Neuronal Migration Defects in the *Dreher* (*Lmx1a*) Mutant Mouse: Role of Disorders of the Glial Limiting Membrane

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Dreher **(*dr^J*) is an autosomal recessive mutation in the newly identified LIM homeobox gene, *Lmx1a*. The homozygous mutant phenotype includes misplaced neurons (heterotopia) in the cerebral cortex, cerebellum and hippocampus, which mimic the mild end of the spectrum of neuronal migration disorders in humans. Heterotopic neurons are found mainly in the normally cell-sparse layer I within the cerebral hemispheres of *dr^J* homozygotes. Neu-N immunostaining confirms the neuronal nature of these heterotopic cells, while bromodeoxyuridine-birthdating shows that the misplaced neurons are generated predominantly during the late stages of corticogenesis (E15–E17), suggesting an over-migration of neurons destined for layer II. Immunohistochemistry for laminin, and staining of reticulin fibres, reveals disruption of the glial limiting membrane specifically overlying the areas of heterotopic neurons. Factor VIII (von Willebrand factor) staining shows an abnormal vascular network in layer I, associated with the fragmented glial limiting membrane. Layer I astrocytes, recognized by immunostaining for glial fibrillary acidic protein, exhibit attachment of their end feet to the fragmented glial limiting membrane. We suggest that disruption of the glial limiting membrane is central to the pathogenesis of heterotopic neurons in *dreher*, perhaps via defective radial glial-guided neuronal migration.**

Introduction

Recent years have seen major advances in our understanding of the genetic basis of neuronal migration disorders. Mutations in the human genes *doublecortin* and *LIS1* (Hattori *et al*., 1994; Lo Nigro *et al*., 1997; Des Portes *et al*., 1998; Gleeson *et al*., 1998), and in the mouse genes *reeler* and *disabled* (D'Arcangelo *et al*., 1995; Goffinet, 1997) have been implicated in the etiology of gross disorders of the layered structure of the cerebral cortex. In humans, these developmental defects are associated with severe neurological disease, including mental retardation and intractable epilepsy.

The genetic basis of defects at the mild end of the spectrum of neuronal migration disorder is also emerging. Localized groups of misplaced neurons (heterotopia) may be found in a periventricular location, as in the X-linked condition resulting from mutation in the *filamin-1* gene (Fox *et al*., 1998). Alternatively, heterotopic neurons may be present in the marginal zone as in type II (cobblestone) lissencephaly, a condition present in Fukuyama disease, which results from a mutation affecting the fukutin protein (Kobayashi *et al*., 1998), and in the genetically distinct condition Walker–Warburg syndrome (Williams *et al*., 1984; Chadani *et al*., 2000).

A possible mouse model for type II lissencephaly is provided by the *dreher* mutant mouse (Sekiguchi *et al*., 1994). *Dreher* mice are characterized by circling behaviour, balance abnormalities, hyperactivity and deafness (Lyon, 1961; Sweet and Wahlsten, 1983; Washburn and Eicher, 1986), with hypoplasia of the cerebellum and abnormalities of hippocampal structure (Sekiguchi *et al*., 1992). Neocortical abnormalities in homozygotes for the *dr^J* allele particularly involve heterotopic neurons within the marginal zone (layer I) (Sekiguchi *et al*., 1994).

Recently, *dreher* was identified as a mutation of *Lmx1a*, a LIM homeobox gene that is expressed in the roof plate of the developing spinal cord, the neural crest and the rhombic lip of the hindbrain, as well as more generally in the posterior aspect of the developing cerebral hemispheres (Millonig *et al*., 2000). As a preliminary to determining the role of the *Lmx1a* gene in corticogenesis, we studied the origin and pathogenesis of the localized neuronal heterotopias in the brains of *dreher* mutant mice. We show that the neuronal heterotopias in *dr^J/dr^J* mice result from over-migration of neuroblasts destined for layer II of the cerebral cortex, and are closely associated with defects of the glial limiting membrane, providing clues to the possible pathogenesis of this category of neuronal migration disorder.

Materials and Methods

Dreher Mice and Genotyping

Heterozygous *dr^J* mice of the B6C3Fe-a/a-*dr^J* strain were purchased from the Jackson Laboratory (Bar Harbor, USA). Timed matings were performed overnight between pairs of *dr^J* heterozygotes, with the day of finding a copulation plug designated as embryonic day 0.5 (E0.5). Genotype at the *dr^J* locus was determined using the flanking polymorphic genetic markers *D1Mit452* and *D1Mit15* (mapbase@genome.wi.mit.edu). Genomic DNA was prepared from tail tip biopsies (Estibeiro *et al*., 1990) and amplified by polymerase chain reaction using 2 µl DNA, 1.0 mM $MgCl_2$, 0.1 mM each dNTP, 0.5 mM each primer and 1 unit of Taq polymerase (Bioline) in NH_4 buffer (16 mM ammonium sulphate, 67 mM Tris–HCl and 0.01% Tween 20 per reaction). Polymerase chain reactions (PCRs) involved initial denaturation at 95°C for 5 min, 35 amplification cycles of 92°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final step of 72°C for 10 min. Ethidium bromide-stained PCR amplification products were separated by agarose gel electrophoresis.

Preparation of Brains for Microscopic Analysis

Brains were perfusion-fixed *in vivo* prior to removal from the skull. Terminal anaesthesia in juveniles and adults was by i.p. injection of 0.1 ml/g body wt of 25% Hypnovel® (midazolam 5 mg/ml), 25% Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml) in sterile distilled water. Mice were perfused via the left ventricle with a flush of phosphate-buffered saline (PBS), then a 5–10 min perfusion of 4% paraformaldehyde (PFA) in PBS. Following fixation, brains were removed from the skull, fixed in 4% PFA for 2 h at room temperature, cut coronally into four standard slices and processed for paraffin wax embedding. Sections, 10 µm thick, were mounted on slides subbed with 1% aqueous 3-aminopropyltrietoxysilane (TESPA, Sigma) and stained with either hematoxylin and eosin or cresyl violet acetate (Sigma). Anatomical levels studied in wild-type and *dr^J/dr^J* mice (n = 4 in each case) were (A) anterior part of the frontal cortex, rostral to the corpus callosum; (B) anterior part of the corpus callosum and the anterior commissure; (C) anterior part of the hippocampus; (D) posterior part of the hippocampus; (E) posterior tip of the hippocampus and the occipital cortex; (F) posterior to the hippocampus, through the occipital cortex. At each level, the presence or absence of heterotopic neurons, and disruption of the

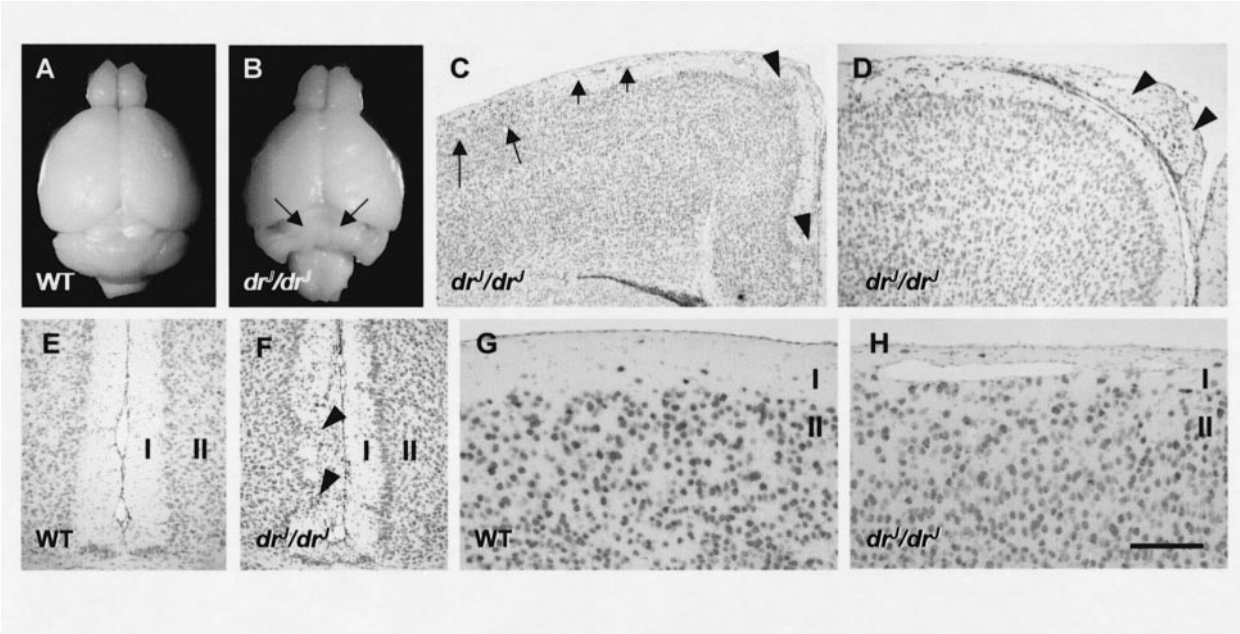

Figure 1. Abnormalities including neuronal heterotopias in the brains of dr^J homozygotes. Whole fixed adult brains (*A*,*B*), and coronal sections through the dorsal parietal (*C*,*D*: cresyl violet; *G*,*H*: NeuN immunostaining) and interhemispheric (*E*,*F*; cresyl violet) regions of the P21 cerebral cortex of wild-type (*A*,*E*,*G*) and dr^J/dr^J (*B–D*,*F*,*H*) brains. (*A*,*B*) The reduction in cerebellar size, particularly affecting the midline vermis, is apparent in the dr^J/dr^J brain (arrows in *B*) whereas the gross structure of the olfactory lobes, cerebral hemispheres and brain stem appears normal. (*C*,*D*) Layer I heterotopias in dr^J/dr^J brains are heterogeneous in nature. At some sites there is generalized hypercellularity of layer I (long arrows in *C*), whereas elsewhere distinct nodules apparently protrude from layer II into layer I (arrowheads in *C*). In some cases, heterotopic cells occupy the leptomeningeal space (arrowheads in *D*). Linear membranous structures, corresponding to the disrupted glial limiting membrane, run within layer I, roughly parallel to the pial limiting membrane (short arrows in *C*). (*E*,*F*) The cell-sparse nature of layer I is clearly seen in the wild-type interhemispheric cortex, whereas layer I is hypercellular in dr^J/dr^J, with apparent projections of layer II cells into layer I (arrowheads in *F*). (*G*,*H*) NeuN immunochemistry ($n = 8$ for both wild-type and dr^J/dr^J) reveals layer I of the wild-type brain to be almost devoid of positively staining nuclei, whereas NeuN positive cells are present throughout layer I in the dr^J/dr^J brain, confirming the neuronal nature of heterotopias in *dreher* brains. Scale bar in (*H*) represents: 7 mm in (*A*,*B*); 0.56 mm in (*C*); 0.24 mm in (*D*); 0.32 mm in (*E*,*F*); 0.12 mm in (*G*,*H*).

glial limiting membrane, were scored separately in each of the cortical areas defined by Caviness (Caviness, 1975).

Histochemistry and Immunohistochemistry

PFA-fixed sections were stained for reticulin fibres by a modification of the method of Gordon and Sweet (Bradbury and Rae, 1996). Sections were treated with 0.25% acidified potassium permanganate for 5 min, bleached in oxalic acid, then treated with 5% iron alum for 15 min, freshly filtered ammoniacal silver solution for 2 min, 10% formalin in tap water for 1 min, 0.2% gold chloride for 30 s and 5% sodium thiosulphate for 1 min. Distilled water washes followed each step. Sections were counterstained with 0.05% Nuclear Fast Red in 5% aqueous aluminium sulphate.

Immunohistochemistry was performed on PFA-fixed sections using antibodies specific for NeuN (mouse monoclonal, 1:1000 dilution, Chemicon MAB377), laminin (polyclonal rabbit anti-mouse, 1:50, Sigma L-9393), Factor VIII (polyclonal rabbit anti-human, 1:1000, DAKO A082) and glial fibrillary acidic protein (GFAP; polyclonal rabbit anti-cow, 1:500, DAKO Z0334). Hydrated sections were treated with 3% hydrogen peroxide, incubated for 1 h at room temperature, or overnight at 4°C, in primary antibody diluted in blocking serum (of the species in which the secondary antibody was raised), exposed to biotinylated secondary antibody for 30 min at room temperature, and incubated for 30 min with an avidin–biotin horseradish peroxidase solution (ABComplex/HRP, DAKO). Sections were exposed to 0.7 mg/ml diaminobenzidine, 0.7 mg/ml urea hydrogen peroxide and 0.06 M Tris buffer in distilled water, followed by counterstaining with cresyl violet acetate or haematoxylin. For anti-NeuN and anti-laminin immunostaining, sections were also microwaved in 0.01 M citrate buffer (pH 6.0) for 10 min and incubated with blocking serum for 30 min, immediately before adding primary antibody. For anti-Factor VIII immunostaining, sections were digested for 10 min with protease (type XXIV, Sigma, 0.02% in PBS) at 37°C, prior to incubation with blocking serum and primary antibody. Controls, in which the primary antibody was omitted, were negative in all cases.

BrdU Birthdating and Morphometric Analysis

Pregnant $dr^J/+$ females, time-mated with $dr^J/+$ males, received a single i.p. injection of 70 µg bromodeoxyuridine (BrdU)/g body wt at 12.00 h on E11.5, 13.5, 15.5 or 17.5. Progeny were genotyped between P6 and P10 (P0 = day of birth), then anaesthetized and perfusion-fixed on P21. Paraffin sections (thickness 10 µm) from brain level C (see above) were immunostained with an anti-BrdU antibody, as described by Gillies and Price (Gillies and Price, 1989). A camera lucida was used to plot the position of all BrdU-positive cells within a standardized 0.76 cm wide area of parietal cortex. Cells with dense staining of more than half the nucleus were considered BrdU-positive (Del Rio and Soriano, 1989; Gillies and Price, 1993; Price *et al.*, 1997) and were counted in a pair of adjacent sections, using the 'dissector' method (Sterio, 1984). Endothelial cell nuclei, identified on the basis of their elongated, fusiform shape and localization in vessel walls, were not counted. Cells in cortical layers IV, V and VI were clearly distinguishable, in both wild-type and dr^J/dr^J brains, whereas no obvious border could be discerned between layers II and III, in either wild type or dr^J/dr^J mice, as described previously for normal mouse parietal cortex (Caviness, 1975). Moreover, the presence of layer I heterotopias made it difficult to differentiate between layers I and II in dr^J/dr^J mice. Hence, cell counts were pooled, in both genotypes, for cortical layers I, II and III. The mean birthdate of heterotopic layer I cells in dr^J/dr^J brains was obtained from the BrdU-labelling index of heterotopic cells, specifically in regions of disrupted glial limiting membrane. In order to avoid including layer II cells in this estimate, cell counts were restricted to a ribbon of neocortex immediately beneath the pial surface. The thickness of this neocortical ribbon of neocortex corresponded to the outer 2/3 of layer I, as judged from areas of normal neocortex (i.e. lacking heterotopias) in the same brain.

Results

Gross examination of the brains of dr^J/dr^J mice revealed a relatively normal appearance of the cerebral hemispheres, but a

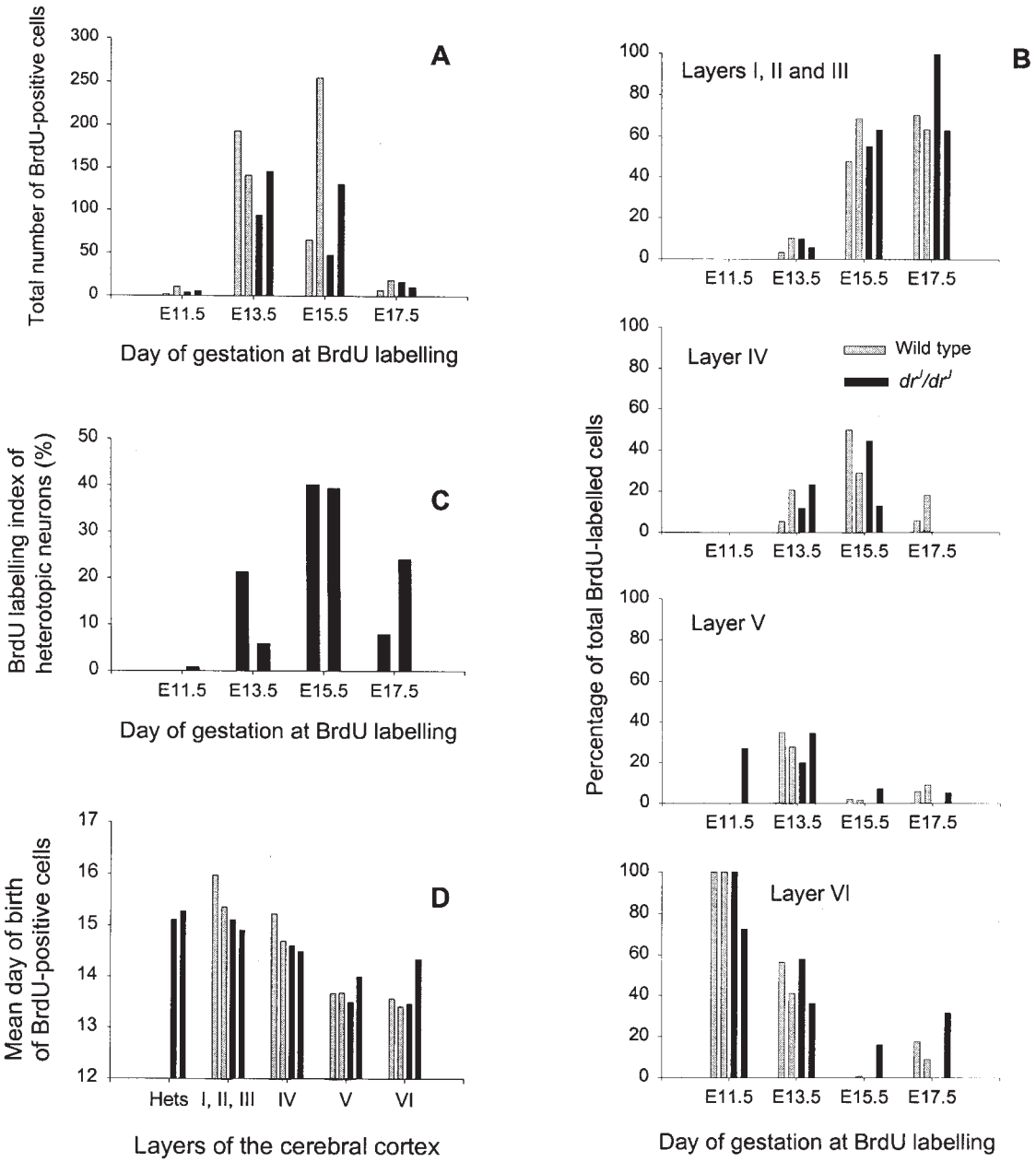

Figure 2. BrdU birthdating analysis of *dreher* corticogenesis. Pregnant females were given a single injection of BrdU at E11.5, 13.5, 15.5 or 17.5 and their offspring were analysed at P21. BrdU-labelled cells were counted in a 0.76 cm wide region of parietal cortex and data are plotted separately for each of the two brains of each genotype analysed for each gestational day of injection. (*A*) The total number of labelled cells does not differ between the cerebral cortex of wild-type (grey bars) and dr^J homozygous (black bars) mice (two-way analysis of variance; $P = 0.316$) whereas the number of BrdU-positive cells varies significantly between gestational days of injection ($P = 0.013$). (*B*) The distribution of BrdU-labelled cells among the different cortical layers, calculated as a percentage of total BrdU-labelled cell count, does not differ between wild-type (grey bars) and dr^J homozygous (black bars) mice (two-way analysis of variance; $P > 0.05$), whereas there is significant variation in the percentage of labelled cells between gestational days of injection ($P < 0.025$ for each layer). (*C*) Heterotopic neurons in dr^J/dr^J brains are born relatively late in corticogenesis. BrdU labelling index (number of labelled cells/total cell number $\times$ 100) of heterotopic layer I neurons varies significantly with gestational day of BrdU labelling (analysis of variance, $P = 0.038$), with the highest labelling index seen after injection at E15.5. (*D*) The mean day of birth of BrdU-labelled heterotopic neurons in layer I of dr^J/dr^J brains (Hets) is ~15 days, closely resembling cells of the pooled layers I–III, and differing markedly from cells in layers V and VI (wild-type, grey bars; dr^J/dr^J, black bars). Mean birthdate was calculated as: (number of cells labelled on a particular gestation day $\times$ gestation day/total labelled cells) summed across all gestation days studied.

severe reduction in cerebellar mass, particularly affecting the midline vermis (Fig. 1*A*,*B*). Dilatation of the fourth ventricle and distortion of the midbrain colliculi was observed in some cases. Histological examination of the cerebellar vermis revealed a disorganized structure, with a frequent admixture of cells belonging to the molecular, Purkinje and granule cell layers (data not shown). The hippocampus also exhibited histological abnormalities, including a rudimentary dentate gyrus, splitting of the pyramidal cell layer, and fusion of the infrapyramidal blade with the thalamus (data not shown), confirming previous observations on *dreher* mutants (Sekiguchi *et al.*, 1992).

Heterotopias in Layer I of the Dreher Cerebral Cortex

Heterotopic cells were observed in layer I (the marginal zone) of

the cerebral cortex in 3 weeks postnatal (P21) dr^J/dr^J mice, as described previously (Sekiguchi *et al.*, 1994). In affected regions, the upper border of layer II appeared indistinct, or ruffled, and heterotopic cells were either distributed diffusely throughout large areas of layer I (hypercellularity) or, more commonly, occurred in small clusters, with relatively normal areas of layer I intervening between heterotopias (Fig. 1*C*,*F*). In some cases, the appearance was of finger-like expansions from layer II into layer I, abutting onto the surface of the brain (Fig. 1*C*,*F*). Collections of heterotopic cells were occasionally seen invading the leptomeningeal space (Fig. 1*D*). In contrast to the layer I abnormalities, we could detect no consistent differences between wild-type and dr^J/dr^J mice in the organization of layers II–VI of the cerebral cortex.

The spatial distribution of layer I neuronal heterotopias was determined in four typical P21 wild-type and dr^J/dr^J brains. The wild-type brains were normal throughout, whereas all four dr^J/dr^J brains exhibited abundant layer I heterotopias in dorsal aspects of the frontal cortex [areas 4, 6 and 8 of Caviness (Caviness, 1975)] and parietal cortex (area 3). In contrast, more ventrally located areas of frontal cortex (areas 10 and 11) and parietal cortex (area 40) were not affected by heterotopias in any of the dr^J/dr^J brains. Two of the four brains also showed heterotopias in rostral cortical areas bordering the interhemispheric fissure (area 24). In contrast, the occipital lobe was only affected in a single brain, while the temporal lobe and ventral regions of the frontal and parietal cortex were not affected in any of the brains. Hence, the defects show a definite preponderance in rostral and dorsal cerebral cortex, close to or including the midline.

Neuronal Nature of Heterotopic Cells in dr^J Homozygous Brains

Heterotopic layer I cells exhibited a neuronal phenotype in cresyl violet-stained preparations, with oval or pyramidal cell bodies and large nuclei. This morphology resembled layer II neurons and was quite distinct from the bipolar, horizontal Cajal–Retzius cells that normally populate layer I. To examine an alternative possibility – that the heterotopic cells are glial in nature – we immunostained sections of P21 brains with an antibody to NeuN, a general marker of neurons (Wolf *et al.*, 1996). Wild-type brains contained almost no NeuN-positive cells in layer I (Fig. 1*G*), whereas heterotopic cells stained positively for NeuN in dr^J/dr^J brains, so that immunopositive cells could often be seen extending near to the pial surface (Fig. 1H). We conclude that the heterotopic cells are neuronal, with a morphology resembling layer II cells.

Heterotopic Neurons in Layer I of dr^J Homozygotes are Born Late in Cerebral Corticogenesis

To determine the stage of corticogenesis at which layer I heterotopias are generated in dr^J/dr^J brains, we labelled wild-type and dr^J/dr^J fetuses with a single maternal injection of BrdU, on E11.5, 13.5, 15.5 or 17.5, and harvested brains from the offspring when they reached P21. Neurons in all layers of the cerebral cortex were counted within a 0.76 cm wide region of parietal cortex, which was chosen to include layer I regions exhibiting abnormalities of the glial limiting membrane in dr^J/dr^J brains (see below). Injections of BrdU on different gestational days produced strikingly different numbers of labelled cells, with injections at E13.5 and E15.5 producing many more labelled cells than injections at E11.5 or E17.5 (Fig. 2*A*). However, the total number of cells labelled with BrdU did not differ between wild-type and dr^J/dr^J brains (Fig. 2*A*) suggesting that the rate of birth of post-mitotic neuroblasts during corticogenesis is not disturbed in *dreher* mice.

When the allocation of BrdU-labelled cells to the different layers of the cerebral cortex was analysed, we again found striking differences between brains labelled on different gestational days (Fig. 2*B*). E11.5 injections labelled predominantly layer VI cells, E13.5 injections labelled cells in all layers, E15.5 labelled cells mainly in the pooled layers I–III (mainly layers II and III) and in layer IV, and E17.5 injections labelled cells predominantly in the pooled layers I–III. Hence, our findings confirmed the 'inside-out' generation of cerebral cortical layers (Angevine and Sidman, 1961; Caviness, 1982). Importantly, we did not observe any abnormalities of this overall pattern of neuroblast generation in dr^J/dr^J brains (Fig. 2*B*), suggesting that the orderly allocation of post-mitotic neuroblasts to layers of the cerebral cortex is not grossly disrupted in *dreher* mice.

Next, we specifically analysed the BrdU-labelling profile of heterotopic neurons in dr^J/dr^J brains. Layer I cells exhibited maximal labelling in dr^J/dr^J following injection of BrdU at E15.5, whereas heterotopic neurons in brains from E11.5 injections were very rarely labelled (Fig. 2*C*). BrdU injection at E13.5 and E17.5 labelled intermediate numbers of heterotopic layer I cells. The mean day of birth of the heterotopic neurons was ~15 days, similar to the value obtained for the pooled cells of layers I, II and III in both wild-type and dr^J/dr^J brains (Fig. 2*D*). Layer IV neurons exhibited a mean birth date of ~14.5 days, in both genotypes, whereas the values for layer V and VI neurons were around 13.5 and 13.25 respectively (Fig. 2*D*). We conclude that heterotopic neurons in layer I of dr^J/dr^J brains resemble layer II and III neurons in being generated towards the end of corticogenesis.

Abnormalities of the Glial Limiting Membrane and Abnormal Layer I Vessel Organization in dr^J Homozygotes

Abnormalities of the glial limiting membrane, and an abnormal vascular network, were present in association with heterotopic neurons in layer I of dr^J/dr^J brains on P21 (Fig. 3). These defects were intimately related with each other and had the same spatial distribution as heterotopic layer I neurons. Defects of the glial limiting membrane were seen in sections stained with cresyl violet (Fig. 1*C*) or haematoxylin and eosin in all 11 dr^J/dr^J brains studied, but in none of eight age-matched wild-type brains. Fine membranous structures were observed in the outer two-thirds of layer I, as broken lines usually running parallel to the brain surface (Fig. 3*B*). They were either isolated, continuous with vessel walls, or associated with the external surface of the brain (i.e. with the normal localization of the pial–glial interface), in which case they assumed a more oblique direction (e.g. Fig. 3*D*). These structures were unlabelled with anti-Factor VIII antibodies (Fig. 3*E*,*F*), but they were highlighted with a reticular fibre impregnation technique (Fig. 3*A*,*B*) and with anti-laminin antibodies (Fig. 3*C*,*D*), suggesting that they represent a broken, abnormally positioned glial limiting membrane. In support of this idea, reticulin staining also demonstrated a focally discontinuous pial–glial membrane at the surface of the brain in affected cortical regions of dr^J/dr^J mice (arrows in Fig. 3*B*), in contrast to the continuous, even-stained pial–glial membrane of wild-type brains (Fig. 3*A*).

The vascular abnormalities in layer I of dr^J/dr^J brains were highlighted by immunolabelling sections of the parietal cortex

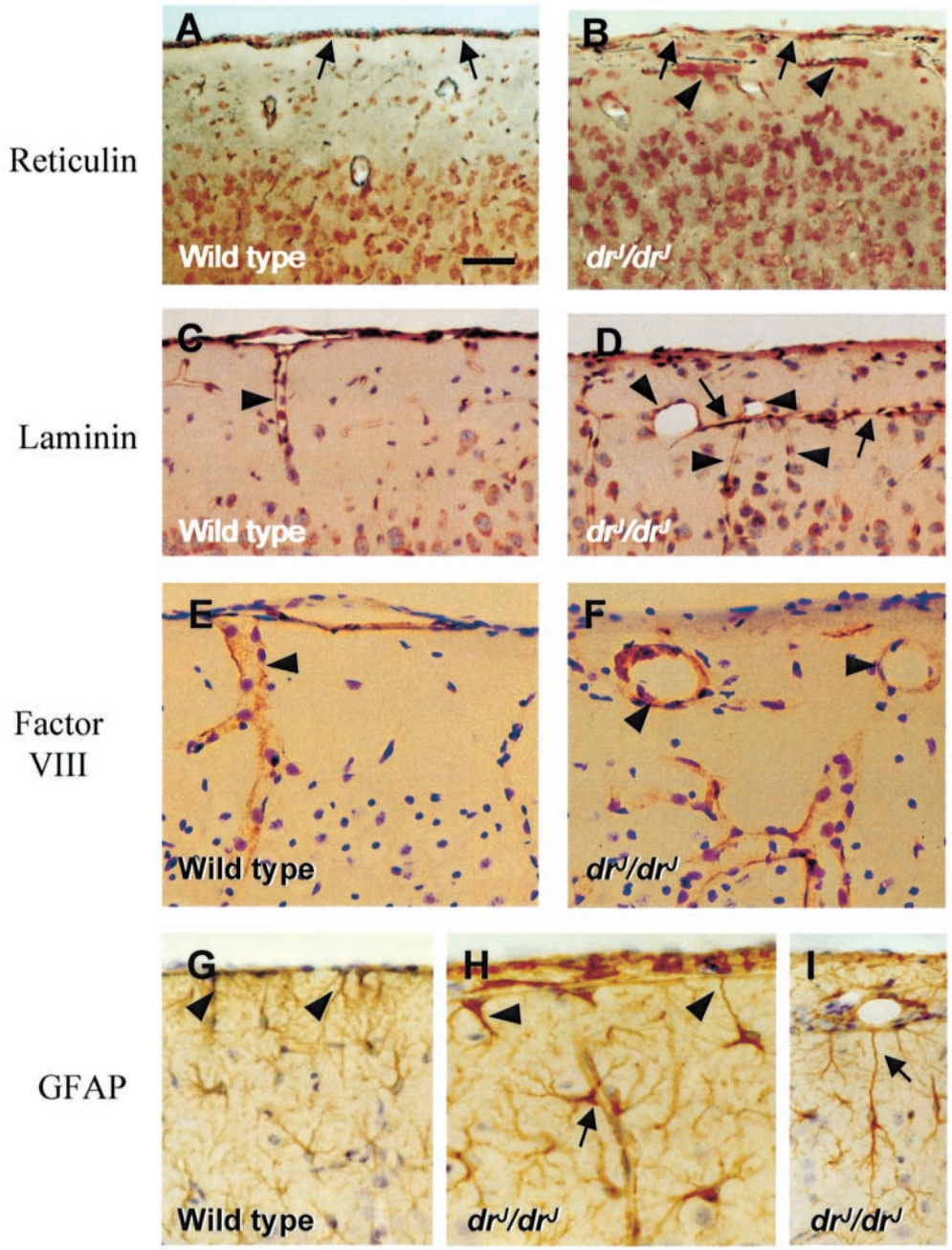

Figure 3. Abnormalities of the glial limiting membrane, and abnormal vascular network, in layer I of the cerebral cortex of dr^J homozygotes, in association with neuronal heterotopias. Coronal sections through the parietal cortex of wild-type (*A*,*C*,*E*,*G*) and dr^J/dr^J (*B*,*D*,*F*,*H*,*I*) mice at P21. (*A*,*B*) Reticulin fibre staining by the Gordon and Sweet method (stained grey to black; $n = 8$ for both wild-type and dr^J/dr^J). The pial–glial junction is continuous and evenly stained in the wild-type brain (arrows in *A*), whereas it exhibits focal disruption in the dr^J/dr^J brain, with discontinuous staining (arrows in *B*) and membranous profiles within layer I (arrowheads in *B*) running parallel to the brain surface. (*C*,*D*) Immunostaining of basement membranes (stained dark red) using an anti-laminin antibody ($n = 8$ for both wild-type and dr^J/dr^J). In the wild-type brain, the pial–glial surface exhibits strong, continuous staining. A perforating blood vessel (arrowhead in *C*) is visible, originating from the pial surface. In contrast, the dr^J/dr^J brain exhibits weaker staining of the pial surface, with additional immunoreactivity in ectopic membranous profiles within layer I (arrows in *D*). Vessels with immunopositive walls can be seen running in various directions within layer I (giving the appearance of open spaces), particularly in association with the ectopic membrane profiles (arrowheads in *D*). (*E*,*F*) Immunostaining with an antibody specific for Factor VIII (von Willebrand factor; $n = 3$ for both wild-type and dr^J/dr^J), a marker of vessel walls. A perforating vessel can be seen in the wild-type brain originating from the pial surface (arrowhead in *E*), whereas the dr^J/dr^J brain exhibits vessels running within layer I, with no apparent relationship to the pial surface (arrowheads in *F*). (*G*–*I*) Immunostaining with an anti-GFAP antibody ($n = 5$ for both wild-type and dr^J/dr^J). Immunopositive astrocytes can be seen in the wild-type brain, adhering to the inner surface of the glial limiting membrane (arrowheads in *G*). In dr^J/dr^J brains, astrocytes are detected attaching to the ectopic glial limiting membrane (arrowheads in *H*) and to abnormally positioned vessels (arrows in *H*,*I*) within layer I. Scale bar in (*A*) represents 100 μm in (*A*,*B*); 45 μm in (*C*,*D*); 30 μm in (*E*,*F*,*H*,*I*); 70 μm in (*G*).

using an antibody specific for Factor VIII, a general vascular marker. Normal layer I vessels are usually perforating vascular channels that originate in the leptomeninges, and penetrate the brain approximately at right angles to the pial surface. These vessels were immunostained in wild-type brains by both the anti-Factor VIII and anti-laminin antibodies (Fig. 3*C*,*E*). The con-

tinuous pial–glial membrane was also labelled by the anti-Factor VIII antibody (Fig. 3*E*). In the brains of dr^J/dr^J mice, however, vascular profiles appeared more numerous and were more often arranged parallel, rather than perpendicular, to the pial surface (Fig. 3*F*). These features were seen exclusively in areas with an abnormal glial limiting membrane and coexisted with heterotopic layer I neurons, which were located either internal or external to the membrane fragments and abnormal vessels. No such vascular network was seen in the brains of normal mice or in areas of normal-looking cortex in dr^J/dr^J mice (e.g. temporal neocortex).

Astrocyte Morphology and Distribution in Areas of Abnormal Glial Limiting Membrane

The spatial distribution of astrocytes in layer I of P21 wild-type and dr^J/dr^J brains was studied by immunolabelling with an anti-GFAP antibody. In wild-type brains, layer I astrocytes were usually associated with vessels or the pial surface at the glial limiting membrane (Fig. 3*G*). In constrast, regions of dr^J/dr^J brains containing heterotopias exhibited GFAP-positive astrocytes associated with both the aberrant vascularization of layer I and with the abnormal, disrupted glial limiting membrane, to which some of them were connected (Fig. 3*H*,*I*). Astrocytes not overtly in contact with these structures showed either a fusiform or a stellate appearance with prominent processes. In normal-looking regions from dr^J/dr^J brains, astrocytes were indistinguishable in morphology and distribution from those of wild-type brains.

Discussion

During development of the cerebral cortex in dr^J homozygous mutant mice, a small fraction of neurons become misplaced in the normally cell-poor marginal zone (layer I). Here, we show that these heterotopic neurons derive predominantly from late-migrating neuroblasts, and that these neuronal heterotopias form in close association with disruption of the external glial limiting membrane, suggesting a possible causal connection between these two defects.

Heterotopic Cells are Generated Late During Neurogenesis in Dreher

Using BrdU labelling, we found no major differences between wild-type and dr^J/dr^J mice in the extent, rate and orderly nature of neuronal generation, in terms of their population of the different cortical layers. Moreover, the normal 'inside-out' pattern of genesis of layers II–VI (Angevine and Sidman, 1961; Rakic, 1972; Caviness, 1982) appears to be well preserved in mutant brains. This finding constrasts markedly with birthdating and other studies in mouse mutants such as *reeler*, *scrambler* and *yotari*, in which major abnormalities have been detected in the generation of the cortical plate, with the result that a reversed ('outside-in') pattern of cortical layering is produced (Pinto-Lord *et al*., 1982; Goldowitz *et al*., 1997; Howell *et al*., 1997; Sheldon *et al*., 1997).

BrdU birthdating studies in *dreher* further reveal that heterotopic layer I cells are born at approximately the same stage during gestation as those destined for layers II and III. These findings suggest strongly that heterotopic neurons in layer I of dr^J/dr^J mice do not represent early-born, pre-plate neurons which are normally removed by programmed cell death (Allendoerfer and Shatz, 1994; Price *et al*., 1997), but which might persist under pathological conditions. Failure of programmed cell death is capable of generating heterotopic neurons, as indicated by the excess numbers of layer I neurons observed in the brains of mice homozygous for a null mutation of the *CPP32* gene, in which the intracellular pathway leading to programmed cell death is disrupted (Kuida *et al*., 1996). In *dreher*, however, the generation of heterotopic cells during the late period of neurogenesis is consistent with their origin from cells destined to populate layers II and III, but which 'over-migrate', settling in layer I. This conclusion is supported by our findings that many of the heterotopic cells morphologically resemble neurons of normal layers II and III.

Possible Roles of Disturbed Radial versus Tangential Neuronal Migration in Dreher

The present results are consistent with a pathogenetic mechanism in *dreher* homozygotes involving disturbed radial glial-guided neuronal migration, resulting in 'over-migration' of layer II/III neuroblasts to populate layer I. Radial glial end-feet seem likely to attach to the fragmented pial limiting membrane (as demonstrated by our GFAP staining of their astrocyte derivatives in P21 brains) and this faulty radial glial attachment may disturb glial-guided neuroblast migration, resulting in the population of layer I by cells normally destined for layer II. On the other hand, we cannot exclude an alternative hypothesis that heterotopic cells in layer I might represent tangentially migrating neurons arising from the lateral ganglionic eminence, as has been described for certain subclasses of cortical neurons (Anderson *et al*., 1997; Tamamaki *et al*., 1997). Indeed, Brunstrom *et al*. (Brunstrom *et al*., 1997) described layer I heterotopias induced by neurotrophin 4 resulting from increased numbers of immigrating neurons that resemble cells of the subpial granule cell layer (Gadisseux *et al*., 1992; Meyer *et al*., 1993). If tangential migration were the main pathogenetic mechanism in *dreher*, however, one might expect that the heterotopias would extend beyond the neocortex, along the path for tangential migration, whereas this was not observed. Moreover, an additional mechanism would have to be sought to explain the disruption of the glial limiting membrane and its close spatial association with the areas of heterotopic neurons.

Glial Limiting Membrane Defects as a Possible Factor Leading to Neuronal Heterotopias in Dreher

We found abnormalities of the glial limiting membrane, associated with a network of abnormally arranged vessels, in layer I of the neocortex of dr^J/dr^J brains. These defects exhibited a close spatial correlation with layer I neuronal heterotopias. Although this correlation does not demonstrate a cause-and-effect relationship, it is striking that an association between layer I neuronal heterotopias and abnormalities of the pial–glial interface has been described in several other genetic and non-genetic mouse models. These include the immunodeficient strains New Zealand Black, BXSB and Snell dwarf (Sherman *et al*., 1990), the *Macs* knockout (Blackshear *et al*., 1997), and the brains of newborn mice subjected to minute puncture wounds (Rosen *et al*., 1992) or chemical lesions of the leptomeninges (Supèr *et al*., 1997) prior to the completion of neuronal migration.

Although the association of layer I heterotopias in association with glial limiting membrane defects provides a parallel to the situation we observe in *dreher*, there are notable differences as well. The immune-deficient mice do not exhibit an abnormal arrangement of vessels within layer I, unlike dr^J, while the heterotopias in NZB mice and in the *Macs* knockout are usually single large collections of neurons that protrude into the leptomeningeal space, whereas dr^J homozygotes mainly exhibit

multiple small clusters of heterotopic neurons, or hypercellularity of layer I, with only occasional leptomeningeal protrusions. Moreover, the phenotype in the *Macs* knockout also involves a high frequency of exencephaly, agenesis of brain commissures and cortical lamination defects (Stumpo *et al.*, 1995), not observed in dr^{J}. Thus, *dreher* is unusual in the specificity of its phenotype which comprises defects of late neuronal migration affecting the cerebral cortex, hippocampus and cerebellum, without overall disorganization of neocortical lamination or other major structural brain defects.

Molecular Basis of the dreher Genetic Defect

Millonig and co-workers recently identified *Lmx1a* as the gene mutated in *dreher* mice (Millonig *et al.*, 2000). Members of the *Lmx1* gene family are characterized by possession of a LIM-domain DNA-binding motif, and appear to be responsible for specifying dorsal cell fate in a number of body systems including the developing limb and kidney (Chen *et al.*, 1998). *Lmx1a* is expressed specifically in the dorsal-most cells of the developing mouse neural tube, and diminution of its function, as in *dreher* homozygotes, leads to failure of differentiation of neural crest cells and a subpopulation of dorsal interneurons (Manzanares *et al.*, 2000; Millonig *et al.*, 2000). While the pattern of expression of *Lmx1a* during cerebral corticogenesis has not yet been described in detail, transcripts are reported mainly in the midline of the developing forebrain (Millonig *et al.*, 2000).

We found neuronal heterotopias, in P21 *dreher* brains, confined predominantly to the dorsal aspects of the frontal and parietal lobes, and to cortex bordering the interhemispheric fissure, consistent with a requirement for *Lmx1a* in the dorsal cerebral cortex. On the other hand, we frequently observed heterotopias, in parietal cortex, at a considerable distance from the midline. Heterotopic neurons could undergo lateral translocation, within layer I, after they have taken up their ectopic positions, but we do not favour this explanation because we invariably observed a disrupted glial limiting membrane, and abnormal vascularization of layer I, in association with the heterotopic neurons, even at locations far from the midline.

An alternative possibility is that *Lmx1a* expression is necessary for normal development of the glial limiting membrane, perhaps by promoting a trophic effect of dorsal forebrain cells on formation of the pial–glial basement membrane, or by promoting attachment of radial glial end feet to the pial surface, a necessary step in formation of the glial limiting membrane (Sievers *et al.*, 1994; Struckhoff, 1995). Such effects may depend on molecular events considerably downstream of the action of the Lmx1a transcription factor, and could involve diffusible signalling molecules or trophic factors. This may account for our finding of abnormalities in layer I of *dreher* brains, relatively distant from the midline.

Notes

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References

Allendoerfer KL, Shatz CJ (1994) The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. Annu Rev Neurosci 17:185–218.

Anderson SA, Eisenstat DD, Shi L, Rubenstein JLR (1997) Interneuron migration from basal forebrain to neocortex: dependence on *Dlx* genes. Science 278:474–476.

Angevine JB, Sidman RL (1961) Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. Nature 192:766–768.

Blackshear PJ, Silver J, Nairn AC, Sulik KK, Squier MV, Stumpo DJ, Tuttle JS (1997) Widespread neuronal ectopia associated with secondary defects in cerebrocortical chondroitin sulfate proteoglycans and basal lamina in MARCKS-deficient mice. Exp Neurol 145:46–61.

Bradbury P, Rae K (1996) Connective tissues and stains. In: Theory and practice of histological techniques (Bancroft JD, ed.), pp. 113–138. London: Churchill Livingston.

Brunstrom JE, Gray-Swain MR, Osborne PA, Pearlman AL (1997) Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. Neuron 18:505–517.

Caviness VS (1975) Architectonic map of neocortex of the normal mouse. J Comp Neurol 164:247–263.

Caviness VS (1982) Neocortical histogenesis in normal and reeler mice: a developmental study based upon tritiated thymidine autoradiography. Dev Brain Res 4:293–302.

Chadani Y, Kondoh T, Kamimura N, Matsumoto T, Matsuzaka T, Kobayashi O, Kondo-Iida E, Kobayashi K, Nonaka I, Toda T (2000) Walker–Warburg syndrome is genetically distinct from Fukuyama type congenital muscular dystrophy. J Neurol Sci 177:150–153.

Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL (1998) Limb and kidney defects in *Lmx1b* mutant mice suggest an involvement of *LMX1B* in human nail patella syndrome. Nature Genet 19:51–55.

D'Arcangelo G, Miao GG, Chen S-C, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 374:719–723.

Del Rio JA, Soriano E (1989) Immunocytochemical detection of 5′-bromodeoxyuridine incorporation in the central nervous system of the mouse. Dev Brain Res 49:311–317.

Des Portes V, Pinard JM, Billuart P, Vinet MC, Koulakoff A, Carrie A, Gelot A, Dupuis E, Motte J, Berwald Netter Y, Catala M, Kahn A, Beldjord C, Chelly J (1998) A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. Cell 92:51–61.

Estibeiro JP, Copp AJ, Cockroft DL, Brown NA, Clarke DO (1990) Extraction of macromolecules from embryonic material. In: Postimplantation mammalian embryos: a practical approach (Copp AJ, Cockroft DL eds), pp. 173–204. Oxford: IRL Press.

Fox JW, Lamperti ED, Eksioglu YZ, Hong SE, Feng Y, Graham DA, Scheffer IE, Dobyns WB, Hirsch BA, Radtke RA, Berkovic SF, Huttenlocher PR, Walsh CA (1998) Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron 21:1315–1325.

Gadisseux JF, Goffinet AM, Lyon G, Evrard P (1992) The human transient subpial granular layer: an optical, immunohistochemical, and ultrastructural analysis. J Comp Neurol 324:94–114.

Gillies K, Price DJ (1989) The use of bromodeoxyuridine to label proliferating cells in the nervous system of the fetal mouse. J Physiol 420:4P(abstract).

Gillies K, Price DJ (1993) The fates of cells in the developing cerebral cortex of normal and methylazoxymethanol acetate-lesioned mice. Eur J Neurosci 5:73–84.

Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA (1998) Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell 92:63–72.

Goffinet AM (1997) Developmental neurobiology — unscrambling a disabled brain. Nature 389:668–669.

Goldowitz D, Cushing RC, Laywell E, D'Arcangelo G, Sheldon M, Sweet HO, Davisson M, Steindler D, Curran T (1997) Cerebellar disorganization characteristic of reeler in scrambler mutant mice despite presence of reelin. J Neurosci 17:8767–8777.

Hattori M, Adachi H, Tsujimoto M, Arai H, Inoue K (1994) Miller–Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor. Nature 370:216–218.

Howell BW, Hawkes R, Soriano P, Cooper JA (1997) Neuronal position in the developing brain is regulated by mouse *disabled-1*. Nature 389:733–737.

Kobayashi K, Nakahori Y, Miyake M, Matsumura K, Kondo-Iida E, Nomura

Y, Segawa M, Yoshioka M, Saito K, Osawa K, Hamano K, Sakakihara Y, Nonaka I, Nakagome Y, Kanazawa I, Nakamura Y, Tokunaga K, Toda T (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature 394:388–392.

Kuida K, Zheng TS, Na SQ, Kuan CY, Yang D, Karasuyama H, Rakic P, Flavell RA (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368–372.

Lo Nigro C, Chong CS, Smith AC, Dobyns WB, Carrozzo R, Ledbetter DH (1997) Point mutations and an intragenic deletion in LIS1, the lissencephaly causative gene in isolated lissencephaly sequence and Miller–Dieker syndrome. Hum Mol Genet 6:157–164.

Lyon MF (1961) Linkage relations and some pleiotropic effects of the dreher mutant of the house mouse. Genet Res 2:92–95.

Manzanares M, Trainor PA, Ariza-McNaughton L, Nonchev S, Krumlauf R (2000) Dorsal patterning defects in the hindbrain, roof plate and skeleton in the *dreher* (*dr*^{J}) mouse mutant. Mech Dev 94:147–156.

Meyer G, Gonzalez Hernandez T (1993) Developmental changes in layer I of the human neocortex during prenatal life: a DiI-tracing and AChE and NADPH-d histochemistry study. J Comp Neurol 338:317–336.

Millonig JH, Millen KJ, Hatten ME (2000) The mouse *Dreher* gene *Lmx1a* controls formation of the roof plate in the vertebrate CNS. Nature 403:764–769.

Pinto-Lord MC, Evrard P, Caviness VS Jr (1982) Obstructed neuronal migration along radial glial fibers in the neocortex of the reeler mouse: a Golgi-EM analysis. Dev Brain Res 4:379–393.

Price DJ, Aslam S, Tasker L, Gillies K (1997) Fates of the earliest generated cells in the developing murine neocortex. J Comp Neurol 377: 414–422.

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol 145:61–84.

Rosen GD, Sherman GF, Richman JM, Stone LV, Galaburda AM (1992) Induction of molecular layer ectopias by puncture wounds in newborn rats and mice. Dev Brain Res 67:285–291.

Sekiguchi M, Shimai K, Guo H, Nowakowski RS (1992) Cytoarchitectonic abnormalities in hippocampal formation and cerebellum of dreher mutant mouse. Dev Brain Res 67:105–112.

Sekiguchi M, Abe H, Shimai K, Huang G, Inoue T, Nowakowski RS (1994) Disruption of neuronal migration in the neocortex of the dreher mutant mouse. Dev Brain Res 77:37–43.

Sheldon M, Rice DS, D'Arcangelo G, Yoneshima H, Nakajima K, Mikoshiba K, Howell BW, Cooper JA, Goldowitz D, Curran T (1997) *Scrambler* and *yotari* disrupt the *disabled* gene and produce a *reeler*-like phenotype in mice. Nature 389:730–733.

Sherman GF, Morrison L, Rosen GD, Behan PO, Galaburda AM (1990) Brain abnormalities in immune defective mice. Brain Res 532:25–33.

Sievers J, Pehlemann FW, Gude S, Berry M (1994) Meningeal cells organize the superficial glia limitans of the cerebellum and produce components of both the interstitial matrix and the basement membrane. J Neurocytol 23:135–149.

Sterio DC (1984) The unbiased estimation of number and sizes of artibrary particles using the disector. J Microsc 134:127–136.

Struckhoff G (1995) Cocultures of meningeal and astrocytic cells — a model for the formation of the glial-limiting membrane. Int J Dev Neurosci 13:595–606.

Stumpo DJ, Bock CB, Tuttle JS, Blackshear PJ (1995) MARCKS deficiency in mice leads to abnormal brain development and perinatal death. Proc Natl Acad Sci USA 92:944–948.

Supèr H, Martínez A, Soriano E (1997) Degeneration of Cajal–Retzius cells in the developing cerebral cortex of the mouse after ablation of meningeal cells by 6-hydroxydopamine. Dev Brain Res 98:15–20.

Sweet HO, Wahlsten D (1983) Shaker short-tail (sst). Mouse News Lett 69:26.

Tamamaki N, Fujimori KE, Takauji R (1997) Origin and route of tangentially migrating neurons in the developing neocortical intermediate zone. J Neurosci 17:8313–8323.

Washburn L, Eicher EM (1986) A mutation at the dreher locus (dr2J). Mouse News Lett 75:28–29.

Williams RS, Swisher CN, Jennings M, Ambler M, Caviness VS (1984) Cerebro-ocular dysgenesis (Walker–Warburg syndrome): neuropathologic and etiologic analysis. Neurology 34:1531–1541.

Wolf HK, Buslei R, Schmidt-Kastner R, Schmidt-Kastner PK, Pietsch T, Wiestler OD, Bluemcke I (1996) NeuN: a useful neuronal marker for diagnostic histopathology. J Histochem Cytochem 44:1167–1171.