Dissection of the Cellular and Molecular Events that Position Cerebellar Purkinje Cells: A Study of the math1 Null-Mutant Mouse

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Granule cell precursors in the external germinal layer (EGL) of the cerebellum have been proposed to be a major player in the migration and positioning of Purkinje cells through the expression of the Netrin-like receptor Unc5h3 and the extracellular matrix molecule Reelin. To explore the role of the EGL on these processes, we made use of the math1 null-mutant mouse in which the EGL does not form. In the absence of the EGL, we find three populations of ectopic Purkinje cells. First, we find 1% of all Purkinje cells in a supracerebellar position at the dorsal midline. Second, we find 7% of all Purkinje cells in the inferior colliculus, similar to what is seen in the Unc5h3 mutant. Our finding that Unc5h3 expression is not disrupted in these cells supports the proposed role of EGL granule cell precursors in establishing the anterior cerebellar boundary through the expression of Unc5h3. Third, we find 20% of all Purkinje cells positioned deep to the cerebellar cortex as seen in the reeler mutant. However, unlike the reeler mutant, where 5% of the Purkinje cells migrate successfully, we find that in the math1 1 null that 72% of the Purkinje cells migrate successfully. This finding demonstrates that Purkinje cell migration is not solely dependent on Reelin signaling from the EGL and is likely caused by Reelin signals emanating from the nuclear transitory zone or the ventricular zone, or both.

Key words: Math1; Reelin; Disabled-1; Unc5h3; migration; EGL; granule cell

A fundamental goal in developmental neurobiology is to determine the controlling elements that guide the migration of neurons from their site of genesis to their normal position in the mature nervous system. In the cerebellum, the migratory path of Purkinje cells, from the ventricular zone (VZ) to their final position in the Purkinje cell layer (PCL) in the cerebellar cortex, has been well documented (Miale and Sidman, 1961; Yuasa et al., 1991; Altman and Bayer, 1997). However, the molecular mechanisms underlying their migration and positioning are just beginning to be discovered. Through the study of neurological mutant mice, several molecules involved in this process have been identified (D'Arcangelo et al., 1995; Ohshima et al., 1996; Ackerman et al., 1997; Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999). One such molecule is the extracellular matrix molecule Reelin (D'Arcangelo et al., 1995).

In the homozygous reeler mutant mouse (rl/rl), which contains a mutation in the gene encoding Reelin, ~95% of the total Purkinje cell population is ectopic, deep to the cerebellar cortex (Heckroth et al., 1989). Previous reports have indicated that Reelin signaling from the external germinal layer (EGL) is essential for the migration of Purkinje cells (Miyata et al., 1997; Curran and D'Arcangelo, 1998; D'Arcangelo and Curran, 1998; Rice and Curran, 1999). However, the EGL is not the sole source of Reelin. Reelin is also expressed in the nuclei of the nuclear transitory zone (NTZ), which will form the cerebellar nuclei in establishing the anterior cerebellar boundary through the expression of Unc5h3. Third, we find 20% of all Purkinje cells positioned deep to the cerebellar cortex as seen in the reeler mutant. However, unlike the reeler mutant, where 5% of the Purkinje cells migrate successfully, we find that in the math1 1 null that 72% of the Purkinje cells migrate successfully. This finding demonstrates that Purkinje cell migration is not solely dependent on Reelin signaling from the EGL and is likely caused by Reelin signals emanating from the nuclear transitory zone or the ventricular zone, or both.

Key words: Math1; Reelin; Disabled-1; Unc5h3; migration; EGL; granule cell

Materials and Methods

Animals and determination of genotype. Heterozygous math1 mice (math1^+/gal^+), which contain a β-galactosidase (β-gal) reporter gene in place of the math1 open reading frame (originally obtained from Dr. Huda Y. Zoghbi, Baylor College of Medicine, Houston, TX) (Bermingham et al., 1999), and reeler mice (originally obtained from the Jackson Laboratory, Bar Harbor, ME) were maintained at the University of Tennessee Health Science Center animal care facility. Mice were kept on a 14–10 hr light/dark cycle with food and water ad libitum. All of the mice were treated in accordance with The Society for Neuroscience policy on the use of animals in research.

Because math1^−/gal^− mice are neonatal lethal, all math1^−/gal^− mice and math1^+/gal^− mice used in this study were generated from matings between math1^−/gal^− breeding pairs. Reeler mice were generated from matings

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between heterozygous (+/h) males and either +/h or homozygous (h/h) females. Females were examined each morning for the presence of a vaginal plug. Once a plug was detected, the female was removed from the cage, and noon of that day was designated as embryonic day (E) 0.5. Embryos were collected on E13.5, 15.5, 17.5, and the day of birth (P0).

To determine genotype, DNA was isolated from either the tail or yolk sac. The math1 genotype was determined by PCR using the following primers: math1-forward 5’-TAAACGGCAGATGGCC3’-math1-reverse 5’-CTAACAAGCTACCCAGAC-3’. With the PCR conditions described in (Jensen et al., 2003). Adjacent sections were hybridized onslides (Fisher Scientific, Pittsburgh, PA). ET0/AA-fixed tissue was placed in 70% ethanol overnight, followed by dehydration in a series of ethanols and clearing in xylene. Tissue was embedded in paraffin, and 6 µm sagittal sections were mounted on Superfrost Plus slides (Fisher Scientific). Immunochemistry. For each time-point and genotype, adjacent paraffin sections were processed for immunohistochemistry with antibodies against Calbindin (anti-Calbindin D28K 1:50; Chemicon), and 20 µg sagittal cryosections were mounted on Superfrost Plus slides (Fisher Scientific). ET0/AA-fixed tissue was rinsed in 0.1 M PBS solution, pH 7.3, followed by fixation with either 4% paraformaldehyde or a 3:1 solution of 95% ethanol and acetic acid (EtOH/AA). Mice younger than E17.5 were immersion fixed in either 4% paraformaldehyde or ET0/AA; 4% paraformaldehyde fixed tissue was rinsed with PBS and cryoprotected overnight in a solution of 30% sucrose in PBS. Tissue was embedded in tissue-freezing medium (TBS, Triangle Biomedical Sciences, Durham, NC), and 20 µm sagittal cryosections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). ET0/AA-AA-fixed tissue was rinsed with 0.2 M NaOH for 20 min. Slides were rinsed with 0.2 M acetic acid and 0.1 M triethanolamine. Slides were rinsed with 0.2× SSC and dehydrated with graded alcohols and exposed to Biomax MR film (Kodak, Rochester, NY) for 3 days at 80°C. Slides were dipped in Kodak NTB-2 emulsion and exposed at 4°C for 2 weeks. Slides were developed with Kodak D-19 developer and counterstained with cresyl violet.

RESULTS

Purkinje cell positioning in the math1 null cerebellum

In the cerebellum, the primary target of the math1 null mutation is the cells of the rhombic lip resulting in the failed formation of the EGL (Ben-Arie et al., 1997). However, initial analysis of the E18.5 mutant also revealed Purkinje cell ectopia (Ben-Arie et al., 1997), despite the lack of Math1 expression in these cells (Akazawa et al., 1995; Helms and Johnson, 1998). This indirect effect of the math1 null mutation on Purkinje cells suggests the importance of the EGL in Purkinje cell development. To examine the influence of the EGL on Purkinje cells, we performed a detailed analysis of Purkinje cell positioning in the math1 null cerebellum during the time of Purkinje cell migration between E13 and P0. Using antibodies against the Purkinje cell markers Calbindin and Disabled-1, we compared Purkinje cell placement in the math1-/- cerebella at E13.5, 15.5, 17.5, and P0.

At E13.5 in the math1-/- cerebellum, Purkinje cells were aligned in a radial manner migrating outward from the VZ. By E15.5 there were increased cohorts of migrating Purkinje cells present in both the math1-/- and wild-type cerebellum, and at the primitive cortex a Purkinje cell plate (PCP) had begun to form. At these two time points, there were no obvious differences in Purkinje cell placement between the two genotypes. In addition, Nestin immunohistochemistry, used to highlight radial glia, revealed no difference in glial morphology between the mutant and wild-type cerebellum (data not shown).

At E17.5, there were notable differences in Purkinje cell positioning between the two genotypes. In the wild-type cerebellum, the PCP was well defined, and initial fissure formation was evident dividing the Purkinje cells into loosely arranged clusters (Fig. 1D–F). In the math1-/- cerebellum, the PCP was not as well defined as in the wild-type cerebellum, and no fissure formation was evident (Fig. 1A–C). In addition, in the anterior region of the cerebellum, Purkinje cells were observed migrating into the mesencephalon (Fig. 1C), and in some cases, Purkinje cells had migrated dorsally past the pial surface of the cerebellum at the midline (Fig. 1A, inset).

By P0, initial foliation had occurred in the wild-type cerebellum, and with the exception of a few Purkinje cells still migrating toward the anterior portion of the cerebellar cortex, almost all of the Purkinje cells reached the P0 (Fig. 1J–L). In the P0 math1-/- cerebellum, initial fissure formation was evident, but no foliation had occurred (Fig. 1G–I). In addition, the PCP was well defined by the presence of large clusters of Purkinje cells (Fig. 1G–I). Despite the increase in normally placed Purkinje riboprobes generated from a plasmid containing Reelin nucleotides 5818–5973 (gift from Tom Curran, St. Jude Children’s Research Hospi
tal, Memphis, TN) and a plasmid containing 582 bp of the coding region of UnSh3 (gift from Sue Ackerman, The Jackson Laboratory, Bar Har
bor, ME). Riboprobes were labeled with [35S]UTP or [35S]UTP (Amersham Biosciences, Piscataway, NJ) by in vitro transcription according to the manufacturer’s instructions (Promega, Madison, WI). E13.5 and P0 cryosections were fixed with 4% paraformaldehyde followed by pretreatment with 0.25% acetic anhydride and 0.1 M triethanolamine. Slides were rinsed with 0.2× SSC and dehydrated with graded alcohols. Sections were prehybridized for 2 hr at room temperature followed by hybridization with riboprobes at 50°C overnight. Sections were rinsed with 2×, 1×, and 0.5× SSC and digested in 20 µg/ml RNase A (Sigma, St. Louis, MO). Sections were washed in 1× RNAse buffer, 2×, 1×, and 0.5× SSC at room temperature, and in 0.1× SSC overnight at 45°C. Sections were again rinsed with 0.2 M NaOH for 30 min. Slides were rinsed with PBS/T and incubated with Alexa Fluor 488-conjugated antibodies against BrdU (anti-G4G3 1:4; Developmental Studies Hybridoma Bank) overnight at room temperature. After three, 10 min rinses with PBS/T, slides were incubated with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) for 2 hr at room temperature. After thorough rinsing, slides were coveredslipped with a 2:1 PBS/glycerol solution.

In situ hybridization. RNA in situ hybridization was performed using
Figure 1. Purkinje cell positioning in the math1β-Cael/b-Gal cerebellum. Sagittal view of medial to lateral sections of an E17.5 math1β-Cael/b-Gal (A–C), E17.5 math1+/− (D–F), P0 math1β-Cael/b-Gal (G–I), and P0 math1+/− (J–L) cerebellum immunostained for the Purkinje cell marker Calbindin and counterstained with cresyl violet. Three ectopic populations of Purkinje cells can be seen in the math1β-Cael/b-Gal cerebellum that are not present in the wild-type cerebellum. At the midline (A, G, and inset) Purkinje cells are found in a supracerebellar position streaming through the dorsal aspect (asterisk) of the cerebellum. In more lateral sections (C, H, I), Purkinje cells are found migrating into the inferior colliculus (double arrows). At the midline (G) and mid-hemisphere (I) of the P0 math1β-Cael/b-Gal cerebellum, ectopic Purkinje cells (E) are in clusters deep to the PCP (P). In all sections, at P0 (G–I) numerous Purkinje cells are also found in their proper position at the PCP. Scale bar (shown in L): A–L, 200 μm; A, G, inset, 70 μm.

Table 1. Purkinje cell number in the math1β-Cael/b-Gal and math1+/− cerebellum

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Purkinje cell numbera</th>
<th>Number and regional distribution of ectopic Purkinje cellsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC</td>
<td>SCB</td>
</tr>
<tr>
<td>math1β-Cael/b-Gal</td>
<td>54,859 ± 5192</td>
<td>3796 (6.9%)</td>
</tr>
<tr>
<td>math1+/−</td>
<td>54,848 ± 4539</td>
<td>None</td>
</tr>
</tbody>
</table>

aMean number of Purkinje cells per half cerebellum calculated for n = 3 ± SD. Numbers determined as described in Materials and Methods.
bMean number of ectopic Purkinje cells within the inferior colliculus (IC), supracerebellar region (SCB), and intracerebellar region (ICB) calculated for n = 3 mice.

Quantitative analysis of math1 null cerebellum

To determine whether the absence of the EGL has an effect on Purkinje cell number, we counted Purkinje cells in P0 math1β-Cael/b-Gal and math1+/− cerebella. We determined the total number of Purkinje cells for one-half of the cerebellum (see Materials and Methods). We found no significant difference in the total number of Purkinje cells between the two genotypes (unpaired t test; p > 0.1) (Table 1). We also calculated the percentage of ectopic Purkinje cells and found that 28% of the total Purkinje cell population is in ectopic positions in the math1β-Cael/b-Gal cerebellum, and only 2% is found in ectopic positions in the wild-type cerebellum (Table 1). Of the 28% ectopic Purkinje cells in the math1β-Cael/b-Gal cerebellum, 7% were located within the inferior colliculus, 1% were located at the dorsal midline in a supracerebellar position, and the majority, 20%, were found deep to the PCP in an intracerebellar position (Table 1). The 2% of ectopic Purkinje cells in the wild-type cerebellum appeared to still be in the migratory phase of development, with their cell bodies oriented toward the PCP. However, this was not the case in the mutant cerebellum where the majority of ectopic Purkinje cell bodies had no consistent directionality, and in many cases were oriented away from the PCP.

Birth-dating analysis of ectopic Purkinje cells

In the math1β-Cael/b-Gal cerebellum, the anterior PCP was not as well defined as the posterior PCP. During normal Purkinje cell development, it is the latest born population of Purkinje cells, E13.0 in the mouse, that are destined for the anterior lobe of the cerebellum (Altman and Bayer, 1997). To determine whether the ectopic Purkinje cells within the math1β-Cael/b-Gal cerebellum represent the latest born population, birth-dating analysis was performed using BrdU. Timed pregnant females were injected with BrdU at either the start of Purkinje cell genesis (E11 and E11.5) or the final stage of Purkinje cell genesis (E12.5 and E13.0), and their offspring were examined at P0 for Calbindin and BrdU
immunoreactivity. In the mutant cerebellum there were many double-labeled ectopic Purkinje cells that were born at both early and late time points. A–D, Confocal images of P0 math1/B-gal/cerebella that are double immunolabeled for the Purkinje cell marker Calbindin and BrdU. A, C, Pregnant dams were injected with BrdU at E11 and 11.5 and killed at P0 for immunocytochemistry. B, D, Pregnant dams were injected with BrdU at E12.5 and 13 and killed at P0 for immunocytochemistry. C, D, Two regions of ectopic Purkinje cells are shown in greater detail to demonstrate double-labeled cells (arrows). Arrowheads point to single-labeled Calbindin-positive Purkinje cells. Scale bar (shown in B): A, B, 200 μm; C, D, 25 μm.

Figure 3. Unc5h3 is expressed in all math1/B-gal/cerebellar Purkinje cells. Sagittal view of P0 math1/B-gal/cerebella (A) and math1+/+ (B) cerebella probed with antisense Unc5h3. In the math1+/+ cerebellum (B), Unc5h3 is expressed in the cells of the external germinal layer (EGL) and Purkinje cell plate (PCP). In the math1/B-gal/cerebellum (A), Unc5h3 is expressed in the PCP and in ectopic Purkinje cells (arrowheads) in the inferior colliculus (IC). Scale bar (shown in B for A and B): 200 μm.

Unc5h3 signaling in the math1 null cerebellum

The migration of Purkinje cells into the inferior colliculus in the math1/B-gal/cerebellum is reminiscent of the Purkinje cell ectopia found in the Unc5h3 mutant brain (Przyborski et al., 1998). Unc5h3 is expressed in cerebellar granule and Purkinje cells and is necessary for the establishment of the cerebellar territory (Ackerman and Knowles, 1998; Przyborski et al., 1998). In the Unc5h3 null-mutant mouse, both neuronal types migrate beyond the cerebellar boundary into the inferior colliculus (Przyborski et al., 1998). To determine whether the extracerebellar ectopia in the math1/B-gal/cerebellum is caused by altered Unc5h3 expression, we examined Unc5h3 mRNA expression at P0 by in situ hybridization. In the wild-type cerebellum, Unc5h3 was expressed in the EGL and the Purkinje cell population (Fig. 3B). In the math1/B-gal/cerebellum, all Purkinje cells, including the ectopic population in the inferior colliculus, also showed high levels of transcript (Fig. 3A), indicating that Unc5h3 expression does not appear to be affected. As found in the chimera analysis of the Unc5h3 null cerebellum (Goldowitz et al., 2000), this finding would indicate that in Purkinje cells, Unc5h3 does not function in setting the rostral cerebellar boundary.

Reelin signaling in the math1 null cerebellum

Reelin signaling from the EGL is thought to be a critical factor in the migration of Purkinje cells (Miyata et al., 1997; Curran and D’Arcangelo, 1998; D’Arcangelo and Curran, 1998; Rice and Curran, 1999). In the absence of Reelin, in the rlh mouse, only 5% of the Purkinje cells migrate successfully (Heckroth et al., 1989). However, in the absence of the EGL in the math1/B-gal/cerebellum, we found that 72% of the Purkinje cells migrate...
successful migration of the majority of Purkinje cells. This finding demonstrates that Reelin signaling is only present in the NTZ and VZ. In a lateral section from the same E13.5 cerebellum, Reelin-immunopositive cells (arrows) are seen migrating away from the region of the VZ closest to the rhombic lip (RL) and throughout the NTZ (arrowheads). Scale bar (shown in C for A–C) 150 µm.

**Figure 5.** Reelin expression in the math1β-Gal/β-Gal cerebellum. Sagittal views of a E13.5 math1+/+ (A) and math1β-Gal/β-Gal (B) cerebellum probed with antisense Reelin and a math1β-Gal/β-Gal (C) cerebellum immunostained for Reelin. In the math1+/+ cerebellum (A), Reelin transcript is detected in the external germinal layer (EGL), the nuclear transitory zone (NTZ), and the ventricular zone (VZ). In the math1β-Gal/β-Gal cerebellum (B), Reelin mRNA is only present in the NTZ and VZ. In a lateral section from the math1β-Gal/β-Gal cerebellum (C), Reelin-immunopositive cells (arrows) are seen migrating away from the region of the VZ closest to the rhombic lip (RL) and throughout the NTZ (arrowheads). Scale bar (shown in C for A–C) 150 µm.

**Figure 4.** Comparison of Purkinje cell placement in the math1β-Gal/β-Gal and rl/rl cerebellum. Sagittal view of the P0 math1β-Gal/β-Gal (A) and rl/rl (B) cerebellum immunostained for the Purkinje cell marker Calbindin and counterstained with cresyl violet. In the math1β-Gal/β-Gal cerebellum (A), most of the Purkinje cells are located at the Purkinje cell plate (PCP). In the rl/rl cerebellum (B), most of the Purkinje cells are located deep to the Purkinje cell plate. The dashed line is 100 µm interior to the surface of the cerebellum in A and 100 µm interior to the inner EGL in B. Scale bar (shown in B for A and B): 200 µm.

Thus, to further assess the Reelin signaling pathway, we investigated the intracellular protein Disabled-1, which functions downstream of Reelin (Howell et al., 1997; Sheldon et al., 1997). In the absence of Reelin, as demonstrated in the rl/rl mouse, there are increased levels of Disabled-1 (Rice et al., 1998). We sought to examine the expression of Disabled-1 in the math1β-Gal/β-Gal cerebellum to determine whether Disabled-1 was altered in ectopic Purkinje cells. Our prediction was that there would be increased levels of Disabled-1 in the ectopic Purkinje cells because they did not receive an adequate Reelin signal. We compared Disabled-1 expression in math1β-Gal/β-Gal and math1+/+ cerebellum by immunohistochemistry (Fig. 6C,D). At all ages examined before P0 there was no obvious difference in Disabled-1 immunoreactivity between the math1β-Gal/β-Gal and math1+/+ cerebellum (Fig. 6A,B). At P0, we found that there was an increase in Disabled-1 immunoreactivity in the math1β-Gal/β-Gal cerebellum (Fig. 6C). Interestingly, the increased immunoreactivity was not limited to the ectopic Purkinje cells but was also seen in Purkinje cells that had migrated successfully.

**DISCUSSION**

The math1 null mutant mouse provides a fascinating model system to examine the importance of the EGL in cerebellar morphogenesis and Purkinje cell migration and positioning. It has allowed us to critically examine the influence of Reelin signaling from the EGL in Purkinje cell migration and to validate the role of the EGL in establishing the rostral cerebellar boundary through the expression of Unc5h3.

The absence of Reelin expression in the math1 null mouse demonstrates the critical role of prenatal granule cell precursors as the motive force in this process. Previous studies in which the EGL was disrupted during the prenatal period, using irradiation or chemical insult, found that foliation is EGL dependent, whereas initial fissure formation occurs independently of the EGL (Chen and
immunoreactivity between the Purkinje cells. At E17.5, there is no obvious difference in Disabled-1 staining to highlight the EGL and its relative position to Disabled-1 immunostained for Disabled-1. Sections were counterstained with cresyl violet.

Sagittal views of the E17.5 null Purkinje cells. Math1 expression is increased in the P0 math1β-Gal/β-Gal cerebellum and the P0 math1β-Gal/β-Gal (C) and math1+/+ (D) cerebellum immunostained for Disabled-1. Sections were counterstained with cresyl violet to highlight the EGL and its relative position to Disabled-1-positive Purkinje cells. Sections were counterstained with cresyl violet.

Figure 6. Disabled-1 is increased in P0 math1β-Gal/β-Gal Purkinje cells. Sagittal views of the E17.5 math1β-Gal/β-Gal (A) and math1+/+ (B) cerebellum and the P0 math1β-Gal/β-Gal (C) and math1+/+ (D) cerebellum immunostained for Disabled-1. Sections were counterstained with cresyl violet to highlight the EGL and its relative position to Disabled-1-positive Purkinje cells. Sections were counterstained with cresyl violet.

math1 null mutant, where the EGL is completely eliminated throughout embryonic development, confirm these authors’ interpretation of the role of the EGL in formatting cerebellar foliation.

The principal rationale behind these current studies was to better define the relationship between the developing EGL and Purkinje cell migration. We have identified three distinct ectopic populations of math1 null Purkinje cells that are obvious as early as E17.5. A first population of ectopic Purkinje cells (representing only 1% of the total Purkinje cell number) is found in a supra-cerebellar position at the dorsal midline in ~50% of mutant brains. This abnormality is most likely related to the absence of the EGL at the dorsomedial aspect of the cerebellum, where final fusion of the cerebellar primordia occurs. In the math1 null, we found that this fusion event does not occur, leaving a crevice at the dorsal midline that is visible during gross dissection of the cerebellum. This crevice separates two large populations of Purkinje cells that would normally become aligned as the cerebellar primordia expand and fuse. Instead, in the mutant, Purkinje cells remain concentrated under, and lateral to, the crevice. It is speculated that some variable feature of the mutant cerebellum (such as a disrupted pial lining) permits this unusual accumulation of Purkinje cells, in their migratory phase of development, to stream outside the cerebellum.

A second population of ectopic Purkinje cells (representing ~7% of the total Purkinje cell number) was always found in the inferior colliculus. Mutations in the Netrin-like receptor Unc5h3, which functions in establishing cerebellar boundaries, also results in a similar phenotype (Ackerman and Knowles, 1998; Przyborski et al., 1998). Unc5h3 is expressed in Purkinje cells and granule cell precursors in the cerebellum. Our finding that there is no disruption in Unc5h3 expression in ectopic Purkinje cells implies that Unc5h3 expression in these cells does not inhibit their migration outside the cerebellum. This is in agreement with the result of Unc5h3 null chimera (Unc5h3/Unc5h3 ↔ /+ ) studies, where both +/+ and Unc5h3/Unc5h3 Purkinje cells ignore the anterior cerebellar boundary (Goldowitz et al., 2000). In chimeras, the only ectopic granule cells were genotypically unc5h3/unc5h3, indicating that wild-type granule cell precursors are the effective cells in reading the Unc5h3 mediated signal (Goldowitz et al., 2000). Given that these cells do not exist in the math1 null, our current findings support the role of EGL granule cell precursors in establishing the anterior cerebellar boundary through the expression of Unc5h3.

The third ectopic population of Purkinje cells is found deep to the PCP. This population is by far the most numerous, representing ~20% of the total Purkinje cell number. This ectopia is most prominent at the midline and mid-hemisphere and is consistently seen among mutants. It is likely that ~2% of these cells would ultimately reach the PCP as seen in +/- control brains. Thus, 18% of the Purkinje cells appear to be dependent on the EGL for proper migration.

An alternate interpretation of these results is that the ectopic Purkinje cell population is developmentally delayed and would ultimately migrate to the PCP if the math1 null mouse had survived into the postnatal period. This is an unlikely possibility because some ectopic Purkinje cells are from the earliest born cohorts (at E11.0), which would be expected to have migrated the farthest. Furthermore, all mutant animals show exuberant extracerebellar migration into the mesencephalon, and ~50% show supracerebellar migration at the midline. Finally, our analysis of math1β-Gal/β-Gal → /+ chimeras, which survive as late as P21, still have mutant Purkinje cells in ectopic positions (P. Jensen and D. Goldowitz, unpublished results). These findings contraindicate any delay in the migratory process.

Thus, the most likely interpretation is that the EGL is necessary for the migration of these ectopic Purkinje cells in the math1 null cerebellum, and the most likely feature of the EGL that is critical to successful migration is the signaling molecule Reelin. The fact that ectopic math1 null Purkinje cells demonstrate increased levels of Disabled-1, which functions downstream of Reelin and is increased in the absence of Reelin signal (Rice et al., 1998; Trommsdorff et al., 1999), suggests that these cells are not receiving adequate Reelin signal. It is interesting that there is also an increase in Disabled-1 expression in normally placed Purkinje cells in the P0 math1β-Gal/β-Gal cerebellum. This increase in Disabled-1 is not seen before P0. These findings suggest that either Reelin signaling from the EGL may have a second function in addition to guiding the migration of Purkinje cells, or that a “yet-to-be-identified” signaling mechanism is responsible for the accumulation of Disabled-1.

In the absence of the Reelin signal in the reeler mouse, 95% of the Purkinje cells are ectopic (Heckroth et al., 1989). Our finding that only approximately one-fifth of the Purkinje cells are dependent on the EGL for successful migration has significant ramifications with regard to how we view Reelin signaling in the cerebellum. Our results indicate that in the absence of the EGL there is another signaling mechanism to guide the migration of Purkinje cells. All current evidence points to Reelin as this signaling mechanism. That is, there is no alternative or compensatory mechanism to promote the migration of Purkinje cells in
the complete absence of Reelin signal as evident in the reeler mutant and the VLDLR and ApoER2 double knock-out mice (Rice et al., 1998; Trommsdorff et al., 1999). Thus, on the basis of our current understanding of Reelin signaling, another source of Reelin (other than the EGL) provides a key signal for Purkinje cell migration. It has typically been assumed that Reelin signaling from the EGL is essential for Purkinje cell migration, although it has been well established that the cells of the NTZ and VZ also express Reelin (D’Arcangelo et al., 1995; Miyata et al., 1996; Schiﬀmann et al., 1997; Rice et al., 1998; our current ﬁndings). Considering the tempo-spatial relationships during cerebellar development, the cells of the NTZ and the VZ are likely candidates for Reelin signaling during early Purkinje cell migration. During the earliest phase of Purkinje cell migration, E13, the EGL is just beginning to form. At this time, only those Purkinje cells arising from the most lateral neuroepithelium are in proximity to the forming EGL. Most of the newly born Purkinje cells are more medially placed. These cells are most likely inﬂuenced by the cells of the NTZ, which are the earliest generated neurons of the cerebellum (E10) and migrate in a circuitous manner in the superﬁcial cerebellar primordia just above the migrating Purkinje cells (Altman and Bayer, 1997). Their temporal and spatial juxtaposition to the migrating Purkinje cells make them a prime candidate as the primary source of Reelin during early Purkinje cell migration.

The speciﬁc features of Purkinje cells that are EGL-responsive are unknown. Two obvious factors that could determine responsiveness are the time and place of birth. The EGL does not begin to form until E13. Thus, it might be expected that the latest born Purkinje cells (i.e., those born at E12.5–13) are targets of signals arising from the EGL. However, we ﬁnd that this is not the case, because those Purkinje cells that are ectopic are born throughout the entire neurogenic period. Therefore, some other factor, such as the site of genesis within the neuroepithelium, or the course and distance of migration to their ﬁnal position, may play an important role in determining the effective source of Reelin signal.

Our present ﬁndings have deﬁned at least two sources of Reelin involved in the successful migration of Purkinje cells. The baseline phenotype when the Reelin signal is completely absent (as in the reeler mutant mouse) is the ectopic positioning of 95% of all Purkinje cells (Heckroth et al., 1989). In the Math1 null cerebellum, where only the EGL source of Reelin has been removed, ~20% of all Purkinje cells remain ectopic, deep to the PCP. These ectopic Purkinje cells would be, by deﬁnition, the EGL-responsive population. The 70% + that successfully migrate in the Math1 null cerebellum are driven by Reelin from another source. This additional source, analogous to the Cajal-Retzius cells of cortex, is most likely the ﬁrst born neurons of the cerebellum, the nuclear neurons. This was a surprising result in that another effective source of Reelin was identiﬁed, and it had homology to Reelin signaling in cortex. The fact that one population of cells (the Purkinje cells) are under the inﬂuence of at least two different sources of Reelin indicates the complexity and richness of the processes that guide migration in the CNS.

REFERENCES


