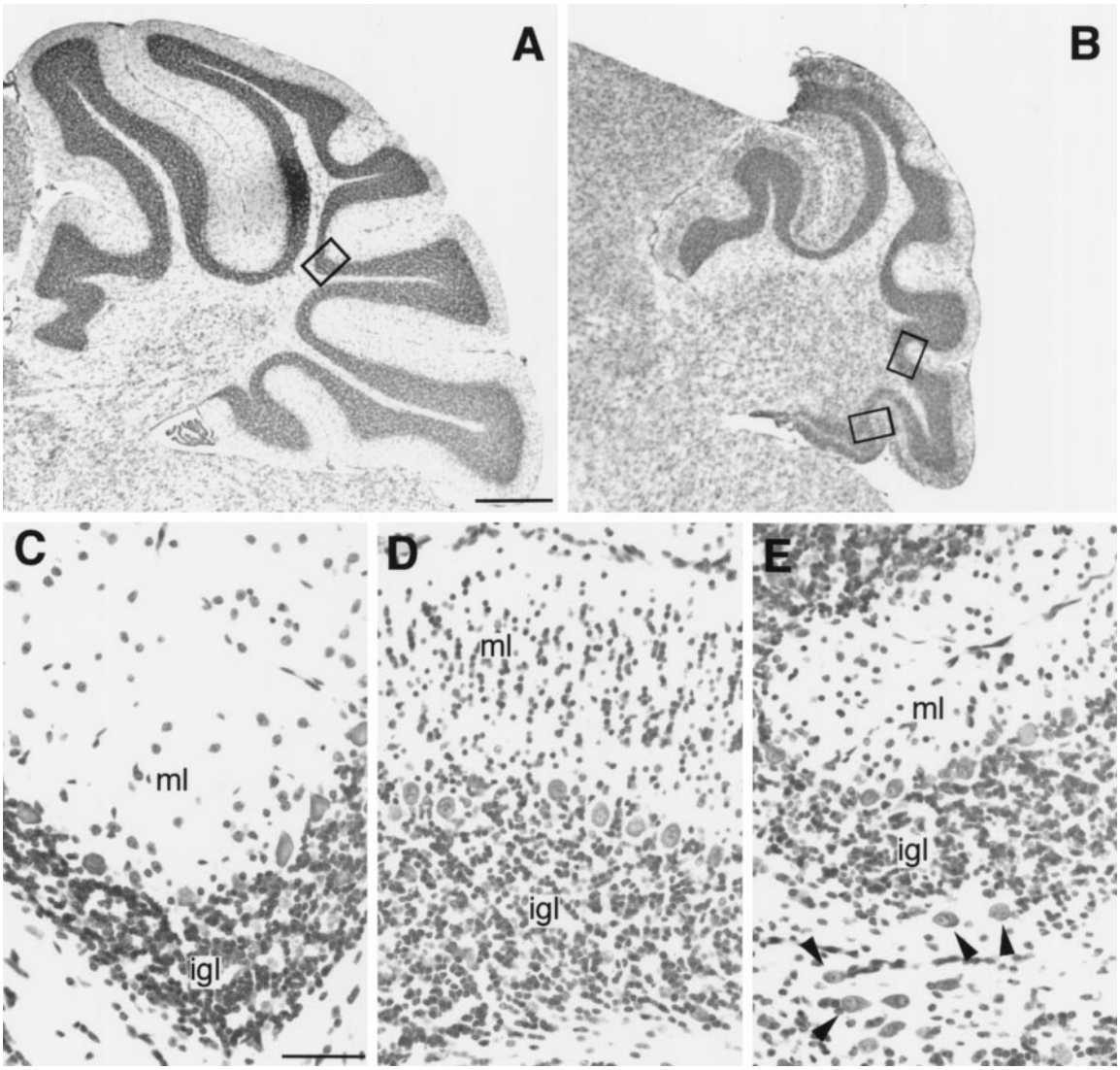

Figure 2. Sagittal cresyl violet-stained sections of adult wild-type (*A*, *C*) and *cdk5*$^{-/-}$ chimeric (*B*, *D*, *E*) cerebellum. The chimeric cerebellum (*B*) is normal in foliation and cellular distribution but is reduced in size compared with wild-type cerebellum (*A*). The molecular layer (*ml*) of normal mice is cell-sparse (*C*, higher magnification of the *boxed area* in *A*). A monolayer of Purkinje cells is found between the molecular layer and the cell dense internal granule cell layer (*igl*). Beneath the internal granule cell layer are the white matter tracts, which contain myelinated axons of Purkinje cells that will synapse on neurons of the deep cerebellar nuclei. Cell density in the molecular layer of chimeras varies from region to region (*D*, *E*, higher magnification of the *boxed areas* in *B*). Despite this, the Purkinje cell layer and internal granule cell layer are normal in appearance. Large cells are seen in aberrant locations (*arrowheads* in the white matter of *E*). Scale bars: (in *A*) *A*, *B*, 200 μm; (in *C*) *C–E*, 50 μm.

examples of DCN neurons that are *cdk5*$^{-/-}$ (Fig. 4*C*,*D*, *arrowheads*). No attempt was made to quantitate the number of *cdk5*$^{-/-}$ neurons because of the difficulties inherent in their identification within the DCN. It is of interest to note that there is a nearly complete segregation of DCN neurons and ectopic Purkinje cells (Fig. 3*B*,*G*,*H*). This phenomena has been observed before in both *reeler* and *scrambler* mice (Goffinet, 1984; Goldowitz et al., 1997). Although ectopic Purkinje cells can be found adjacent to DCN neurons (Fig. 3*G*,*H*, *insets*), they do not exist as a mixture, indicating a developmental mechanism that keeps the populations separate.

Abundant granule cells can be identified in the molecular layer of chimeras, although they are rarely found there in wild-type animals. Antibodies to β2/β3 $GABA_A$ receptor (bd17) were used identify the plasma membrane of granule cells (Ewert et al., 1992; Laurie et al., 1992; Fritschy and Mohler, 1995). Whereas other cell types within the cerebellum may be recognized by bd17, granule cells can be distinguished from all other cell types by their small diameter, 5–8 μm. Because of the very small size of granule cells, confocal microscopy was needed to define their genotype (based on the presence or absence of Cdk5). Wild-type animals show a low but detectable level of Cdk5 protein in all granule cells of adult animals (Fig. 5*A*,*B*). In contrast, the granule cells of the chimeric IGL are a mixture of *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ cells (Fig. 5*C*,*D*). Furthermore, the granule cells found within the molecular layer are nearly all Cdk5-deficient (Fig. 5*E*,*F*). This indicates that the block in cell migration that leaves many granule cells stranded within the molecular layer is intrinsic to *cdk5*$^{-/-}$ neurons. However, the presence of *cdk5*$^{-/-}$ cells correctly located in the IGL indicates that this block is not absolute.

reelin and *disabled-1* mRNA expression is normal

Although it appears that there are distinctions between the cerebellar phenotypes of the *cdk5*$^{-/-}$ and *reeler*/*scrambler* mutants

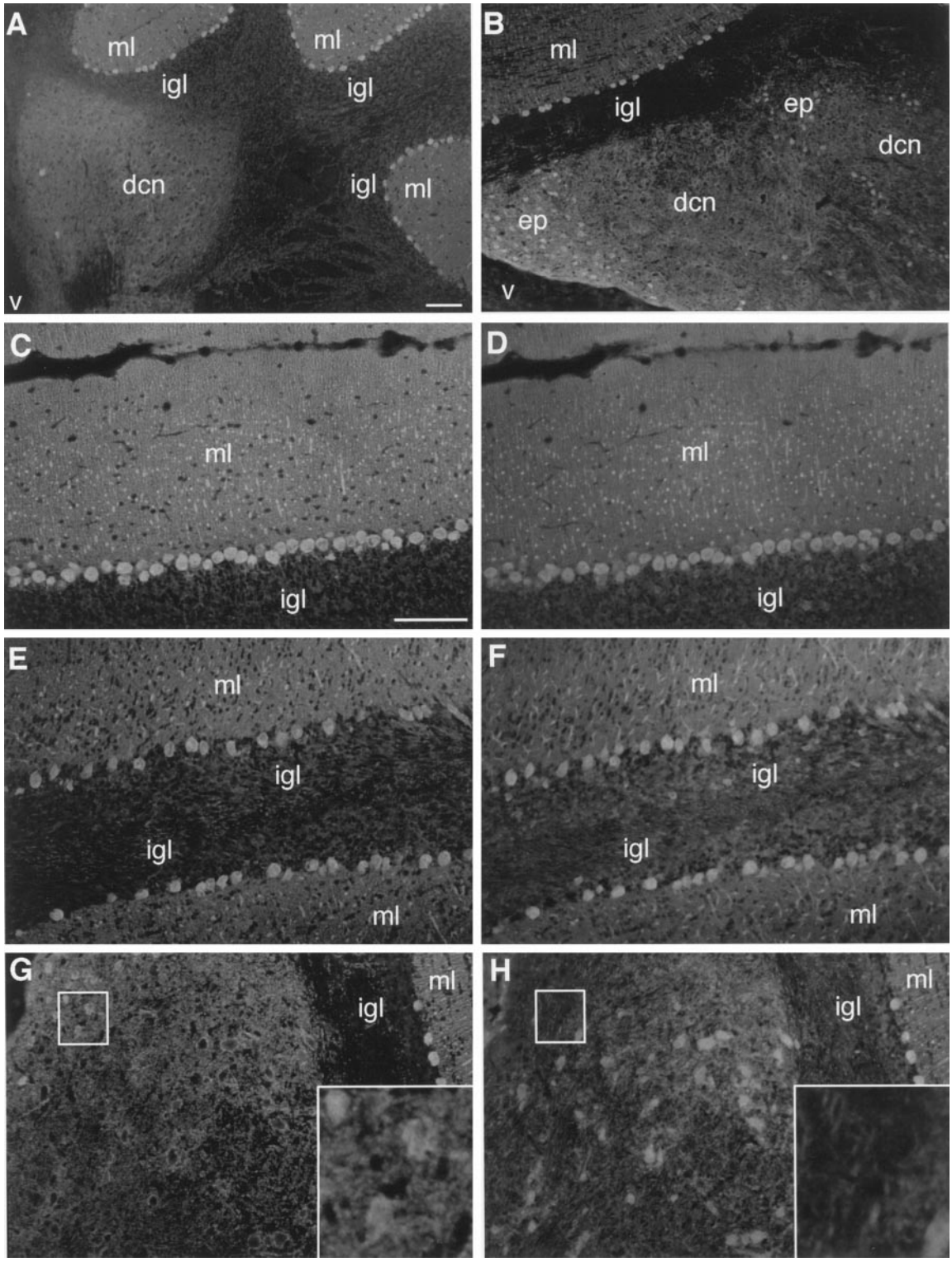

Figure 3. Horizontal sections through deep nuclear region of adult wild-type (*A*, *C*, *D*) and chimeric (*B*, *E–H*) cerebella. Sections were double immunostained for calbindin (*A–C*, *E*, *G*) and Cdk5 (*D*, *F*, *H*). Wild-type cerebellum (*A*) with the ventricle (*v*) to the *left* shows prominent calbindin staining in the Purkinje cell layer of the cerebellar cortex. Only rarely are calbindin-positive neurons found near the ventricles. Calbindin immunostaining of chimeric cerebellum (*B*) indicates that, although correctly located Purkinje cells are calbindin-positive, there are many ectopic Purkinje cells (*ep*) found within the parenchyma of the cerebellum near the regions of the deep cerebellar nuclei (*dcn*). No calbindin-positive neurons are apparent within the internal granule cell layer. In wild-type mice, there is complete congruence between calbindin-positive (*C*) and Cdk5-positive (*D*) neurons. Within the region of the Purkinje cell layer, there are some large neurons in both wild-type and chimeric mice that are Cdk5-positive but not calbindin-positive. These are probably other large neurons of the cerebellar cortex, such as basket, Lugaro, or Golgi cells. All calbindin-positive neurons (*E*) within the Purkinje cell layer of chimeras are wild type, i.e., Cdk5-positive (*F*). However, calbindin-positive Purkinje cells in the chimera (ectopically located near the ventricle in the *top left corner* of *G*) are mutant, i.e., Cdk5-negative (*H*). *Insets* in *G* and *H* are higher magnifications of the areas indicated by the *white boxes*. Scale bars: (in *A*) *A*, *B*, 150 μm; (in *C*) *C–H*, 100 μm.

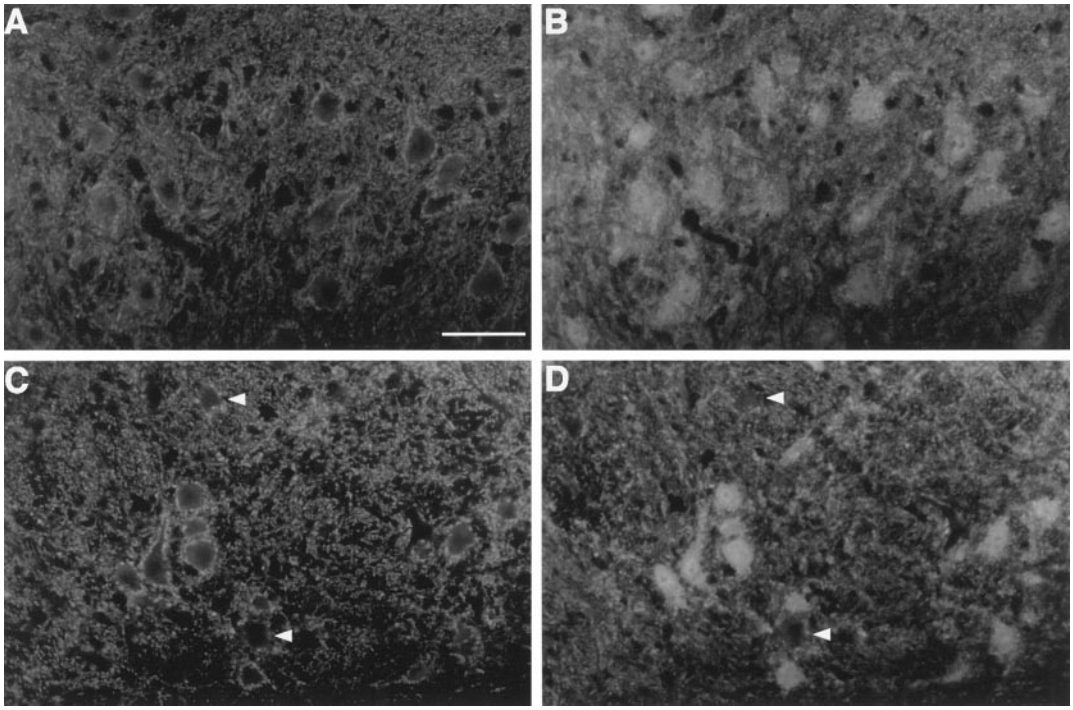

Figure 4. High magnification of deep cerebellar nuclei from wild-type (*A*, *B*) and chimeric (*C*, *D*) cerebella. Sections were double immunostained with calbindin (*A*, *C*) and Cdk5 (*B*, *D*). Wild-type deep cerebellar nuclei neurons can be identified by location and the presence of a calbindin-positive ring surrounding their cell body. The chimeric deep cerebellar nuclei contain both Cdk5-positive and Cdk5-deficient neurons. In the chimera, many deep cerebellar nuclei neurons contain Cdk5; however, some do not (*arrowheads*). Scale bar, 50 μm.

(Goffinet, 1984; Sweet et al., 1996; Goldowitz et al., 1997), they do have many facets in common. The similarities between mutants prompted us to explore the expression of *reelin* and *disabled-1* mRNA (the respective products of these two genes) in the embryonic brains of *cdk5*$^{-/-}$ mice to determine whether alteration of expression of either of them could account for some of the defects found in *cdk5*$^{-/-}$ mice. cDNA fragments were obtained by PCR amplification using primer sets designed from the published GenBank sequence data for *reelin* and *disabled-1*. Correct amplifications were confirmed by sequencing (see Materials and Methods). The labeled fragments were used for Northern blot analysis and for *in situ* hybridization studies. The results illustrate that the mRNA expression levels of *reelin* and *disabled-1* are not changed in the *cdk5*$^{-/-}$ brain (Fig. 6). The distributions of *reelin* mRNA in the brains from *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ mice were also examined by *in situ* hybridization using ^{35}S-labeled riboprobes. As reported in earlier studies (D'Arcangelo et al., 1997; Schiffmann et al., 1997), *reelin* mRNA is distributed in the epithelium of the olfactory bulb, the marginal zone of the cerebral cortex, and the EGL of the cerebellum in the wild-type mice at E16.5 (Fig. 7*A*,*B*). In *cdk5*$^{-/-}$ mice, the distribution and level of *reelin* mRNA remain unchanged (Fig. 7*C*,*D*).

DISCUSSION

The results presented here demonstrate that the cerebellar defects of the *cdk5*$^{-/-}$ mutation are partially rescued by the presence of wild-type cells in *cdk5*$^{-/-}$ ↔ *cdk5*$^{+/+}$ chimeras. Although the cause of perinatal death of *cdk5*$^{-/-}$ embryos remains unknown, it is clear from the long-term survival of the chimeric mice in this study that it is not a result of a toxic effect of *cdk5*$^{-/-}$ cells during development. In most chimeras, the cerebellum is slightly smaller than in wild-type, but the typical trilaminar configuration of the cerebellar cortex is readily apparent, and DCN are present in their proper location. This phenotypic rescue, however, occurs only at the level of cerebellar structure; at the level of the individual cells, there are a number of defects that distinguish the chimeric and wild-type brains. In the chimera, many Purkinje cells are normal in position, but many others are ectopically located deep in the cerebellar parenchyma. Likewise, many granule cells participate in the formation of a well defined IGL, but the molecular layer is dense with ectopic granule cells. The close correlation between abnormal phenotype and mutant genotype leads us to interpret these findings to mean that the defect in migration caused by the absence of the Cdk5 protein is cell autonomous in all affected cells.

The data for the cerebellar Purkinje cells are definitive evidence in support of the conclusion that the migrational defects of the *cdk5*$^{-/-}$ neurons are cell autonomous. At E18.5 in *cdk5*$^{-/-}$ mice, most of the calbindin-positive cells are located deep in the central mass of the cerebellum. The failure of these Purkinje cells to achieve a normal adult position is reminiscent of the defect in cell migration described previously in *cdk5*$^{-/-}$ cerebral cortex

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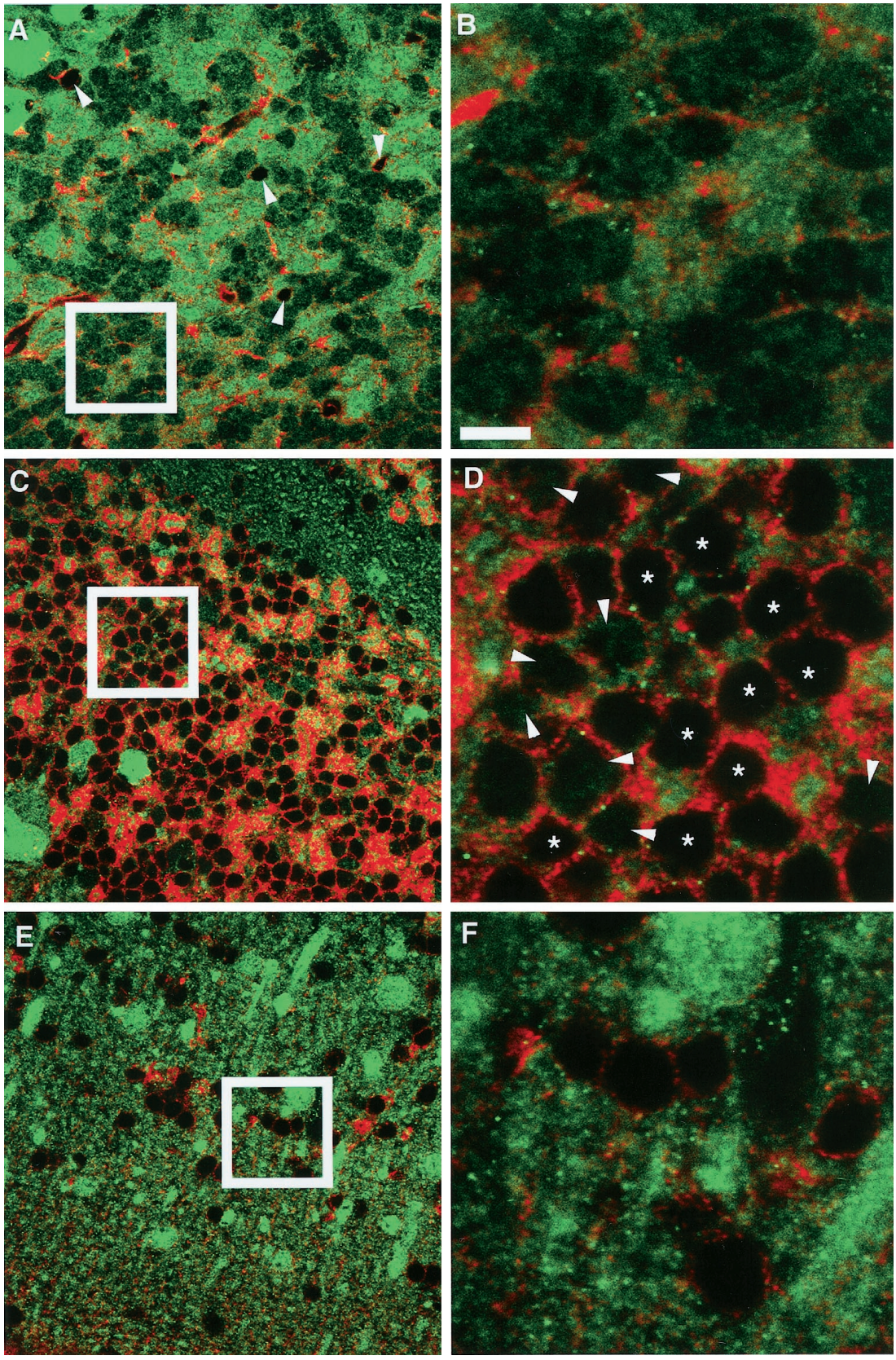
Figure 5. Cdk5 staining of granule cells in wild-type and chimeras visualized with confocal microscopy. Double immunostaining with antibodies β2/β3 $GABA_A$ receptor (bd17) (Ewert et al., 1992) and Cdk5. The cell surface localization of bd17 (*red*) surrounds the Cdk5-positive (*green*) granule cell (Laurie et al., 1992; Fritschy and Mohler, 1995). Higher magnifications of *boxed areas* of *A*, *C*, and *E* are shown in *B*, *D*, and *F*, respectively. Granule cells are identified by membrane staining with bd17 and a diameter of 5–8 μm. The relative Cdk5 staining in granule (*Figure legend continues*)



cells is much lower than in Purkinjecells (*A*, *top left corner*, and *C*, *bottom right corner*). Granule cells of wild-type mice all contain Cdk5 (*A*, *B*). A few capillaries are stained by Cy3-conjugated anti-mouse IgG antibodies (*arrowheads* in *A*) and should not be confused with Cdk5-negative granule cells. The granule cell layer of the chimeric IGL contains both *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ neurons (*C*, *D*). These can be seen at high magnification in *D*, with *cdk5*$^{+/+}$ granule cells indicated by the *arrowheads* and *cdk5*$^{-/-}$ granule cells indicated by *asterisks*. The ectopic granule cell neurons of the chimeric molecular layer are Cdk5-deficient (*E*, *F*). Scale bar: *A*, *C*, *E*, 20 μm; *B*, *D*, *F*, 5 μm.

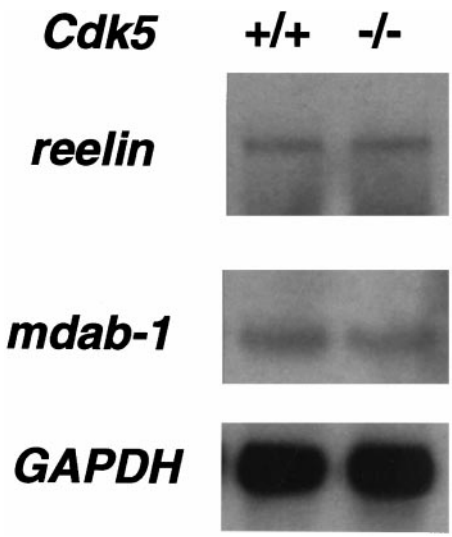


Figure 6. Northern blot analysis of mRNA expression for *reelin*, *disabled-1* (*mdab-1*), and GAPDH in E16.5 brains of *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ mice reveals no difference between wild-type and mutant mice.

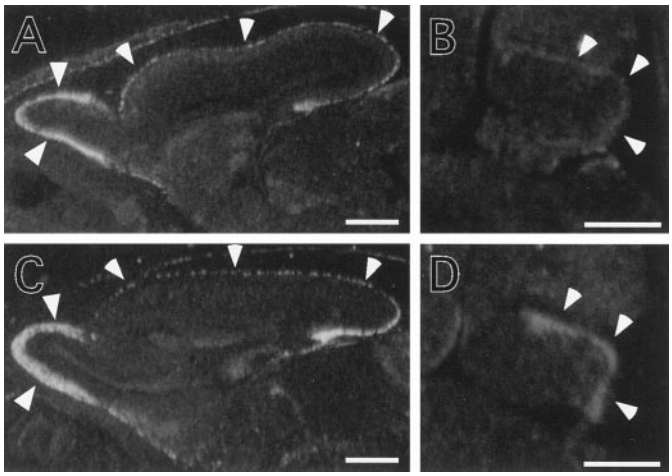


Figure 7. Expression pattern of *reelin* mRNA in sagittal section of cerebrum (*A*, *C*) and cerebellum (*B*, *D*) by *in situ* hybridization. Antisense probes (synthesized from rl-1) hybridized to E16.5 *Cdk5*$^{+/+}$ (*A*, *B*) and *Cdk5*$^{-/-}$ (*C*, *D*) show normal levels and distribution of *reelin* mRNA in the olfactory bulb (*left*, *facing arrowheads*), along with the marginal zone of the cerebral cortex (*arrowheads* along *top*). Note the reelin expression within the external granule cell layer of the cerebellum (*arrowheads*) of wild-type and *Cdk5*$^{-/-}$ mice (*B*, *D*, respectively). No significant hybridization is observed when sense probe is used (data not shown). Two different cDNA fragments (rl-1 and rl-2) were used as probes, and the same hybridization patterns were obtained (rl-2, data not shown). Scale bars: *A*, *C*, 500 μm; *B*, *D*, 800 μm.

(Ohshima et al., 1996b; Gilmore et al., 1998). The tight coupling between Purkinje cell genotype and the migration phenotype in the chimera adds significantly to the overall picture of the migration defect. All Purkinje cells in the normal Purkinje cell layer of the chimera are *cdk5*$^{+/+}$, as revealed by their staining with anti-Cdk5 antibody. In addition, the Purkinje cells that are ectopically positioned in the chimera are all *cdk5*$^{-/-}$. These data show that both the presence of wild-type cells in the chimeric environment does not rescue the migration defect of the *cdk5*$^{-/-}$ Purkinje cells and the presence of *cdk5*$^{-/-}$ mutant cells does not interfere with the normal migration of the neighboring wild-type Purkinje cells.

The normal configuration of the DCN and their mixed genotype composition in the chimera indicate that the cell movements required to form the three nuclear groups (lateralis, interpositus, and medialis) probably occur by a Cdk5-independent process. The DCN neurons migrate along a complex pathway before settling within the three nuclei (Altman and Bayer, 1985). However, it is unclear whether the migration mechanism of the DCN neurons involves glial guidance. Although we cannot exclude the possibility that *cdk5*$^{-/-}$ DCN neurons do not follow the same pathway to reach their final destination as the wild-type neurons, we find this improbable because we have observed many *cdk5*$^{-/-}$ DCN neurons in the correct location, correctly surrounded by Purkinje cell innervation. Our interpretation is that the migration of the DCN neurons is not dependent on Cdk5. This is important because the DCN are generated at a similar time and in the same location as Purkinje cells (Pierce, 1975; Altman and Bayer, 1978). We have shown previously that some of the neurons of the deeper layers of cerebral cortex achieve their normal position even in the nonchimeric *cdk5*$^{-/-}$ mutant (Gilmore et al., 1998). Although there is nothing in the migratory path taken by DCN cells (Altman and Bayer, 1985) that would suggest that the mechanics of migration are different from those used by the Purkinje cells, the *cdk5*$^{-/-}$ mutant and the *cdk5* chimera plainly identify this difference. This reinforces the conclusion of our earlier work in cerebral cortex (Gilmore et al., 1998) that important yet unidentified differences exist in the mechanics of neuronal migration across different cell types.

The behavior of the granule cells in the chimera is also consistent with this idea. Although the IGL is made up of both *cdk5*$^{-/-}$ and *cdk5*$^{+/+}$ granule cells, nearly all granule cells in the molecular layer are Cdk5-deficient. Thus, although many mutant granule cells are incapable of completing migration, others are seemingly unaffected. One possible explanation for this finding is that there are distinct granule cell populations, each with a different requirement for Cdk5 activity. Another possibility is that wild-type granule cells are capable of rescuing some mutant granule cells. Such a rescue has been reported when granule cells from in the cerebellar mutant *weaver* are transplanted to a wild-type EGL or cultured *in vitro* with wild-type cells (Gao et al., 1992; Gao and Hatten, 1993). We favor an alternative explanation, however, one that assumes the existence of compensatory mechanisms in the granule cell population that reduces the impact of the Cdk5 deficiency. The nature of these mechanisms is unclear, but the milder phenotype of the *p35*$^{-/-}$ mouse would fit well with this explanation (Chae et al., 1997). The implication of this hypothesis is that the ability of granule cells to migrate is near some threshold level in the *cdk5*$^{-/-}$ mutant. It is worth noting that the long tangential migration of the granule cell precursors from the rhombic lip to the anterior-most and medial-most regions of the cerebellar surface appears undisturbed by the absence of the Cdk5 kinase and is likely, therefore, to be Cdk5-independent. Thus, once again, the *Cdk5* mutation partitions neuronal migration into different types: an early Cdk5-independent form and a later Cdk5-dependent form. The granule cell data suggest that a single cell type can use both types sequentially during development.

The close chromosomal proximity of loci for *cdk5* (Ohshima et al., 1995) and *reeler* on chromosome 5 (Dernoncourt et al., 1991; Goffinet and Dernoncourt, 1991) raised concerns that the *reelin* gene could be downregulated because of an insertion-induced

alteration in a long-distance enhancer located in the vicinity of *cdk5*. This might reduce the levels of Reelin, thus possibly contributing to the observed phenotype. To address this, we analyzed the expression levels and pattern of *reelin* mRNA in wild-type and *cdk5*$^{-/-}$ mouse brains and found no difference. Furthermore, the expression levels of *disabled-1* were also found to be similar to wild type, suggesting overall that the migration defect of *cdk5*$^{-/-}$ neurons is not mediated by changes in the expression of either of these two phenotypically related genes.

The characterization of the *cdk5*$^{-/-}$ cellular phenotype in the adult cerebellum allows the first direct comparison of the effect of the mutation with those of null alleles of the Cdk5 activator p35. In *p35*$^{-/-}$ cerebella, there is a normal Purkinje cell layer and a slight increase in the density of granule-like cells in the molecular layer (Chae et al., 1997). Our results contrast with this picture. The *cdk5*$^{-/-}$ Purkinje cells are totally blocked in their migratory ability, and the granule cell block is much more severe. These observations, as well as those reported previously in cerebral cortex, suggest that nearly all of the differences between *cdk5*$^{-/-}$ and *p35*$^{-/-}$ mice, including the final location of subplate (Ohshima et al., 1996b; Chae et al., 1997; Gilmore et al., 1998; Kwon and Tsai, 1998), are caused by compensatory actions of multiple activators of Cdk5, such as p39. Therefore, the differences found in the cerebellar phenotypes, along with previously characterized differences of cerebral cortical phenotypes, lead to the hypothesis that alternative Cdk5 activators can substitute when p35 is not present to induce Cdk5 activity. This hypothesis predicts that the *p35*$^{-/-}$ phenotypes are a subset of the *cdk5*$^{-/-}$ phenotypes.

It is of interest to compare the cell migration phenotypes of the *cdk5*$^{-/-}$ mouse with those found in other cerebellar mutants. In both *reeler* and the *mdab-1* mutant *scrambler*, a small but significant number of mutant Purkinje cells successfully complete migration to the Purkinje cell layer (Mariani et al., 1977; Goldowitz et al., 1997). In contrast, none of the *cdk5*$^{-/-}$ Purkinje cells in the chimeras successfully migrate to the cerebellar cortex. This suggests that the *cdk5*$^{-/-}$ mutation causes a more severe arrest of Purkinje cell migration than either the *reeler* or *scrambler* mutation. A far more dramatic difference is that no granule cell migration defect is reported in *reeler*/*scrambler* mice, yet Cdk5-deficient mice have significant cell-autonomous defects in granule cell migration. The *cdk5*$^{-/-}$ granule cell migration deficiency is most reminiscent of *weaver* heterozygous mice, *wv*/+. Mice homozygous for the *weaver* gene *wv*/*wv* have extensive granule cell pathology (Rakic and Sidman, 1973a,b; Herrup and Trenkner, 1987; Smeyne and Goldowitz, 1989). In heterozygous *wv*/+ mice, the cerebellar structure is primarily normal, yet a subset of granule cells remain trapped in the molecular layer, even while most granule cells successfully migrate into the IGL (Rakic and Sidman, 1973a). Studies of chimeric mice have indicated that this migration block is a cell-autonomous defect (Goldowitz and Mullen, 1982). However, other studies have indicated that the migration ability of the *wv*/*wv* granule cells, although normally deficient, can be rescued through interaction with wild-type neurons (Gao et al., 1992; Gao and Hatten, 1993). It is unclear how the genetic defect in *weaver* mice, a point mutation in the inwardly rectifying potassium channel *girk2*, leads the granule cell migration defect (Patil et al., 1995; Rossi et al., 1998). It would be of interest to determine, however, if the block in *weaver* granule cells is in any way related to Cdk5 activity in the deficient granule cells.

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