Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum

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Received for publication 15 May 2003; revised 29 January 2004; accepted 5 March 2004

Available online 24 April 2004

Abstract

The signals that promote regional growth and development of the brain are not well understood. Sonic hedgehog (Shh) is produced by Purkinje cells of the cerebellum and is a potent inducer of granule cell proliferation. Here, we demonstrate that Shh protein is present in the murine cerebellum during late stages of embryogenesis and is associated with Purkinje cell bodies and their processes. To better determine the role of Shh during cerebellar development, we genetically removed Shh activity specifically from Purkinje cells and the cerebellar anlage of the mouse embryo. We show that Shh is required for expansion of the granule neuron precursor population, but not for the subsequent differentiation of these cells. In addition, the loss of Shh activity influences Purkinje cell development and the formation of folia in the cerebellum. A role for Shh in compartmentalization of the cerebellum is also suggested by the more severe rostral defects observed in the absence of Hedgehog signaling. Together, these findings provide additional evidence for Shh’s key regulatory role in controlling growth of the cerebellar primordium.

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Keywords: Sonic hedgehog; Cerebellum; Patterning; Cancer; Granule cell; Purkinje cell

Introduction

Growth and patterning of the cerebellum is a distinct event in brain morphogenesis as it involves a dramatic increase in volume (over a thousand fold) that primarily occurs during neonatal development. This increase in size is predominantly due to rapid proliferation and expansion of granule cells, which are the most abundant neuronal population of the mature brain.

The cortex of the mature cerebellum consists of several well-defined neuronal cell populations that are positioned in distinct layers due to the precise coordination of cell proliferation, differentiation, and migration events that occur during development (reviewed by Altman and Bayer, 1996; Goldowitz and Hamre, 1998). These layers consist of a molecular layer that is principally populated by granule cell axons and Purkinje cell dendrites, a Purkinje cell monolayer, and the internal granule layer (igl) consisting primarily of granule cell neurons. During development, two distinct proliferative zones populate the cerebellum: the ventricular neuroepithelium and the external granule layer (egl). Purkinje cells exit the ventricular zone between E11 and E13 in the mouse to form a temporary plate-like structure. About this time, granule cell precursors that make up the external
granule layer (egl) exit the rhombic lip, a neuroepithelial structure located slightly caudal and dorsal of the region where Purkinje cells arise, and migrate subpially to eventually populate the cerebellar surface. Postnatally, granule cells proliferate in the egl and then exit this transient layer to migrate inward past the developing Purkinje cells, which reside beneath the egl, to generate the igl. This radial migration of post-mitotic granule neurons is thought to be aided by Bergmann glia, whose cellular bodies reside immediately below the Purkinje cell layer (pcl) and fibers extend through the egl. During this process, Purkinje cells extend their dendrites toward the egl and begin to form a monolayer. By the end of the third postnatal week in the mouse, the egl has ceased to exist and the shape of the cerebellum has been transformed from its embryonic flat, oval form to a more complex structure with deep fissures and large folia.

The mechanisms underlying control of granule cell proliferation and transition to terminal differentiation are starting to be uncovered. Shh is expressed by Purkinje neurons and plays an important role in granule cell development, as demonstrated by the ability of a neutralizing anti-Shh antibody to perturb granule cell maturation (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Notably, Sonic hedgehog (Shh) is a potent inducer of granule cell proliferation and the expression of two Shh transcriptional targets in the egl is consistent with Shh directly regulating the expansion of granule cell precursors (Dahmane and Ruiz i Altaba, 1999; Kenney and Rowitch, 2000; Wallace, 1999; Wechsler-Reya and Scott, 1999). Further evidence for the importance of Shh signal regulation in the cerebellum comes from the identification of inactivating mutations in the Shh receptor Ptcl that constitutively activate the pathway and are associated with the formation of cerebellar tumors, thought to be of granule cell origin, in humans and mice (Goodrich et al., 1997; Hahn et al., 1996; Johnson et al., 1996).

Recent studies have sought to better define the mechanisms underlying Shh activity during cerebellum development. The mitogenic effects of Shh on granule neuron precursors appear to be regulated by downstream genes, such as cyclinD1 and Nmyc, whose expression is specifically up-regulated in response to Shh (Kenney et al., 2003; Zhao et al., 2002). However, the promotion of G1 cyclin expression by Shh appears to be indirect and may be regulated by intermediary factors (Kenney and Rowitch, 2000; Zhao et al., 2002). In addition, the identification of other signals that can modify granule cell responses to Shh has provided some clues as to how granule cells might stop proliferating and begin terminal differentiation while migrating toward the source of Shh in the Purkinje cell layer before finally settling in the underlying igl. Stromal-cell-derived factor (SDF-1α) appears to act as a chemoattractant for granule cell precursors and also enhances granule cell proliferation responses to Shh (Klein et al., 2001). Since this chemokine is localized to the pia mater during embryonic development and its receptor CXCR4 is expressed in the egl (McGrath et al., 1999), it appears that SDF-1α has dual roles in localizing granule cell precursors to a proliferative environment and synergizing with Shh to induce proliferation. In contrast, the extracellular matrix glycoprotein vitronectin has been shown to directly interact with Shh, inhibit Shh-induced proliferation, and promote granule cell differentiation through phosphorylation of cyclic-AMP responsive element-binding protein (CREB) (Pons and Marti, 2000; Pons et al., 2001). These findings, along with the expression of vitronectin in zones where granule cells differentiate, suggest that inhibition of the Shh proliferative signal allows granule cell differentiation to occur.

Despite these studies, it remains unclear whether Shh has a direct role to play in Purkinje cell biology, Bergmann glia differentiation, and the process of cerebellar foliation (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999), or whether these processes are regulated by factors expressed by granule cells themselves. To better define the roles of Shh during development of the cerebellum, we have closely examined the expression and activity of Shh in the mouse. We demonstrate regional differences in Shh expression and the presence of Shh protein in Purkinje cell dendrites that are in close proximity to the egl during postnatal development. Further, we have used two complimentary genetic models to prevent Shh signaling by Purkinje cells. Our results demonstrate that Shh is required for correct patterning of the cerebellum and the proliferative expansion of granule neuron precursors, but Shh activity is not absolutely required for the terminal differentiation of granule cell neurons or for Bergmann glia differentiation.

Material and methods

Generation of the Shhc allele and transgenic mouse strains

The gene targeting construct used to generate the Shhc allele contained a single loxP site and floxed PGK-Neo cassette inserted upstream and downstream, respectively, of exon 2 sequences. An HSV-tk cassette was also included at the 3’ end of the targeting vector to select against random integration events. Embryonic stem cells (ES) that had undergone homologous recombination of the targeting vector at the Shh locus were selected for resistance to G418 and sensitivity to ganciclovir, as described previously (Lewis et al., 2001). Recombinant ES cells were screened for the correctly targeted Shh allele by Southern and PCR analysis (refer to supplementary information for further details). Cre recombinase was transiently expressed in the correctly targeted ES cell clones using the pMC-Cre plasmid (kindly supplied by D. Drouin) and clones were selected that had undergone excision of the PGK-Neo cassette to generate the Shhc allele, which possessed two loxP sites flanking exon 2. Screening for the Shhc allele was performed by Southern and PCR analysis (refer to supplemenary information for further details). PCR primer pairs
Fig. 1. Shh signaling during embryonic and postnatal stages of cerebellum development. Whole mount β-galactosidase (β-gal) staining of brains from ShhlacZ/ + and Ptc1lacZ/ + mice. Dorsal (A, C, E, G, I, K, M, O, Q, S, U, W), lateral (B, N), and midsagittal (D, F, H, J, L, P, R, T, V, X) views of the cerebellum are shown. (A–D) β-gal activity from the ShhlacZ allele is not detected at E14.5 and E16.5. (E, F) Weak activity is first seen at E17.5 in rostral and caudal regions of the cerebellar anlage underlying the egl (arrowheads). (G–J) By P0, higher levels of Shh expression are seen in rostral regions that correlate well with the pcl. Distinct parasagittal stripes are seen either side of the midline. (K, L) At P2, higher levels of Shh expression are seen throughout the pcl and more parasagittal bands. Note that β-gal activity detected in the choroid plexus was above the background levels seen in wild-type animals at all stages examined. (M, N) β-gal activity from the Ptc1lacZ allele is not detected at E14.5. (O, P) Low levels of Ptc1 expression are detected at E16.5 throughout the cerebellar anlage and in the egl (arrow). (Q, R) By E17.5, Ptc1 expression has increased in the rostral region underlying the egl that corresponds to the pcl. Staining can also be seen in the ventricular epithelium (white arrowhead). (S–X) During later stages of development, Ptc1 expression is maintained throughout the cerebellum with highest levels seen in the egl and pcl. Note that no activity from the Ptc1 allele is observed in dorsal regions of the midbrain that are adjacent and rostral to the cerebellum. Dotted gray lines in dorsal views indicate the plane of view shown in the corresponding lower panel. Abbreviations are cb, cerebellum; mb, midbrain; egl, external germinal layer; pcl, Purkinje cell layer. Scale bar represents 100 μm.
used to detect the upstream loxP site were GGACAC-CATTCTATGCAGGG (primer 1; 5' to 3') and TCT-CTGCGAAGTTGTCCGGG (primer 2; 5' to 3'). PCR primers used to detect the downstream loxP site in the Shh allele were ATGCTGGCTGGCCTGGTGGAGA (primer 3; 5' to 3') and GAAGAGATCAAGGCAA-GCTCTGCAG (primer 4; 5' to 3'). The PCR primer pairs used to detect the Shh allele were primer 1 and primer 4; those used to detect the floxed PGK-Neo cassette in the recombiant Shh-loxP allele were primer 3 and TACC-GGTGATGGGAATGTGTCGG (primer 5; 5' to 3').

A ShhShh homozygous stock was generated after first outcrossing the Shh allele onto a Black Swiss (Harlan) background. The L7-Cre transgene was generated by placing the L7-ΔAUG promoter enhancer (Smye et al., 1995) upstream of Cre in the pSP-13Cre plasmid (supplied by M. Rudnicky). The transgene was released by EcoRI and HindIII restriction enzyme digests. The Pax2-Cre transgene was generated using a previously identified Pax2-regulatory region (Rowitch et al., 1999) Generation of mice possessing the Ptc-lacZ and Shh alleles have been described elsewhere (Goodrich et al., 1997; Lewis et al., 2001). Mice carrying the ShhShh allele were generated by targeting an IRES nuclear lacZ construct into the 3' untranslated region of Shh. This leaves Shh coding sequences intact. This strain was generated by A. Kottmann and kindly provided by Dr. T. Jessell (data not published, for further details, contact T.M. Jessell). Generation of mice for analysis in this study was achieved by interbreeding transgenic males that possessed a single Shh allele (+/Shh, Pax2-Cre and +/Shh, L7-Cre) with Shh homozygous females.

β-Galactosidase staining

Brains were either dissected in half or the cerebellum was isolated intact and β-galactosidase was detected by whole mount staining as described previously (Whiting et al., 1991). Tissue for sectioning was obtained after transcardial perfusion of mice with PBS followed by 2% paraformaldehyde before the collection of brain tissue and overnight fixation. After fixation, one half of each brain was embedded in paraffin while the other half was immersed in 30% (w/v) sucrose at 4°C until submerged when it was embedded in OCT (Tissue-Tek) for frozen sectioning.

Histology, immunocytochemistry, and BrdU analysis

Pregnant mice at embryonic (E) day 18.5 and postnatal (P) day 5 mice were injected intraperitoneally with 50 μg/g body weight of a 5 mg/ml solution of BrdU (Sigma, St. Louis, MO) in saline. Three hours after the final injection, brains were removed from the animals and fixed overnight in 4% paraformaldehyde at 4°C. Neonatal and adult mice were transcardially perfused with PBS followed by 4% paraformaldehyde before the collection of brain tissue and overnight fixation. After fixation, one half of each brain was embedded in paraffin while the other half was immersed in 30% (w/v) sucrose at 4°C until submerged when it was embedded in OCT (Tissue-Tek) for frozen sectioning.

Analysis of the cerebellum was restricted to within 100–200 μm of the midline. Paraffin-embedded brains were serially sectioned (5 μm) and sections, spaced at 75-μm intervals, were processed for histology using Nissl stain. Sections spaced at 75-μm intervals were also used for the immunocytochemical detection of either BrdU (1:100 anti-BrdU monoclonal antibody, DAKO, Carpenteria, CA) or PEP-19 (1:5000, generous gift of Dr. James Morgan). Primary antibody labeling was detected with either mouse or rabbit biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). Staining was visualized using Avidin conjugated to either horseradish peroxidase or alkaline phosphatase (Vector Laboratories) and DAB substrate. To evaluate cell death, sections spaced 75 μm apart were assayed for apoptotic cells using the Dead End Colorimetric Detection System (Promega, Madison, WI) according to the manufacturer’s instructions. Images were captured using a Kontron Digital Camera Model 3012.

Immunofluorescence was performed on frozen sections (25 μm) with the following primary antibodies: anti-BLBBC polyclonal (1:1000; Feng et al., 1994), anti-Calbindin-D28K polyclonal (1:1000, Swant), anti-β-Galactosidase monoclonal (1:1000, Promega), anti-GFAP polyclonal (1:400, Chemicon) or monoclonal (1:400, Sigma), anti-Math1 polyclonal (1:100; Helms and Johnson, 1998), 4D7 anti-TAG1 polyclonal (1:4, supernatant, provided by the Developmental Studies Hybridoma Bank), TuJ1 anti-neural tubulin monoclonal (1:1000, BAbCO), and anti-Zic1 rabbit polyclonal (1:400, Rockland). Primary antibody labeling was visualized with either anti-mouse or anti-rabbit Alexa-la-
beled secondary antibodies (A488 and A568, Molecular Probes). In the case of the anti-TAG1 antibody, a rhodamine-labeled anti-goat (IgM) secondary antibody was used. Sections were mounted with VectaShield (Vector Laboratories) and images were captured on a Zeiss Axioplan2 confocal microscope using a digital camera.

All other immunohistochemistry was performed on brain tissue collected intact, fixed in Sainte Marie's solution, embedded in paraffin, and processed for immunohistochemistry essentially as described previously (Gritli-Linde et al., 2001). The primary rabbit polyclonal antibodies used on serial sections through the vermis of these samples were
Ab80 anti-Shh (1:300–1:800; Bumcroft et al., 1995), anti-Calbindin-D28K (1:3500, Chemicon), anti-phospho-histone H3 Ser10 (1:600, Cell Signaling Technology/New England Biolabs), anti-Pax2 (1:3000, Zymed), and anti-Zic1 (1:400, Rockland). Sections were counterstained with azuII-methylene blue.

Quantification

Estimates of the number of Purkinje cells were determined for each of the genotypes at E18.5, P0, P5, P13, and P28 using a previously described method (Smythe and Goldowitz, 1990). Paraformaldehyde-fixed brains were dissected into two halves and one half was embedded into paraffin for sectioning. Serial sections of Purkinje cells were identified by PEP-19 immunoreactivity as well as their classical appearance in Nissl-stained sections. Cell number was estimated at 75-μm intervals. Uncorrected total estimates (U) for each animal were obtained through summation of the individual section counts (n) multiplied by the sampling frequency (f):

\[ U = \sum n \times f \]

where: \( U \) = uncorrected cell estimate; \( \Sigma n \) = sum of the PEP-19/Nissl-positive cells counted for any animal; \( f \) = the frequency of samples sectioned, that is, every 25th section in this case.

Corrected total cell numbers (N) were obtained by applying a correction value to each of the uncorrected total estimates. The correction value was obtained for each animal by first estimating the average nuclear diameter (D) of a random pool of 100 PEP-19-positive cells and then dividing this average diameter by the sum of the average diameter plus the average section thickness (t):

\[ N = U \times \left( \frac{D}{D + t} \right) \]

where: \( N \) = corrected total cell estimate; \( U \) = uncorrected cell estimate; \( D \) = average cell diameter; \( t \) = average section thickness.

The number of corrected cells per section were then plotted from the most lateral aspect of the cerebellar hemisphere (minus the paraflocculus) to the most medial aspect of the cerebellar vermis. To generate the total number of Purkinje cells per cerebellum, uncorrected number of Purkinje cells was summed, multiplied by 15 (sections were sampled at 75-μm intervals), and the calculated correction factor/individual brain was applied.

Cerebellar volumes

The volume for each was computed using the Bioquant Image Analysis System (R & M Biometrics, Nashville, TN). Briefly, the image of individual sections of the cerebellum spaced 75 μm apart were captured by a computer. The area of each section of the cerebellum was then computed and then a 3-D reconstruction was made using the Bioquant Image Analysis Topographer Program. Once a 3-D image was generated, the volume of the cerebellum was computed.

Results

Shh signaling occurs during embryonic, postnatal, and adult stages of cerebellum development

To determine when and where the Shh signaling pathway might function during cerebellar development, \( \text{Shh}_{\text{lacZ}} \) and \( \text{Ptc1}_{\text{lacZ}} \) alleles were examined to identify Shh producing and Shh responding cell populations, respectively. Expression of β-galactosidase from the \( \text{Shh}_{\text{lacZ}} \) allele appears to correlate well with endogenous Shh expression in all comparisons that have been made in the CNS and elsewhere in the embryo (A.P.M, unpublished observations). The \( \text{Ptc1}_{\text{lacZ}} \) allele has previously been described and used to identify Shh responsive cells (Goodrich et al., 1997). Low levels of \( \text{Shh}_{\text{lacZ}} \) were first detected in the developing cerebellum at E17.5 (Figs. 1E, F) and by P2 expression could be seen throughout the pcl, with higher levels of expression observed in distinct parasagittal domains (Figs. 1G–L, 2A, C). Interestingly, higher levels of \( \text{Shh}_{\text{lacZ}} \) were observed in rostral lobes than in caudal regions. During all later stages of cerebellar development and in the adult, \( \text{Shh}_{\text{lacZ}} \) expression was always associated with Purkinje cells (Figs. 2F, G, J, K, N, O, R–V). Low levels of \( \text{Ptc1}_{\text{lacZ}} \) expression were first detected in the developing cerebellum at E16.5 in the egl and more broadly in the anlage (Figs. 1O, P). At E17.5, \( \text{Ptc1}_{\text{lacZ}} \) expression was up-regulated in the egl and pcl (Figs. 1Q, R). \( \text{Ptc1}_{\text{lacZ}} \) was also observed in the ventricular neuroepithelium at this stage. By P2, high levels of \( \text{Ptc1}_{\text{lacZ}} \) were observed in the egl and pcl, however, lower levels of expression were also observed throughout the igl (Figs. 1S–X, 2B, D, E). Subsequently, \( \text{Ptc1}_{\text{lacZ}} \) expression continued to be associated with these cell layers, Purkinje cells, and scattered cells within the molecular layer (Figs. 2H, I, L, M, P, Q).

To verify that these sites of Shh expression and activity correlate well with the presence of Shh protein and to further determine the role of Shh signaling, the localization of Shh protein was examined in the developing cerebellum. At E18.5, Shh protein levels were clearly elevated in the pcl (Figs. 3A, B) but significant levels of protein were also present in the egl and deeper regions of the anlage. At P14 and in the adult, Shh protein was detected in Purkinje cells extending into the molecular layer and within the igl (Figs. 3D, G). A closer inspection revealed a punctate pattern of immunostaining, characteristic of intracellular vesicles, in Purkinje cell bodies and their dendrites (Figs. 3E, H). Shh immunostaining in the igl did not appear to be associated with the cell bodies of granule cell neurons and was most likely present in Purkinje cell axons that extend through this
region though a more detailed analysis will be required to address this issue.

Together, these findings indicate that Purkinje cells from late embryonic stages of development through to adulthood produce a Shh signal. Shh protein was associated with Purkinje cell bodies and their cellular extensions (dendrites and most likely axons), the movement of Shh in this way may provide at least a partial explanation for the up-regulation of an apparent signaling response (Ptc1 expression) in cells of the egl, pcl, and igl.

Fig. 3. Shh protein localization during development of the cerebellum. Shh immunohistochemistry on sections of the cerebellum. (A) At E18.5, Shh is predominantly detected in the pcl with low levels of protein seen throughout the cerebellum. Slightly higher levels of protein are detected in rostral lobes. (B) High-power view of a rostral lobe from panel A is shown. (C) High-power view of rostral lobe in the absence of primary antibody showing absence of signal. Arrow in B and C indicates egl. (D, G) By P14 and adulthood, Shh protein can be seen in Purkinje cell bodies (arrows), throughout the ml and in the igl. (E, H) High-power views at P14 and in the adult showing punctate staining for Shh in Purkinje cell bodies (arrows) and dendrites extending toward the pial surface of the cerebellum (arrowheads). Shh protein can also be seen within the igl, but is not associated with granule cells. (F, I) High-power views at P14 and in the adult in the absence of primary antibody, showing that no signal is detected. Arrows point to Purkinje cell bodies. Scale bar represents 200 μm (A), 50 μm (B, C, D, H), and 20 μm (E, F, H, I).
Removal of Shh from the cerebellum using a conditional allele

As a first step towards determining the role of Shh during cerebellum development in the mouse, we generated a Shh conditional null allele (Shhc) by flanking exon 2 and some neighboring intronic sequences with loxP sites (Supplementary Fig. 1). The Shhc allele was demonstrated to have a wild-type function by the observations that mice homozygous for the Shhc allele alone or heterozygous for the Shhc and Shhn alleles developed and reproduced normally (data not shown; Dassule et al., 2000; Lewis et al., 2001). Effective Cre-mediated recombination of the Shhc allele to generate the Shhn allele has previously been demonstrated in the mouse embryo (Dassule et al., 2000; Lewis et al., 2001).

To generate transgenic mice that express Cre in Purkinje cells of the cerebellum during different stages of development, we used the promoter/enhancer sequences for the Pax2 (Rowitch et al., 1999) and L7 (Smeyne et al., 1995) genes. The Pax2 promoter/enhancer has previously been shown to drive expression of a β-galactosidase transgene throughout the mid-hindbrain region, from which the cerebellum is derived, during early stages of embryogenesis (Rowitch et al., 1999). Later, expression is largely confined to Purkinje cells and the egl of the fetal and neonatal cerebellum. In contrast, the L7 promoter/enhancer has been demonstrated to drive expression of a β-galactosidase transgene in parasagittal bands of the cerebellum at E17 (Oberdick et al., 1994). These bands of expression subsequently broaden during development until all Purkinje cells express the transgene around birth (Baader et al., 1998; Buffo et al., 1997; De Zeeuw et al., 1998; Smeyne et al., 1995). To verify the activity of the Pax2-Cre and L7-Cre transgenes, transgenic mice were mated with the Rosa26lacZ reporter mouse, which activates β-galactosidase (β-gal) expression after Cre-mediated removal of a translational block (Soriano, 1999). The Pax2-Cre transgene elicited β-gal expression in the mid-hindbrain neuroepithelium from 8.0 dpc and continued to drive expression throughout this region during later stages of development (data not shown). In the cerebellum, which is derived from the mid-hindbrain region, the majority of cells displayed β-galactosidase transgene expression.

Fig. 4. Cre transgene activity in the cerebellum within the Pax2 and L7 expression domains. Staining for β-gal activity within the Pax2 and L7 expression domains in response to Cre activity in transgenic mice that also possess the Rosa26-lacZ reporter allele. Sections (A–D, H, K, L) and whole mounts (E–G, I, J) of the cerebellum are shown. (A, B) The Pax2-Cre transgene displays activity throughout the cerebellum at E16.5 and in the adult. (C) High-power view of rostral lobe shown in B demonstrating Pax2-Cre activity in Purkinje cells (arrows) and many other cells of the cerebellum. (D) High-power view of a caudal lobe from B showing absence of Pax2-Cre activity in some Purkinje cells (arrows). (E–H) The L7-Cre transgene displays activity by E18.5 in a broad region beneath the egl that is the pcl. It is also observed in distinct parasagittal domains in either side of the midline. (I, J) By P2, the parasagittal domains of L7-Cre activity associated with the pcl have become more extensive. (L) In the adult, L7-Cre activity is clearly associated with Purkinje cells and their dendrites within the ml. Sections were counterstained with Nuclear Fast Red. Abbreviations are egl, external germinal layer; igl, internal granule layer; ml, molecular layer; pc, Purkinje cell; pcl, Purkinje cell layer. Scale bar represents 100 μm.
gal activity by E16.5 (Fig. 4A), only a few Purkinje cells in caudal lobes escaped Cre-mediated recombination by adulthood (Figs. 4B–D). These results demonstrate that cells exposed to Pax2-Cre activity and all their ancestors were present throughout the mid-hindbrain region, including the cerebellum, during early stages of development. Importantly, the Pax2-Cre transgene is active in the pcl when Shh expression is first initiated in this region. In comparison, expression of β-gal resulting from L7-Cre activity was present at E17.5 and E18.5 in distinct parasagittal domains of the pcl that lie either side of the midline (data not shown, Figs. 4E–H). Interestingly, the most rostral lobe of the cerebellum did not appear to express β-gal at E17.5 (Fig. 4E), whereas low levels of expression where detected 1 day later (Fig. 4G). By P2, β-gal expression was seen in more extensive parasagittal domains of the pcl (Figs. 4I, J). In the adult, all Purkinje cells examined had undergone L7-Cre-induced activation of the reporter and staining was also observed in dendrites extending into the molecular layer (data not shown, Fig. 4L). Together, these results indicate that the pattern of L7-Cre activity observed here closely resembles that previously observed for the L7-β-galactosidase transgene (Oberdick et al., 1994). Furthermore, they demonstrate that the L7-Cre transgene was active in the pcl by E17.5, when Shh expression was first expressed in the cerebellar anlage.

To investigate the role of Shh signaling during cerebellum development, we generated Shh+/Shhn mice carrying either the Pax2-Cre or L7-Cre transgenes. Pups of the correct genotype were born at Mendelian ratios, indicating no embryonic lethality. Both types of mutant pups displayed ataxia and movement disorders characteristic of abnormal cerebellar function. These behavioral abnormalities where more severe in the Shh+/Shhn, Pax2-Cre pups, which only survived until 4–6 weeks of age. The Shh+/Shhn, L7-Cre animals have survived for at least 1 year.

Since Shh is initially expressed in the pcl of the cerebellum in parasagittal stripes that lie immediately adjacent to the midline, we confined most of our analysis to within 100–200 μm of the midline of the vermis. To determine whether Shh had been effectively removed from this region of the mutant embryos, Shh protein expression was examined at E18.5 (Figs. 5A–L). No Shh protein could be detected in the cerebellum of either the Shh+/Shhn, Pax2-Cre (Figs. 5D–F) or Shh+/Shhn, L7-Cre (Figs. 5G–I) embryos.

Abnormal development of the cerebellum in Shh-deficient cerebellum

To establish whether development of the cerebellum was perturbed in the Shh+/Shhn, Pax2-Cre and Shh+/Shhn, L7-Cre mice, histological sections (Fig. 6) and volumetric analysis (Fig. 7) of the cerebellum were examined from E18.5 through to adulthood. As early as E18.5, the gross cerebellar morphology of the mutant embryos was abnormal with immature fissures and a reduction in the egl was apparent but no significant difference was observed in cerebellar volumes between the mutant and control embryos (Figs. 6A–F, 7A). By P5, the Shh+/Shhn cerebellum like its wild-type counterpart consists of distinct cellular layers that can be morphologically identified as egl, molecular layer, pcl and igl, and the cerebellar volume has increased 6-fold (Figs. 6G, H, 7A). At this time, development of the Shh+/Shhn, Pax2-Cre cerebellum was more severely affected than that of the Shh+/Shhn, L7-Cre pups (Figs. 6I–L). These pups had a reduced number of lobes with no apparent egl or igl, although some scattered granule cells were detected in the cerebellar cortex, and Purkinje cells were seen throughout a broad region of the cerebellum (Figs. 6I, J). The Shh+/Shhn, L7-Cre cerebellum had poorly developed lobes, a drastically reduced egl, an underpopulated igl, and the Purkinje cells were organized into an abnormally thick layer (Figs. 6K, L). Furthermore, the cerebellar volumes of both the Shh+/Shhn, Pax2-Cre and Shh+/Shhn, L7-Cre pups were less than half of those observed for Shh+/Shhn pups (Fig. 7A). By adulthood, the cerebellum has numerous lobes and consists of mature neuronal cell populations that reside in either the molecular layer, Purkinje cell monolayer, or densely populated igl (Figs. 6M, N, 7B). In the mature cerebellum, the egl ceases to exist and the cerebellar volume has increased approximately 4- to 5-fold compared with that observed at P5 (Fig. 7A). A loss of lobes was apparent in the adult Shh+/Shhn, Pax2-Cre cerebellum, although two rudimentary lobes formed caudally (Figs. 6Q, 7C). In addition, Purkinje cells were present in a multicellular band and no igl had formed (Figs. 6O, P). The adult Shh+/Shhn, L7-Cre cerebellum appeared a well-organized miniature of the wild-type cerebellum with a full complement of small lobes containing Purkinje cells in a 1–3 cell diameter layer and a thin igl (Figs. 6Q, R, 7C). Though the mutant cerebellar volumes at adulthood were significantly larger than the cerebellar volumes of the mutants at P5, they were actually smaller than at P13 (Fig. 7A), suggesting continued growth in both genotypes from P5-P13, and an eventual loss of cellular mass during the final stages of cerebellar development.

In summary, in both mutants, the egl was thinner at E18.5 and failed to expand by P5, Purkinje cells were present but failed to form a monolayer, and the igl was absent (Shh+/Shhn, Pax2-Cre) or sparsely populated (Shh+/Shhn, L7-Cre). Interestingly, there was a distinct difference in patterning abnormalities seen in rostral and caudal lobes of the cerebellum. In both mutants, the caudal lobes were better organized than rostral lobes. In the case of the Shh+/Shhn, Pax2-Cre cerebellum, this may reflect the presence of Purkinje cells in caudal lobes that had not undergone Cre-mediated recombination of the Shh+ allele.
However, we did not detect any Shh protein expression in Purkinje cells from caudal lobes at E18.5, P5, and P14 (Fig. 5 and data not shown). It is possible that there are regional differences in patterning of the cerebellum and that other signals co-operate with Shh to pattern the caudal lobes.
Disorganized Purkinje neurons and Bergmann glia

To further examine the morphology of Purkinje cell neurons and their cellular projections in the Shh-deficient cerebellum, expression of the Calbindin-D28 protein was examined at different stages of development (Fig. 8). Calbindin-D28 is commonly used as a marker of Purkinje cell bodies, dendrites, and axonal tracts. At E18.5, Calbindin-D28 could be detected in the Shhc/Shhn cerebellum in what is at this stage a broad pcl immediately beneath the egl (Figs. 8A, B). This same layer of cells was detected in both the Shhc/Shhn;Pax2-Cre and Shhc/Shhn;L7-Cre embryos (Figs. 8C–F). By P5, a thin layer of Purkinje neurons lies beneath the egl projecting short dendrites beneath the egl of the Shhc/Shhn cerebellum (Figs. 8G, H). In contrast, Purkinje cells in the Shhc/Shhn;Pax2-Cre cerebellum form a broad densely packed layer at P5 (Figs. 8I, J), whereas those of the Shhc/Shhn;L7-Cre cerebellum were dispersed in a somewhat thinner layer (Figs. 8K, L). Furthermore, in both mutants it was difficult to distinguish the dendritic projections of the Purkinje cells. In the P14 cerebellum when the egl is normally lost, a well-organized monolayer of Calbindin-D28 producing Purkinje neurons was observed that showed extensive dendritic arbors projecting toward the pial surface of the cerebellum (Figs. 8M, N). In contrast, immunostaining of the Shhc/Shhn;Pax2-Cre cerebellum indicates a mixture of Purkinje cell bodies and disorganized cellular projections (Figs. 8O, P). The Shhc/Shhn;L7-Cre cerebellum shows an intermediate phenotype with a layer of Purkinje neurons that appeared to be better organized and tangles of densely packed dendrites extending toward the pial surface (Figs. 8Q, R). Thus, Purkinje cells were present in the correct location of E18.5 mutants, but failed to assemble into a monolayer with highly organized cellular projections. Further Calbindin-D28 expression correlates well with Shh protein expression in Purkinje neurons and their projections (compare Figs. 3 and 8), except Shh staining was punctate. Importantly, Purkinje cell dendrites do not extend through the egl at any stage of cerebellar development examined.

The number of Purkinje cells in the cerebellum was examined during different stages of development (data not shown). These findings indicated that at E18.5, Purkinje cells were reduced by 25% in Shhc/Shhn;Pax2-Cre embryos, whereas Shhc/Shhn;L7-Cre and Shhc/Shhn embryos had similar numbers. By P5, Purkinje cell numbers were reduced by 50% in Shhc/Shhn;Pax2-Cre embryos and 20% in Shhc/Shhn;L7-Cre embryos. This reduction in Purkinje cells continued into adulthood when a 65% and 35% reduction was apparent, respectively. It is most likely that the loss of Purkinje cells observed during postnatal development and in the adult is a secondary effect due to loss of trophic support from differentiated granule cells. However, TUNEL analysis
on both mutants at different stages of cerebellum development did not indicate any increases in cell death most likely reflecting a very gradual loss of these cells (data not shown). The differences in Purkinje cell numbers were not observed in more lateral regions of the cerebellum of mutant and control animals (data not shown).

To determine whether Shh is required for other aspects of cerebellar development in particular for the Bergmann glia, BLBP and GFAP expression were examined (Supplementary Fig. 2). BLBP-expressing cells were detected in the cerebellum of both \textit{Shhc/Shhn}, \textit{Pax2-Cre} and \textit{Shhc/Shhn};\textit{L7-Cre} embryos at E18.5 and P5. During subsequent stages of development, the organization of Bergmann glia was more severely disrupted in the \textit{Shhc/Shhn};\textit{Pax2-Cre} pups. This was most likely a secondary effect due to disorganization of the Purkinje neurons and possibly a lack of granule cell’s parallel fibers, evidenced by the presence of GFAP-expressing glial fibers that surrounded Calbindin-D28-expressing Purkinje neurons at P5 and P13. We also examined Pax2 protein expression in the cerebellum and found this population of mature neurons to be present in both mutants (data not shown).

Removing Shh signaling from the cerebellum blocks granule neuron development

To investigate how the development of granule neurons was affected in the cerebellum of mice lacking Shh, expression of several granule cell-specific markers was examined (Fig. 9). At E18.5, Math1 was present in mitotic granule neuron precursors that reside in the outer egl of \textit{Shhn}/\textit{Shhn} embryos (Figs. 9A, B) and was also detected in both mutants, albeit at lower levels (Figs. 9C–F). A small number of post-mitotic, premigratory granule cells that synthesize TAG1 and Zic1 reside in the inner egl at this time (Figs. 9G, H). Zic1 was also seen in post-mitotic granule cells within deeper regions of the cerebellum (Figs. 9G, H). In the E18.5 \textit{Shhn}/\textit{Shhn};\textit{Pax2-Cre} cerebellum, no TAG1 and very few Zic1 producing cells were detected (Fig. 9I), although a few TAG1-positive cells were observed in caudal lobes (Fig. 9J). However, TAG1 and Zic1 producing granule cells were observed in the E18.5 \textit{Shhn}/\textit{Shhn};\textit{L7-Cre} cerebellum (Figs. 9K, L). By P5, when granule cell proliferation is at its peak in the normal cerebellum, a large zone of TAG1- and Zic1-positive post-mitotic granule cells were seen residing in the inner egl and Zic1-expressing granule cells were present in the developing igl of the \textit{Shhn}/\textit{Shhn} cerebellum (Figs. 9M, N). At a later stage of cerebellum development, the number of granule cells entering the inner egl declined and the majority of granule cells had populated the igl where they continued to express Zic1 (Figs. 9S, T). In contrast, the postnatal \textit{Shhn}/\textit{Shhn};\textit{Pax2-Cre} cerebellum had no post-mitotic granule cells (TAG1, Zic1 positive) in the egl and very few granule cells (Zic1 positive) were observed within the igl (Figs. 9O, U, V). Interestingly, at P5, some TAG1- and Zic1-expressing inner
egl cells were detected in caudal lobes of these mutants (Fig. 9P). In the Shhc/Shhn, L7-Cre cerebellum, reduced numbers of TAG1- and Zic1-positive cells were present in the inner egl in both rostral and caudal lobes (Figs. 9Q, R, W, X). Thus, removal of Shh from the cerebellum does not appear to block granule neuron migration and differentiation, but prevents expansion of granule cell precursors and formation of the post-mitotic, premigratory granule neuron population.

Proliferation of granule precursor cells is reduced in the Shh-deficient cerebellum

The absence of premigratory, post-mitotic granule cell precursors may reflect the failure to expand granule cell progenitors in the egl. To determine whether these cells were capable of proliferating in the absence of Shh, we examined cells in S phase of the cell cycle by BrdU incorporation (Fig. 10) and those in M phase by immunostaining for Phospho-Histone H3 (PH3)), (data not shown). As in the wild type, at E18.5, BrdU-immunopositive cells were located throughout the egl and inner cerebellar cortex of Shh+/Shhn embryos (Fig. 10A). These latter proliferating cells most likely represent astrocytes though double-labeling experiments will be required to confirm their identity. However, no BrdU-labeled cells were seen in the egl of the Shh+/Shhn, Pax2-Cre cerebellum at this time, although numerous BrdU-labeled cells were present in the cerebellar cortex (Fig. 10B). This is in contrast to the Shh+/Shhn, L7-Cre cerebellum, where a small number of BrdU-positive cells were identified in the egl at E18.5 (Fig. 10C). By P5, the wild-type egl has increased in thickness and numerous BrdU-labeled cells were observed in this layer of the Shh+/Shhn cerebellum as well as in more centrally located astrocytes (Fig. 10D). By P5, the egl has been lost from the Shh+/Shhn, Pax2-Cre cerebellum and no BrdU-positive cells were seen in the superficial layers of the cerebellum (Fig. 10E). However, several immunopositive cells were observed below this region and scattered throughout the cerebellar cortex. We cannot exclude the possibility that some of these BrdU-labeled cells were inappropriately...
Fig. 9. Granule neuron precursors and differentiated granule neurons in mice lacking Shh. Immunohistochemistry for cellular markers of mitotic granule cell precursors (Math1); post-mitotic, premigratory granule cells (TAG1); and post-mitotic, differentiated granule cells (Zic1). The pan-neuronal cell marker TuJ1 was also used. Analysis was performed on sections of the cerebellum from Shhc/Shhn, Shhc/Shhn;Pax2-Cre, and Shhc/Shhn;L7-Cre mice at different stages of development. Rostral (A, C, E, I, K, M, O, Q, S, U, W) and caudal lobes (B, D, F, H, J, L, N, P, R, T, V, X) are shown. (A–F) Mitotic granule cells expressing Math1 are seen in the outer egl of all embryos at E18.5, although reduced levels of staining are seen in the mutants. All other neuronal cells in the cerebellum express TuJ1. (G, H) Low numbers of post-mitotic granule cells expressing TAG1 (arrows) are seen in the inner egl of Shhc/Shhn embryos at E18.5. Post-mitotic granule cells expressing Zic1 are also seen in the inner egl and scattered through deeper regions of the cerebellum. (I, J) The E18.5 Shhc/Shhn;Pax2-Cre cerebellum has no TAG1-expressing granule cells in rostral lobes and only low levels present caudally (arrow). Zic1-expressing granule cells are present. (K, L) TAG1- (arrows) and Zic1-expressing post-mitotic granule cells are present in the Shhc/Shhn;L7-Cre cerebellum at E18.5. (M, N) At P5, a thick layer of TAG1-expressing post-mitotic granule cells (arrows) is present in the inner egl of Shhc/Shhn embryos. Zic1 is also expressed in these cells and those that contribute to the igl. (O) Rostral lobes of the Shhc/Shhn;Pax2-Cre cerebellum at P5 have no TAG1- or Zic1-positive cells superficially, although a small number of Zic1-positive cells are seen in deeper regions of the cerebellum. (P) In caudal lobes, reduced numbers of TAG1- and Zic1-expressing granule cells are seen. (Q, R) The Shhc/Shhn;L7-Cre cerebellum at P5 also has reduced TAG1- and Zic1-expressing cells. Rostral lobes are more severely affected than caudal lobes. (S, T) In the Shhc/Shhn cerebellum at P13, a thin layer of TAG1-expressing granule cells is only present at the periphery of rostral lobes and numerous Zic1-expressing neurons are found in the igl. (U–X) At P13, no TAG1 and very few Zic1-expressing granule cells are seen in the mutants. Dotted lines demark cerebellar lobe boundaries in samples where they are difficult to visualize. Sections were counterstained with DAPI. Scale bar in A is the same for all panels and represents 50 μm.
positioned granule cells, but the position and irregular shape of most of these cells suggest they are of glial lineage. Similarly, the Shhc/Shhn, L7-Cre cerebellum at P5 has a greatly reduced egl and very few BrdU-positive cells were seen in this region (Fig. 10F). PHH3-immunopositive cells were also observed in the cerebellum of both mutants at E18.5, but were lost by P5 co-incident with loss of the egl (data not shown). Together, these results indicate that before birth a proportion of granule cell precursors were able to enter M phase of the cell cycle in the absence of Shh, but that Shh is required for full proliferative expansion of the egl during the immediate prenatal and postnatal period.

Discussion

Shh and patterning of the cerebellum

In this study, we have provided a detailed spatiotemporal picture of the normal role of Shh during cerebellar development. Shh expression is first detected at E17.5 in distinct parasagittal domains located rostrally and either side of the midline, domains that overlap with a reporter driven by the Purkinje-cell-specific promotor L7 and therefore corresponds to the Purkinje cell precursors. In contrast to an earlier report that detected Shh expression within the egl, we find no evidence for Shh expression in any cell type other than Purkinje cell precursors and mature Purkinje cells (Dahmane and Ruiz i Altaba, 1999). By E17.5, Purkinje cells have settled in a broad plate beneath the egl, which has migrated across the sub-pial surface of the cerebellar anlage. Cells up-regulating Ptc1, which may therefore be responding to Shh, were observed within the egl, pcl, and throughout the anlage, suggesting that before birth this signal might play a role not only in granule cell biology, but in the development of other cell types in the cerebellum. After birth, the domain of Shh-expressing Purkinje cells extends progressively along the entire rostral–caudal axis of the cerebellum and in an increasing number of parasagittal stripes that extend laterally from the midline. The majority of cells responding to Shh over this time are granule cells found within the egl and newly formed igl. Interestingly, Purkinje cells may both produce Shh protein and respond to Shh signaling. The presence of Shh in vesicular structures of Purkinje cell dendrites demonstrates that the protein can be transported to the close vicinity of the egl, although dendrites never penetrate this layer. This suggests that Shh might be transported from Purkinje cell dendrites to act on the egl, where the Shh-responsive granule cell precursors reside. The Shh signal is certainly capable of travelling across a field of responding cells in the mouse embryonic limb bud (Lewis et al., 2001), but the mechanism(s) by which this could be achieved in the cerebellum are not understood. It will be interesting to determine whether the obligatory cholesterol modification on the N-terminal Shh signaling moiety that is produced by autocatalytic processing plays any role in this trafficking process.

The higher levels of Shh expression in rostral lobes of the embryonic and neonatal cerebellum appears to correspond with elevated levels of Shh protein and Ptc1 expression.

Fig. 10. Reduced granule cell proliferation during early stages of cerebellar development in the absence of Shh. BrdU labeling was performed on sections of the cerebellum from Shhc/Shhn (A, D), Shhc/Shhn;Pax2-Cre (B, E), and Shhc/Shhn;L7-Cre (C, F) mice at E18.5 (A–C) and P5 (D–F). (A, D) BrdU labeling in the Shhc/Shhn cerebellum indicates numerous proliferating cells in the egl and other cells in the putative igl (iglp). (B, E) The Shhc/Shhn;Pax2-Cre cerebellum has no BrdU-positive cells in the egl, whereas some BrdU-labeled cells are seen in the igl. (C, F) The Shhc/Shhn;L7-Cre cerebellum has very few proliferating cells in the egl, whereas BrdU-labeled cells are seen below this layer. Arrowheads indicate regions in the P5 cerebellum where an egl would be expected to be found, but is absent or rudimentary in the mutants. Scale bar represents 30 μm.
observed in rostral lobes of the cerebellum before birth. However, only subtle differences were detected after birth. Whether there is a functional significance to this transient rostral–caudal difference is unclear. The cerebellum is thought to be compartmentalized along the medial–lateral and anterior–posterior axis based on the restricted expression of many genes and anatomy of the cerebellum (reviewed by Herrup and Kuemerle, 1997; Ozol et al., 1999). However, the functional significance of these boundaries is not well understood and has not been demonstrated. Mice with a reduced dosage of Fgf8 and/or lacking Fgf17 lack the most anterior lobe of the vermis, but this defect is thought to arise from an earlier defect in precursor cell proliferation in the mid-hindbrain junction region where these genes are expressed (Xu et al., 2000). Interestingly, the meander tail mouse mutation causes severe cellular disorganization and a near-total depletion of granule cells in anterior lobes of the cerebellum that is caused by a defect intrinsic to the granule cells (Hamre and Goldowitz, 1997; Ross et al., 1990). The striking regional expression pattern of Shh in the developing cerebellum suggests that Shh may also influence compartmental cellular organization of the cerebellum. Indeed, we have demonstrated that removal of Shh expression from the embryonic cerebellum using a conditional allele results in defects that are more severe in rostral lobes. We do not believe that this regional difference in cerebellar phenotype was due to incomplete removal of Shh in the Shhc/Shhn,Pax2-Cre and Shhc/Shhn,L7-Cre mutants, as we did not detect Shh protein in the cerebellum at E18.5 or at any subsequent stage of development examined (data not shown). The most obvious patterning defects observed in these mutants were the loss of granule neurons, reduced size, and abnormal foliation of the cerebellum. The formation of rudimentary lobules in the Shhc/Shhn,L7-Cre mutant cerebellum and their complete absence from rostral regions of the Shhc/Shhn,Pax2-Cre cerebellum suggest a role for Shh in lobe formation. Little is known about this process but it is most likely that defects in foliation are a secondary consequence of the failure in granule cell expansion, which is known to effect lobulation. The Shhc/Shhn,Pax2-Cre and Shhc/Shhn,L7-Cre mutants both had reduced cerebellar volumes, which appeared to be primarily due to the loss of granule cells, and this may have also influenced lobe formation in the mutant cerebellum.

### Shh is required for proliferation of granule neuron precursors

We have demonstrated an essential role for Shh in the normal expansion and proliferation of the egl consistent with the view obtained by antibody blocking experiments and cell culture studies (Dahmane and Ruiz i Altaba, 1999; Kenney and Rowitch, 2000; Wallace, 1999; Wechsler-Reya and Scott, 1999) that Shh is the principle mitogenic input for proliferating granule cell precursors in the egl. However, subtle differences in granule cell development were apparent in the Shhc/Shhn,Pax2-Cre and Shhc/Shhn,L7-Cre mutants that might account for the more severe patterning defects seen in the former. We detected no granule cell proliferation in the cerebellum of Shhc/Shhn,Pax2-Cre mutants, even before birth, whereas the Shhc/Shhn,L7-Cre mutants possessed a small number of granule cell precursors that appeared capable of proliferating. This difference was also apparent in the postnatal cerebellum where the egl was completely lost in the Shhc/Shhn,Pax2-Cre mutants, although some granule cells were still detected in the egl of the Shhc/Shhn,L7-Cre mutants. Thus, the rudimentary igl present in the Shhc/Shhn,L7-Cre cerebellum is likely to have arisen from the differentiation and migration of small numbers of granule cell precursors present in the egl. It is not clear why these precursor cells were still able to proliferate, but it could be due to the presence of low levels of Shh protein that Purkinje cells may produce before L7-Cre activity that are beyond the limits of detection in this study. Alternatively, another signal that on its own is only capable of inducing low levels of granule cell proliferation may be differentially affected in the two models. SDF-1α has been implicated in granule cell proliferation; however, this cytokine only has an effect in the presence of Shh (Klein et al., 2001). A more likely candidate is Jagged-1 which appears to maintain granule cell proliferation independent of Shh (Solecki et al., 2001). The possible action of other mitogenic factors may also contribute to the enhanced cerebellar development that is evident caudally in both mutants. In summary, our data indicate that the process of granule neuron maturation and migration are most likely independent of Shh signaling but that the eventual loss of precursor cells from the egl limits the period over which postmitotic granule cell progenitors are generated.

Does Shh play a role in Purkinje cell and Bergmann glia biology?

The final maturation of Purkinje cells occurs in the early postnatal period of cerebellum development, which is evident by the spreading of cells from a multilayered structure to a monolayer and the formation of extensive dendritic arbors and synapses with granule neurons (Goldowitz and Hamre, 1998). Little is known about the signals that govern Purkinje cell differentiation, although granule cells are known to influence these changes, as evidenced by cases in which the granule cell population is disrupted and Purkinje cells are poorly aligned with altered dendritic morphology (Rakic and Sidman, 1973; Ross et al., 1990). We observed similar abnormalities in Purkinje cell development during postnatal life of the mutants described here. It seems most likely that these alterations are secondary to the loss of granule cell precursors. Indeed, a similar disruption in Purkinje cell organization to that observed is Shhc/Shhn,Pax2-Cre embryos is also observed in Math1 mutants where proliferating granule cell precursors are also com-
pletely absent. The less severe Purkinje cell abnormalities observed in the $\text{Shh}^+/\text{Shh}^{-}, L7$-Cre cerebellum at P5 are probably due to the presence of a small number of granule cell precursors that are capable of differentiating. We observed no abnormalities in the Calbindin-D28-expressing Purkinje cell population of both prenatal mutants, which suggests that Shh does not play a role in Purkinje cell differentiation or maturation. Interestingly, differentiating Purkinje cells are also present in Math1-null embryos that completely lack granule neuron precursors (Ben-Arie et al., 1997), demonstrating that Purkinje cell development during embryogenesis is also not dependent on this granule neuron population.

Numerous factors have been shown to be important for Purkinje cell survival (Goldowitz and Hamre, 1998). However, although we observed a reduction in Purkinje cell numbers in the absence of Shh in the cerebellum during postnatal life, we were not able to detect any significant levels of cell death, which might result from an inability to detect the small numbers of Purkinje cells dying at any one time. In the case of the $\text{Shh}^+/\text{Shh}^{-}, L7$-Cre mutants, this effect was most likely due to lack of trophic support from granule neurons since Purkinje cell numbers were not reduced before birth. This explanation most likely also explains the enhanced postnatal phenotype in the $\text{Shh}^+/\text{Shh}^{-}, \text{Pax2-Cre}$ cerebellum. However, these mutants also displayed reduced Purkinje cell numbers before birth. The reasons for this are not clear. Purkinje cells may fail to migrate to the midline region of the vermis where our analysis was mostly confined, but we did not detect elevated numbers of Purkinje cells in more lateral regions of the cerebellum (data not shown). Alternatively, Purkinje cells may have migrated to ectopic extracerebellar locations. Interestingly, some ectopic Purkinje cells were detected in the inferior colliculus immediately adjacent to the most anterior lobes of the cerebellum (data not shown). Alternatively, this effect may have been due to an earlier defect in Purkinje cell development due to removal of Shh activity from the ventricular epithelium of the mid-hindbrain junction, from which Purkinje cells are derived during embryogenesis. The $\text{Pax2-Cre}$ transgene is active in this region and Shh is known to be expressed throughout ventral regions of the brain during this time (P.M.L and A.P.M, unpublished data). Further studies will be required to determine whether Shh is required for the birth, survival, and/or migration of a proportion of the Purkinje cell population during embryogenesis. Interestingly, it has been reported that Shh-null embryos do not appear to have a recognizable cerebellum at E18 and even by E15.5, the cerebellum has reduced morphology (Dahmane et al., 2001). This defect may suggest an earlier role for Shh in the ventricular neuroepithelium of the cerebellum, but it could also be a secondary effect due to disruption of the complex interactions that occur at the mid-hindbrain junction, which is believed to act as an organizing center for patterning this region of brain (reviewed in Wang and Zoghbi, 2001).

Previous studies using hybridoma cells secreting neutralizing Shh antibody injected into the postnatal chick or mouse brain have demonstrated a reduction in egl proliferation, but not differentiation (Wallace, 1999; Wechsler-Reya and Scott, 1999). Another study has also demonstrated that intraventricular injection of Shh antibody-producing hybridoma cells into chick embryos at D4 (Carnegie stage equivalent of E12.5–13 mouse embryo) resulted in a thinner egl with variable abnormal or aborted granule neuron development at D15 (Dahmane and Ruiz I Altaba, 1999). These embryos also had a smaller cerebellum, altered foliation, abnormal Purkinje cell organization, and reduced or absent Bergmann glia. For the most part, our findings are consistent with all of these studies, but we did not observe any noticeable alterations in Bergmann glia differentiation. This could be a species-specific effect with Shh playing a more predominant role in Bergmann glia development in the chick, or complications in the phenotype resulting from these nongenetic ablation studies. Our findings highlight the importance of this signal for granule neuron precursor proliferation and patterning of the cerebellum during both embryonic and postnatal stages of cerebellum development. The role of Shh signaling in the adult cerebellum remains unresolved, but the presence of Shh protein in Purkinje cell dendrites and axons in the adult is consistent with late functions in the neural circuitry.

Acknowledgments

We would like to thank Tom Jessell for generously supplying us with the $\text{Shh}^+/\text{acZ}^{-}$ animals. Thanks also to John Oberdick for providing the L7-ΔAUG promoter enhancer, Jane Johnson for the Math-1 antibody, and Nathan Heinz for BLBP antibody. We acknowledge Carmen Peppicelli for generating the Pax2-Cre transgenic mice and Benoit St-Jacques for his initial contributions toward generating the $\text{Shh-loxP}$ targeting construct while in A.P.M. laboratory. Thanks to Ruby Cook and Ciaran Faherty in the laboratory of R.S for assistance in preparation of histological processing and data collection. Many thanks to Anders Linde for compiling images used in some of the figures. This work was funded by a Postdoctoral Fellowship from the Foundation for Research, Science and Technology of New Zealand awarded to P.M.L. and an NIH grant to A.P.M (NS 33642). A.G.L is supported by the Swedish Research Council (Grants 2789 and 14100). R.S is supported in part by the American Lebanese Syrian Associated Charities (ALSAC).

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