

Sonic hedgehog regulates the growth and patterning of the cerebellum

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SUMMARY

The molecular bases of brain development and CNS malignancies remain poorly understood. Here we show that Sonic hedgehog (Shh) signaling controls the development of the cerebellum at multiple levels. SHH is produced by Purkinje neurons, it is required for the proliferation of granule neuron precursors and it induces the differentiation of Bergmann glia. Blocking SHH function *in vivo* results in deficient granule neuron and Bergmann glia differentiation as well as in abnormal Purkinje neuron

development. Thus, our findings provide a molecular model for the growth and patterning of the cerebellum by SHH through the coordination of the development of cortical cerebellar cell types. In addition, they provide a cellular context for medulloblastomas, childhood cancers of the cerebellum.

Key words: Sonic hedgehog, Cancer, Cerebellum, Bergmann glia, Purkinje neuron, Granule neuron, Medulloblastoma

INTRODUCTION

Cerebellar tumors, or medulloblastomas (MBs), and basal cell carcinomas of the skin (BCCs) are two types of cancers overrepresented in patients with the Basal Cell Nevus or Gorlin's Syndrome (Gorlin et al., 1990). These tumors can arise from activation of the Sonic hedgehog (Shh) signaling pathway through mutations in the membrane receptor components Patched or Smoothed (Hahn et al., 1996, 1998; Johnson et al., 1996; Gailani et al., 1991; Goodrich et al., 1997; Oro et al., 1997; Xie et al., 1998; Reifengerger et al., 1998). In addition, we showed that virtually all sporadic BCCs, representing the most common type of cancer, display activation of the Shh signaling pathway leading to expression of the transcription factor Gli1, a Shh-target and mediator that can itself induce epidermal tumors (Dahmane et al., 1997; Ruiz i Altaba, 1997). Since BCCs are thought to recapitulate steps in hair follicle differentiation normally regulated by SHH (Ackerman et al., 1993; Dahmane et al., 1997; St-Jacques et al., 1998), MBs could recapitulate SHH-regulated ontogenic steps of cerebellar development (e.g. Trojanowski et al., 1992). This idea would be consistent with the expression of *Ptc* and *Gli* genes in the cerebellum (Millen et al., 1995; Goodrich et al., 1996; Traiffort et al., 1998) and the function of SHH as a mitogen, differentiation or survival factor for different cell populations in the CNS (Jensen and Wallace, 1997; Miao et al., 1997; Pringle et al., 1996; Poncet et al., 1996) and other tissues (Fan et al., 1995; Duprez et al., 1998). In addition, the majority of human MBs show expression of ZIC1 (Yokota et al., 1996), a zinc finger protein of the GLI superfamily, and other genes normally expressed in granule neurons or their precursors in the external germinal layer (EGL) (Aruga et al., 1994; Kozmik et al., 1995). However, Shh was not reported to be expressed

in the developing cerebellum and thus, a rational cellular context for MBs was lacking.

The cerebellum contains a large cortical region within which distinct cell types are positioned in a layered fashion. The outermost layer, the EGL, contains dividing granule neuron progenitors. Postmitotic granule cells leave the EGL and migrate inwards to form the internal granular layer (IGL) where granule neurons terminally differentiate. Migrating neurons are guided by Bergmann radial glial fibers through the molecular layer and, before reaching the forming IGL, they pass through the Purkinje neuron layer (PL) containing the cell bodies of Purkinje neurons and Bergmann glia (e.g. Altman and Bayer, 1996). Analyses of cerebellar granule cell development (Hatten and Heintz, 1995; Smeyne et al., 1995; Hatten et al., 1997; Herrup and Kuemerle, 1997) have shown that EGL cells acquire a granule neuron fate from the onset of their migration in the rhombic lip (Alder et al., 1996; Jankovski et al., 1996), that EGL proliferation requires cell contact, is dependent on Purkinje neurons and that this can be potentiated by Insulin-like and Epidermal growth factors (Hatten, 1985; Gao et al., 1991; Smeyne et al., 1995). Moreover, mutual interactions between Purkinje neurons and their future presynaptic regulatory partners, the granule neurons, are required for the normal development of both cell types (e.g. Hatten and Heintz, 1995; Herrup and Kuemerle, 1997). Thus, whereas the cellular interactions involved in cerebellar development have been largely described, their molecular bases remain unknown.

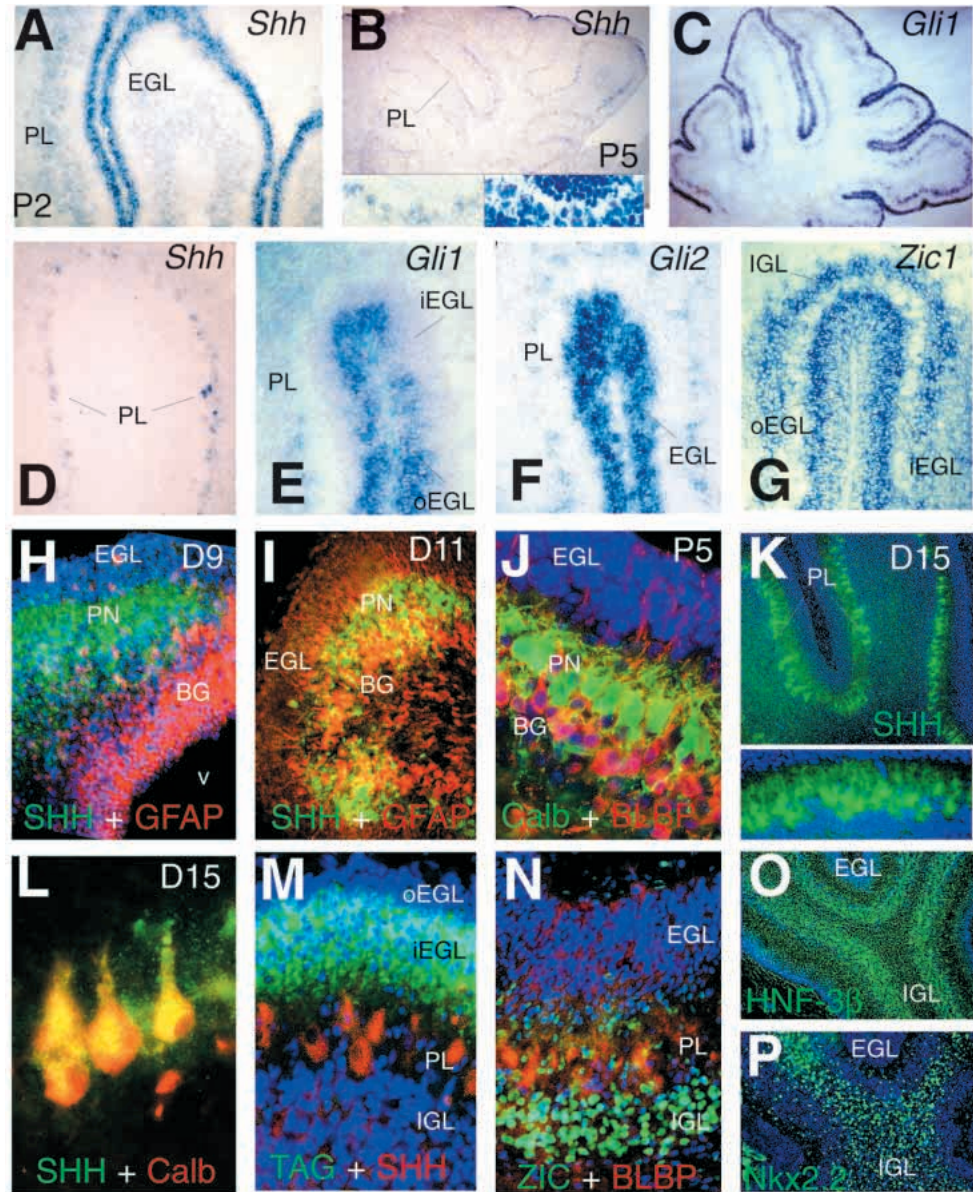
Here, we investigate a possible endogenous role of SHH in cerebellar development. We find that SHH is produced by chick and mouse Purkinje neurons and also transiently by early mouse EGL cells. The results of treating chick cerebellar explants or purified mouse cells with SHH or a blocking anti-SHH antibody show the requirement of SHH in the proliferation of granule

Fig. 1. Localization of *Shh*, *Gli* and *Zic* expression in the developing mouse and chick cerebella and relationship to cell-type-specific markers.

(A-C) Localization of *Shh* (A,B) and *Gli1* (C) mRNA to the mouse PL and EGL at P2 (A) and P5 (B,C). Note the transient expression of *Shh* in the EGL (blue label in A). (Insets in B) Detail of an in situ hybridization (left) and the same section counterstained with hematoxylin (right) showing expression of *Shh* in Purkinje neurons having characteristically large nuclei.

(D-G) Differential gene expression in the P5 mouse PL and EGL. *Shh* is expressed in the PL (D), *Gli1* mostly in the outer EGL (oEGL; E), *Gli2* throughout the EGL (F) and *Zic1* mostly in the inner EGL (iEGL) and IGL (G). (H,I) SHH protein expression in Purkinje neurons (PN) and GFAP in Bergmann glia in D9 (H) and D11 (I) chick cerebella. V, ventricle. (J) Adjacent localization of Purkinje neurons expressing Calbindin and Bergmann glia expressing BLBP in the P5 mouse cerebellum.

(K) Localization of SHH protein in Purkinje neurons in the D15 chick cerebellum. The inset shows a high magnification displaying the typical morphology of developing Purkinje neurons. (L) Co-expression of SHH protein and Calbindin in Purkinje neurons in the D15 chick cerebellum. (M-P) Expression of TAG in the iEGL (M), ZIC proteins in the IGL and migrating cells in the molecular layer in between the EGL and IGL (N), and HNF-3 β in the IGL (O) and Nkx2.2 (P) in deeper cells of D15 chick cerebellum. (H-P) Sections were counterstained with DAPI shown in dark blue to highlight the position of nuclei. The antibodies and colors used in each panel are indicated.



neuron precursors. In addition, glial differentiation is induced by SHH. Blocking SHH signaling *in vivo* leads to the development of hypoplastic cerebella with abnormal foliation, in which Purkinje neurons are abnormally positioned and Bergmann glia and differentiated granule neurons are reduced in number or absent. Together, these *in vivo* and *in vitro* results demonstrate previously unknown functions of SHH in the elaboration of pattern in the maturing central nervous system and provide a molecular model for the coordinate regulation of cortical development in the cerebellum. Moreover, they provide a basis for the development of brain tumors as deregulated or maintained *Shh* signaling *in vivo*, by any mutations activating this pathway, is predicted to lead to the maintained and aberrant proliferation of granule neuron precursors.

MATERIALS AND METHODS

Animals, explant preparation and cell isolation

Embryonic days for mice (E) or chicks (D) were counted starting as

0.5 the morning after conception for mice or the days of incubation for chicks. Mice were obtained from Taconic or Charles River and fertilized chick eggs from Spafas. Embryos or postnatal (P) mice were dissected in L15-air on ice. EGL cerebellar explants were prepared from D10-12 chicks by first isolating the cortical region, which contained the PL with small amounts of IGL, with a surgical scapel and then cutting this into squares. The lateralmost regions were avoided. 50-100 explants were routinely obtained from a single D10 chick cerebellum. Comparing the development of foliation patterns, a D10 cerebellum is roughly equivalent to that of an ~E18 mouse embryo. Chick D10 cerebellar explants were grown in collagen gels (Tessier-Lavigne et al., 1988) with serum-free media (Nothias et al., 1998). Granule neuron precursors and glia were isolated by the percoll gradient method (Hatten, 1985; Gao et al., 1991) followed by sequential platings. Isolated neuronal precursors were allowed to aggregate for 24 hours and isolated glia were treated after 2 days in culture in serum-free media. Each experiment was done at least three independent times unless otherwise stated.

In situ hybridization and immunofluorescence

In situ hybridization was carried out on cryostat sections of fixed by

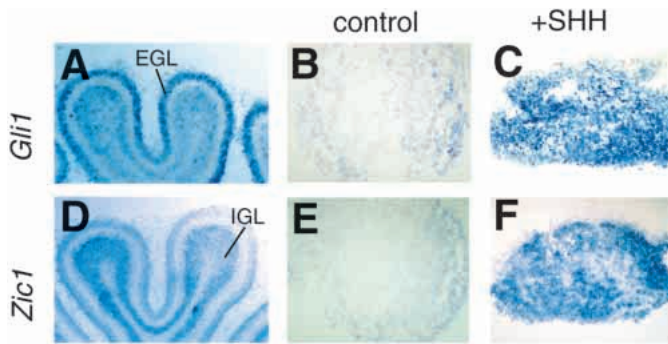


Fig. 2. SHH enhances the expression of granule neuron lineage markers in chick cortical explants. (A,D) Preferential localization of *Gli1* (A) and *Zic1* (D) in the EGL and IGL of D17 chick cerebella. Expression at D11-13 is similar but at lower levels than at D17 (not shown). (B,E) Expression of *Gli1* (B) and *Zic1* (E) in untreated cerebellar explants taken from D11 chicks and grown for 72 hours in vitro; $n=4$ for each. (C,F) SHH-induced enhanced expression of *Gli1* (C) and *Zic1* (F) in sibling explants to those in B,E; $n=4$ for each.

immersion or perfused specimens with 4% paraformaldehyde (Dahmane et al., 1997). Antisense digoxigenin-labelled RNA probes for mouse *Gli*, *Zic* and *Shh* genes were as described (Dahmane et al., 1997; Aruga et al., 1994, 1996). Mouse probes were also successfully used for chick tissue given the high conservation of these genes. In situ hybridization sections were counterstained with hematoxylin (Sigma) to localize transcript expression.

Immunocytochemistry was carried out in 4% paraformaldehyde-fixed/perfused tissue with anti-SHH mAb (5E1; Ericson et al., 1996) obtained from the University of Iowa Hybridoma Bank, anti-BLBP polyclonal antibody (Feng et al., 1994), anti-GFAP polyclonal antibodies (Sigma), anti-Calbindin 28K polyclonal antibodies (Sigma), anti-ZIC proteins mAb (Yokota et al., 1996), anti-TAG polyclonal antibodies (Furley et al., 1990), TuJ1 anti-neural tubulin mAb (Babco), anti-HNF-3 β mAb (Ruiz i Altaba, 1994; Ericson et al., 1996), anti-Nkx2.2 mAb (Ericson et al., 1997) and anti-BrdU mAb (Becton-Dickinson or Harlan-seralab). Primary antibody labeling was visualized with fluorescein- or rhodamine-labeled secondary antibodies (Boehringer Mannheim, TAGO). Blocking antibodies against SHH (5E1; Ericson et al., 1996) were used at 5 μ g/ml. For control experiments, 5E1 antibody was denatured by boiling for 10 minutes. Labeled explants or cell aggregates were mounted under a coverslip and cells labeled by the different markers were counted visually under epifluorescence. Double-labeled cells were counted on confocal micrographs after tilting the stack of images to ensure colabeling.

Growth factors and chemicals

Baculovirus-derived purified SHH (SHH-N) was used at 5 ng/ml. Forskolin and 1,9dideoxyforskolin (Sigma) were used at 50 μ M. BrdU (Sigma) was used at 6 μ g/ml for a 1 hour pulse 24 hours before harvesting the explants. HCl treatment and visualization of BrdU-labelled DNA was done following standard protocols. DAPI (Sigma) was used at 10 ng/ml to counterstain DNA in nuclei. [3 H]thymidine incorporation was measured after incubating purified granule neuron precursor aggregates in media containing 1.5 μ Ci [3 H]thymidine/0.1 ml for 20 hours. In the case of SHH-treated aggregates, these were exposed to [3 H]thymidine after 24 hours of initial incubation with SHH. Hematoxylin and eosin staining on fixed sections was as specified by the manufacturer (Sigma).

Hybridoma injections into developing chick embryos

Hybridoma 5E1 secreting anti-SHH IgG antibody (Ericson et al., 1996), a control hybridoma secreting an IgG antibody unrelated to

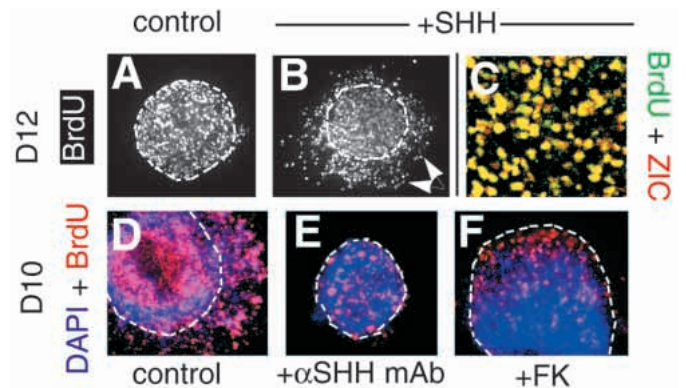


Fig. 3. SHH enhances spreading and proliferation of granule neurons from cerebellar explants. (A,B) Control (A; $n=11$) D12 chick cortical explants grown for 48 hours proliferate but show no significant spreading of newly divided cells into the surrounding collagen. SHH treatment for the first 24 hours of culture induces extensive migration of BrdU-labeled cells (arrows) into the surrounding collagen matrix at 48 hours (B; $n=12$). Treatment with SHH for the first 12 hours of culture resulted in less extensive spreading at 24 hours ($n=8$) as compared to 48 hours, not seen in control ($n=12$) explants. (C) Confocal image of multiple double ZIC+/BrdU+ spreading cells. SHH treatment increased the number of BrdU+ and ZIC+ cells spreading into the collagen gel ($n=6$) as compared to control explants ($n=7$, not shown). (D,E) BrdU labeling of D10 explants grown for 96 hours. A 1 hour BrdU pulse was given 24 hours before harvesting and shows that anti-SHH mAb decreased BrdU incorporation within D10 explants at 48 hours (not shown; $n=8$) and 96 hours (E; $n=6$) as compared to untreated controls (D; $n=8$). The limited spreading of cells into the collagen seen in control explants (D) was also inhibited by anti-SHH mAb (E). (F) Treatment of D12 explants for 24 hours with forskolin followed by a 1 hour pulse of BrdU and another 24 hours of culture decreased BrdU incorporation ($n=10$) as compared to sibling control explants or those treated with the inactive derivative 1,9dideoxyforskolin (not shown; $n=4$). Antibodies and colors are indicated in or next to each panel. Dashed lines depict the boundaries of the explant as determined in Nomarski optics. All explants were counterstained with DAPI.

SHH (unpublished) or the parental myeloma line NS1 were grown under standard conditions. Log-growing cells were collected, washed and resuspended in PBS. Embryos were injected in ovo at D4 into the optic tectum with 5000-10,000 cells in 1 μ l. The egg shells were resealed and the embryos incubated until D15 when they were perfused, sectioned and stained.

RESULTS

Expression of SHH and Shh-responsive genes in the developing cerebellum

To test if *Shh* is endogenously produced in the developing cerebellum, we first defined the precise expression patterns of the *Shh* and SHH-responsive genes in the developing mouse cerebellum. *Shh* mRNA was detected in the cerebellum of E17 embryos (not shown). From E19 to P5 it was continuously detected in Purkinje cells (Fig. 1A,B), a time when *Gli1* was also found in the PL and EGL (Fig. 1C,E; Millen et al., 1995). We also detected transient and variable expression of *Shh* in the EGL at P1-P2 (Fig. 1A). *Gli2* and *Gli3* were expressed also in the EGL and PL although the level of *Gli3* was very low

overall and that of *Gli2* was weak in the PL (Fig. 1F and not shown; Millen et al., 1995). Expression of *Zic1-3* was found at high levels in the IGL, as previously reported (Aruga et al., 1996), but also in EGL cells (Fig. 1G, and not shown). Within the EGL, different members of the Gli/Zic superfamily showed distinct expression domains. *Gli1* was expressed in dividing progenitors in the outer layer (oEGL, Fig. 1E), *Zic1* was predominantly expressed in postmitotic premigratory cells of the inner layer (iEGL; Fig. 1G) and *Gli2* was expressed throughout the EGL (Fig. 1F). Taking *Gli1* and *Gli2* expression as a marker of response to Shh signaling (Marigo et al., 1996; Lee et al., 1997; Ruiz i Altaba, 1998), SHH could thus act in an autocrine manner in early EGL cells. SHH secreted from Purkinje neurons could then act on oEGL cells and in cells within the PL.

The distribution of SHH protein and its localization with respect to a variety of cell-specific markers as prelude for functional studies was carried out in the chick cerebellum, as the pattern of cerebellar cell types is highly conserved. At D9, SHH protein was found in immature Purkinje neurons migrating away from the ventricular zone, where they are born, towards the EGL (Fig. 1H). At this time, glia identified by expression of Glial fibrillary acidic protein (GFAP) or Brain lipid-binding protein (BLBP, Feng et al., 1994) were located close to the ventricular zone where they are also born (Fig. 1H). Unlike in mice, SHH was not detected in the early chick EGL. By D11, Purkinje neurons expressing SHH were adjacent to GFAP+ Bergmann glia, close to the proliferating EGL (Fig. 1I). At D15 in chicks and P5 in mice, SHH+ Purkinje neurons expressing Calbindin and Bergmann glia expressing BLBP were neighbors in the PL (Fig. 1J-L). SHH protein expression in Purkinje neurons is consistent with the detection of *Shh* mRNA (Lin and Cepko, 1998). At this time, postmitotic premigratory cells of the iEGL were marked by TAG1 expression (Fig. 1M; Furley et al., 1990; Kuhar et al., 1993), and IGL as well as a few migrating cells in the molecular layer were marked by the expression of ZIC proteins (Fig. 1N; Yokota et al., 1996). Interestingly, we also found HNF-3 β expression in the IGL (Fig. 1O). Expression of SHH, *Gli1* and HNF-3 β in adjacent cells of the developing cerebellum constitutes a parallel with their expression in the notochord and ventral neural tube (e.g. Echelard et al., 1993; Roelink et al., 1994; Ruiz i Altaba et al., 1995; Lee et al., 1997) and raises the possibility that Shh signaling induces the differentiation of both floor plate cells and granule neurons. In addition, we found that the homeoprotein Nkx2.2, which is expressed in the ventral ventricular zone of the early neural tube (Ericson et al., 1997), is also found in a subpopulation of cells that is more distal to the Purkinje layer than that expressing HNF-3 β (Fig. 1O,P and not shown). Together, these results thus raise the possibility that the patterning of the ventral neural tube and the cerebellar cortex share common inductive mechanisms triggered by SHH.

SHH enhances expression of the granule neuron lineage markers *Gli1* and *Zic1* in cortical cerebellar explants

To begin to investigate the role of SHH in cerebellar development, we first developed an in vitro assay in which chick embryo cerebellar cortical explants are grown in collagen gels in serum-free media. These explants showed the expression of all markers tested except those of Purkinje

neurons (not shown), consistent with the difficulty of growing these neurons in vitro (e.g. Baptista et al., 1994; Dusart et al., 1997). There is, therefore, no maintained endogenous source of SHH in the explant cultures, although it is important to note that explanted EGL cells are likely to have been already exposed to SHH before explant preparation and that SHH lingers in explanted tissues for some time (e.g. Ericson et al., 1996).

As in mice, *Gli1* and *Zic1* in chick embryos were mainly expressed in the EGL and IGL, respectively from D11-D17 (Fig. 2A,D and not shown; Lin and Cepko, 1998). In situ hybridization analysis of sectioned D11 explants grown for 72 hours revealed low levels of *Gli1* and *Zic1* expression (Fig. 2B,E). SHH treatment induced higher expression of these markers (Fig. 2C,F), suggesting an enhancement of the development of granule neurons by Shh signaling.

SHH induces increased migration of granule neurons from cortical explants

D12 chick explants treated with SHH showed dispersion of cells into the surrounding collagen matrix (Fig. 3B). We focused on this migrating or spreading cell population to further test the effects of SHH on explants as it seemed possible that this behavior recapitulated the migration of postmitotic granule cells away from the EGL. Analyses of BrdU incorporation showed that a large number of spreading cells had undergone mitosis (Fig. 3A,B, images show focal planes appropriate for the spreading cells; see legend). Spreading of BrdU+ cells into the surrounding collagen matrix was not observed at 48 hours in control explants (Fig. 3A) although there was limited spreading at 96 hours (Fig. 3D). SHH treatment increased the number of BrdU-labeled spreading cells by ~6-fold on average (Figs 3B, 5A). Changes in BrdU incorporation inside the explants were difficult to quantify given the thickness of the explants and the large number of labeled cells. However, in two experiments, we detected a ~2-fold increase in the number of distinguishable BrdU+ cells in SHH-treated versus control explants (not shown).

Given the upregulation of *Gli1* and *Zic1* observed in explants (Fig. 2), it seemed possible that the majority of spreading BrdU+ cells corresponded to postmitotic ZIC+ IGL granule neurons that would normally be migrating away from the EGL. Treatment of D12 explants with SHH for 48 or 96 hours caused the spreading of ~3-fold more ZIC+ cells on average as compared to untreated controls (Fig. 5C). Confocal imaging revealed that a large fraction of BrdU+ spreading cells in these explants (88%; $n=350$ cells counted in three sections) were also ZIC+ (Fig. 3C). These results could be explained by an enhancement of granule neuron precursor proliferation by SHH within the explant. In this case, an increase in the number of postmitotic ZIC+ cells migrating into the collagen would be proportional to an increase in the rate of proliferation of precursors within the explant. This could reflect a constant percentage of precursors that begin to differentiate at a given time. Alternatively, SHH could act directly or indirectly to induce granule neuron precursor differentiation. We thus proceeded to test whether SHH has a direct proliferative function on granule neuron precursors.

SHH is a mitogen for granule neuron precursors

Because Purkinje neurons are known to regulate the

proliferation of granule neurons (e.g. Smeyne et al., 1995) and the former express SHH (Fig. 1), we specifically tested whether SHH is a mitogen for granule neuron precursors. These cells were isolated from the cerebella of P1-3 mice and were allowed to form aggregates *in vitro*. These aggregates did not contain significant glial contamination as revealed by the absence of GFAP labeling (Fig. 4A) or mature granule neurons (not shown) and showed a modest degree of proliferation as measured by continuous BrdU incorporation after 24 hours in culture (Fig. 4A,B; Hatten, 1985; Gao et al., 1991). In these aggregates, we were able to count every single BrdU+ cell as they are relatively small, providing for careful and reliable quantification. Addition of SHH to the serum-free culture media for 24 hours increased their proliferation by ~2.5-fold on average (Figs 4C, 5D). An analysis of [³H]thymidine incorporation is consistent with this figure (27698 counts/minute for SHH-treated and 14952 counts/minute for control untreated aggregates; not shown). ZIC expression was not induced by SHH treatment (not shown), suggesting that granule cell precursors can proliferate but not differentiate appreciably in these aggregates. The result presented here demonstrates a direct mitogenic effect of SHH on granule neuron progenitors and provides a model for the hyperproliferative behavior of MBs.

Blocking Shh signaling *in vitro* inhibits granule neuron development

To test if Shh signaling is required for granule neuron development, chick cortical explants and mouse granule cell aggregates were incubated with a blocking anti-SHH monoclonal antibody (mAb, Ericson et al., 1996). Because cells in explants may have already been exposed to SHH for some time before explant preparation, we tested the effects of mAb incubation on younger cerebellar explants in which Shh signaling may just be underway. Incubation of D10 explants grown for 96 hours with mAb showed a ~5-fold lower number of both BrdU+ (Figs 3E, 5B) and ZIC+ (Fig. 5C) cells on average as compared to untreated controls (Figs 3D, 5B,C). Treatment with denatured 5E1 antibody gave results indistinguishable from untreated explants (not shown). These results are consistent with the ability of the 5E1 antibody to block motor neuron differentiation in ventral spinal cord explants, lacking an internal source of SHH, if the explants are taken shortly after SHH signaling occurs (Ericson et al., 1996). SHH thus appears to linger on the recipient cells long enough to allow blockage by antibody treatment.

To test more directly for a requirement of SHH in the proliferation of granule cell precursors, these were purified from P1-3 mouse cerebella and treated with anti-SHH mAb. This treatment inhibited the proliferation of purified mouse granule neuron aggregates by ~2-fold on average (Figs 4E, 5D) as compared to untreated controls (Figs 4D, 5D) or ~5-fold on average as compared to SHH-treated aggregates (Figs 4C, 5D).

Requirement of the Shh signaling pathway was also tested by elevating the intracellular levels of cAMP with forskolin and thus enhancing the activity of PKA, a known inhibitor of this pathway. Analysis of BrdU incorporation in D10 or D12 chick cortical explants (Fig. 3F and not shown) showed that forskolin abolished cell spreading and decreased the number of dividing cells inside the explants by ~3-fold on average after treatment for 48 or 96 hours, as compared to that seen in untreated control

explants and those treated with 1,9-dideoxyforskolin, an inactive derivative (Fig. 5A). In addition, isolated mouse granule neuronal precursor aggregates treated with forskolin exhibited ~5-fold lower levels of BrdU incorporation on average (Figs 4F, 5D) than control aggregates (Figs 4D, 5D) or aggregates treated with 1,9-dideoxyforskolin (not shown). The higher inhibition obtained with forskolin versus mAb treatment could be due to the fact that forskolin activates PKA and this acts at a downstream intracellular event that may either be more effectively blocked or is slightly more delayed than extracellular signaling, thus allowing for a greater inhibition. Together, the results with explants and aggregates treated with a blocking anti-SHH mAb or forskolin indicate that SHH is required for proliferation of granule neuron progenitors.

SHH induces the differentiation of Bergmann glia

In our collagen explant assay, we detected an increase in ZIC+ granule neurons spreading into the collagen matrix. Because SHH did not induce the differentiation of purified granule neuron precursors in aggregates, this increase could be due to indirect effects of SHH. We therefore also tested for the effects of SHH on other cell types present in the explants. Double labeling with the pan-neuronal marker TuJ1 and the Bergmann glia marker BLBP (Fig. 6A,B) showed that SHH induced neurite outgrowth, consistent with an enhancement of granule neuron development, but also massive migration of glia into the collagen matrix (Fig. 6B). This was ~35-fold greater on average than that observed in untreated explants (Figs 6A, 5E). Single labeling with BLBP (Fig. 6C) or GFAP (Fig. 6C lower inset) further confirmed this finding, showing that cells invading the collagen matrix in SHH-treated explants had long processes characteristic of radial glia.

To test if SHH enhanced glial proliferation, we analyzed the expression of BLBP in recently divided cells. Double-labeling experiments with BrdU and BLBP followed by quantification of labeled cells in confocal micrographs showed that only a small fraction of BLBP+ cells (5%, $n=440$ cells counted in three sections) were also BrdU+ following treatment with SHH for 24 hours (Fig. 6D and not shown) indicating that SHH does not induce glial proliferation. Spreading BrdU+ cells were often located on top of or near BLBP+ processes (arrows in Fig. 6E), suggesting that these represented granule neurons migrating over radial glia. Thus, spreading BLBP+ cells appear to be postmitotic radial glia that could have been induced to differentiate by SHH.

To test whether SHH directly induces glial differentiation, immature mouse astroglia were isolated from P1-3 mice cerebella and plated *in vitro* (Hatten, 1985). Culture of purified glia at very low density showed that after 4 days in culture these were BLBP negative (Fig. 6F), had a flat morphology and readily divided (Hatten, 1985). In contrast, treatment with SHH at day 2 for 48 hours induced the expression of BLBP in 90% of the cells (Figs 6G, 5D), showing that SHH induces their maturation/differentiation.

Blocking SHH *in vivo* perturbs cerebellar development

To test the relevance *in vivo* of the functions that our *in vitro* experiments have assigned to SHH in cerebellar development, we performed a series of injections of hybridoma cells into the brains of developing chick embryos at D4 and analyzed at D15. This age was chosen as it is late enough to avoid interference

Fig. 4. SHH is required for the proliferation of purified mouse granule neuron progenitors. (A-C) Proliferation of isolated granule neuron precursors in aggregates obtained from P1 mice cerebella (A,B; $n=30$) was enhanced after treatment with SHH for 48 hours (C; $n=30$). These aggregates were mostly free of contaminating GFAP+ glia (A; $n=10$). (D-F) Treatment of purified mouse granule cell aggregates with anti-SHH mAb decreased BrdU incorporation (E; $n=25$) as compared to untreated controls (D; $n=25$). Treatment with forskolin (F; $n=25$) decreased proliferation also as compared to untreated controls. Antibodies and colors are indicated in or next to each panel. FK, forskolin.

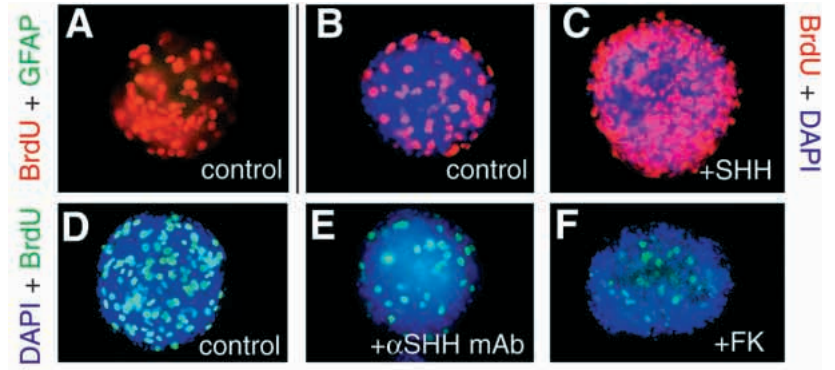
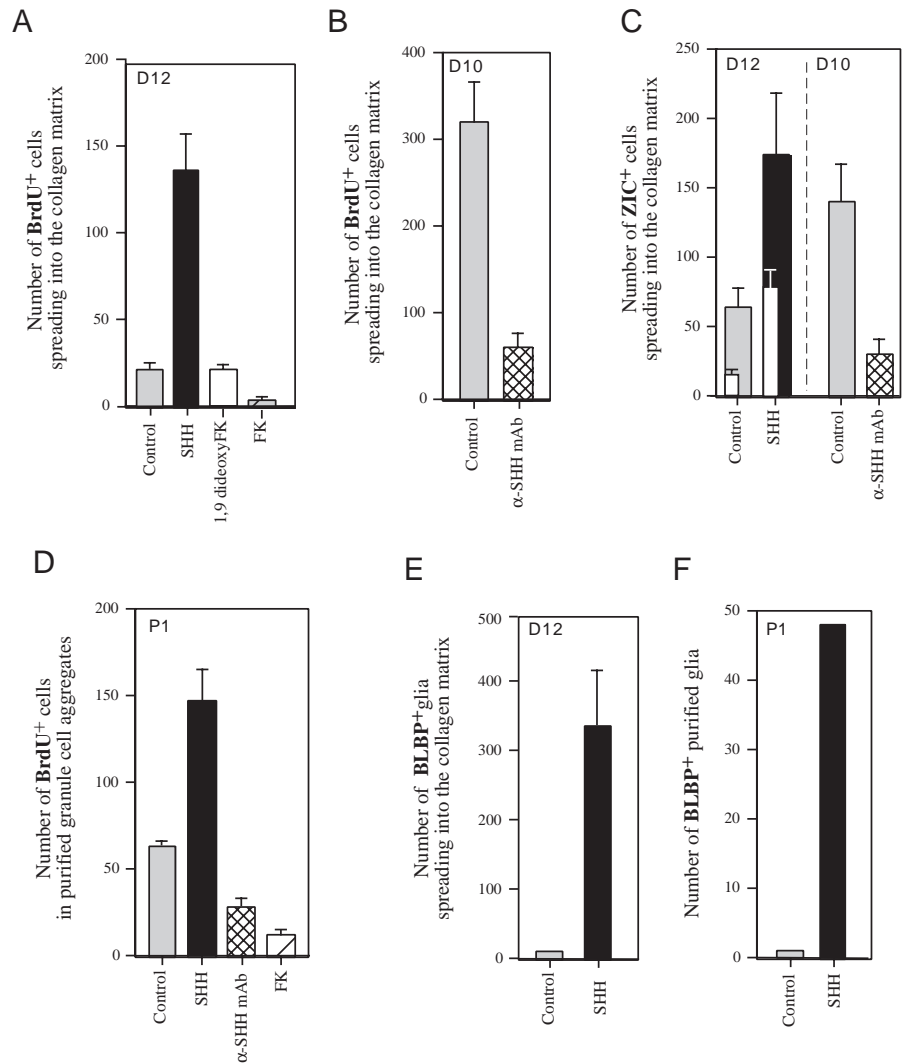


Fig. 5. Quantification of the effects of SHH on granule neuron and glial development in vitro. (A-C) Effects of SHH on chick explants. The panels show the number of BrdU (A,B) or ZIC (C)-positive cells spreading into the collagen surrounding the explants at 96 hours (A-C) and 48 hours (C). Cells in a minimum of four explants were counted in each case.

Histograms show averages \pm s.e.m. of cells per explant. Quantification of BrdU incorporation results at 48 hours (not shown) showed similar relative results as those at 96 hours. 48 hours results in C are shown as small histograms within the larger ones. The stage of explant preparation is given on the top.

(D) Quantification of the effects of SHH on the proliferation of purified mouse granule precursors. BrdU+ cells in a minimum of six aggregates were counted in each case. Histograms show averages \pm s.e.m. of cells per aggregate. Cells were isolated from P1-P3 mice. FK, forskolin. (E,F) Quantitation of the effects of SHH on glial cell spreading in chick explants at 96 hours (E) and on purified mouse glial cells (F). Cells in a minimum of six explants were counted in E and histograms show averages \pm s.e.m. of cells per explant. Quantification of results at 48 hours (not shown) showed similar relative results as those at 96 hours. (F) 50 random cells were scored for BLBP expression and the number of positive cells is depicted in the histograms. The experiment was done twice with similar results. The day of explant preparation or cell isolation is given on the top left.

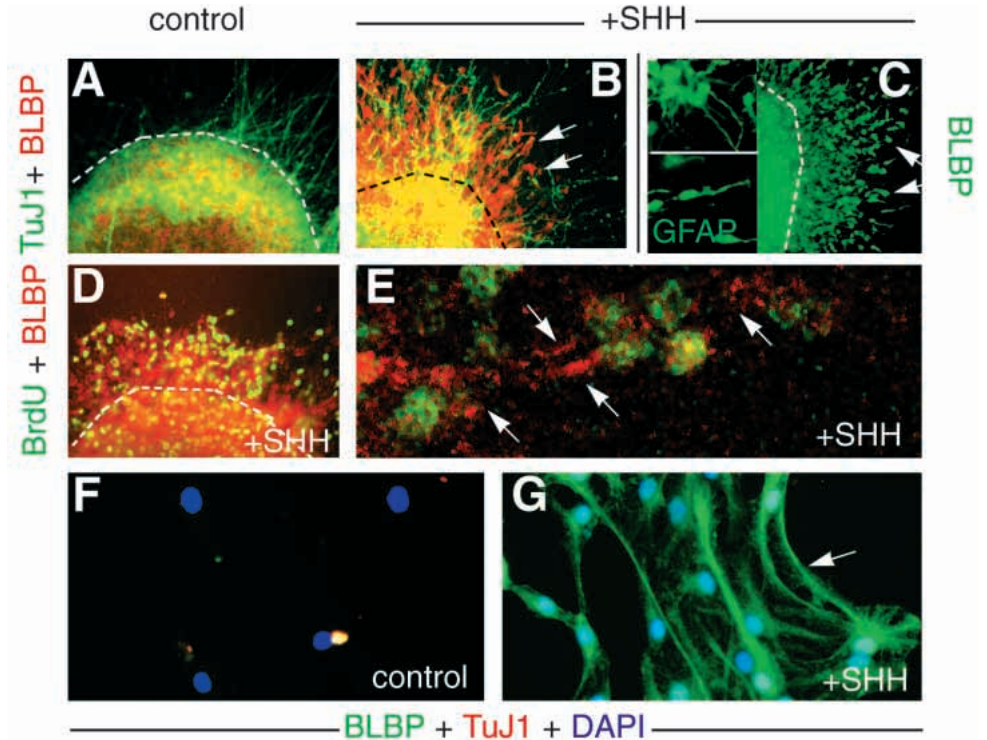
with early dorsoventral patterning of the neural tube and early enough to affect the developing cerebellum. Indeed, the injected cells are in PBS and it takes some time before they recover, proliferate and secrete enough antibody to block endogenous signaling, which could happen a day or two after injection, that is, at D5-D6 at the earliest. Injections at later ages were difficult and yielded large numbers of dead embryos. We injected hybridoma cells secreting anti-SHH blocking antibodies (5E1; Ericson et al., 1996), a control hybridoma



secreting unrelated antibodies of the same isotype or the parental myeloma line (NS1). All cells grew well in the injected embryos and could be detected in the ventricular spaces (Fig. 8H,J and not shown).

Intraventricular injection of 5E1 cells caused a marked reduction in the size of the cerebellum, in comparison to that of the tectum, medulla and cerebral vesicles (Fig. 7B), in half of the injected embryos (16/32) as compared to controls (Fig. 7A). In contrast, hybridoma injections into the circulation

Fig. 6. SHH induces glial spreading from chick explants and the maturation of purified mouse Bergmann glia. (A,B) Control D12 explants grown for 48 hours show minimal TuJ1+ neurite outgrowth and insignificant spreading of BLBP+ glia outside the explant (A; $n=19$). SHH treatment (B; $n=19$) induced neurite outgrowth and glia spreading (arrows), as compared to control explants. (C) Single labeling for BLBP shows the spreading of glia in SHH-treated D12 explants grown for 48 hours (C; $n=27$). SHH induced an increase in BLBP+ cells spreading into the surrounding collagen (arrows) from treated explants ($n=27$) as compared with untreated controls (not shown, $n=24$ explants). Simultaneous addition of SHH and mAb inhibited the action of the former ($n=11$, not shown). At 24 and 96 hours, similar relative differences were observed for untreated and SHH-treated explants ($n=7$ for each case at 24 hours; $n=14$ for each case at 96 hours; not shown). Similar results to those obtained for BLBP labeling were obtained for GFAP labeling at 24, 48 and 96 hours, $n>5$ in each case, not shown. (Insets in C) BLBP+ (top) and GFAP+ (bottom) glia show radial morphology with long endfeet. (D,E) BLBP+ spreading cells, in D12 explants grown for 24 hours before a 1 hour BrdU pulse followed by a 24 hour post-treatment culture, do not incorporate BrdU in response to SHH treatment (D). BrdU+ cells were instead often found on top or near BLBP+ radial glial processes (arrows in E). Similar results were obtained after 4 hour treatment with SHH followed by a 1 hour pulse of BrdU and 24 hour of post-treatment culture ($n=24$ for each case, not shown). (F,G) Isolated immature glia from P1 mouse cerebella do not express BLBP and have a flat morphology (F). (G) SHH treatment induces BLBP expression (arrow) in purified immature glia. The dashed lines mark the boundaries of the explants in collagen gels.



(F,G) Isolated immature glia from P1 mouse cerebella do not express BLBP and have a flat morphology (F). (G) SHH treatment induces BLBP expression (arrow) in purified immature glia. The dashed lines mark the boundaries of the explants in collagen gels.

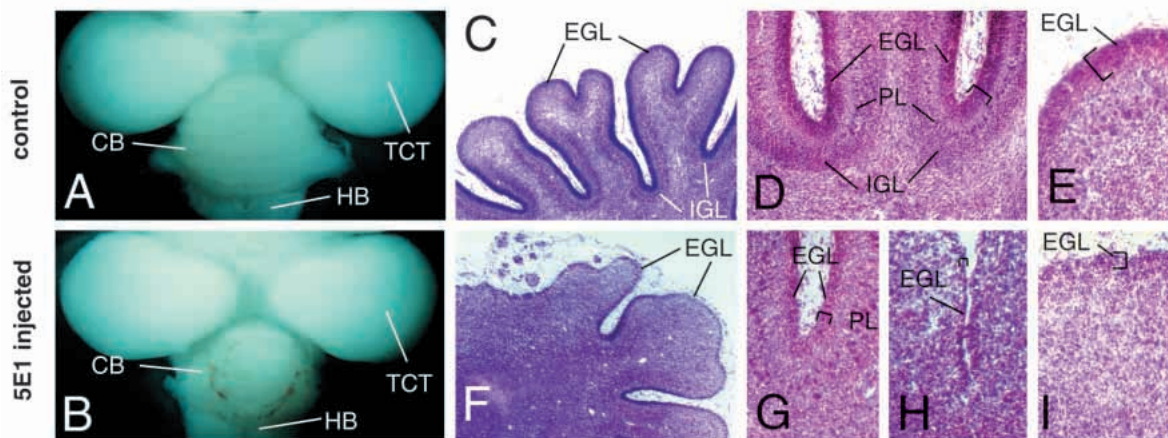


Fig. 7. Morphological defects in the cerebellum after blocking SHH function through hybridoma injections. (A,B) Dorsal view of D15 brains of a control hybridoma-injected embryo (A) or a 5E1-injected embryo (B) showing the normal size of the cerebellum (CB), tectum (TCT) and hindbrain (HB) in the control brain (A) and the reduced size of the cerebellum in the 5E1-injected brain (B). (C-E) Sagittal sections through a control-injected D15 cerebellum showing the normal histology of the cortex stained with hematoxylin and eosin, including normal foliation and laminar organization of the EGL, PL and IGL. (C) Low-power micrograph and (D,E) high-power micrographs in which the lobes and fissures are shown with a thick EGL, an organized PL and an obvious IGL. (F-I) Sagittal sections of 5E1-injected D15 cerebella similar to those shown in C-E showing abnormal foliation (F), absence of a recognizable IGL, abnormal or absent PL and a thin EGL (G-I). Brackets show the thickness of the EGL in D,E,G-I.

resulted in chick embryos that lacked feathers and had abnormal limb and/or craniofacial development (4/4 embryos, not shown). Because these phenotypes are reminiscent of those

seen in mice lacking SHH (Chiang et al., 1996; St.-Jacques et al., 1998), we interpret them as a systemic blockade of SHH function and validate our *in vivo* approach. Control hybridoma

(8/8 embryos) or NS1 (5/5 embryos) cells did not affect the development of the brain or embryo in general. In embryos that received intraventricular injections, dorsal choroid plexus and ventral structures such as the pituitary were observed (not shown), indicating that early dorsoventral patterning of the neural tube was not affected.

Given our *in vitro* results with purified cell populations, we have examined the injected brains for specific defects in cerebellar development. Within the cerebellum, the organization of the cortex was highly abnormal in 5E1-injected embryos. Examination of hematoxylin- and eosin-stained sections showed that foliation patterns were aberrant (Fig. 7F and not shown), as compared to controls (Fig. 7C). No evidence of massive cell death was found as judged by the absence of large numbers of pyknotic nuclei in hematoxylin- or DAPI-labeled sections (not shown). The degree of abnormal development varied, possibly depending on the number of hybridoma cells proliferating *in vivo* and their location within the brain. Histologically, the thickness of the cortex was drastically reduced in 5E1-injected (Fig. 7G-I) versus control-injected (Fig. 7D,E) cerebella: the EGL was uneven and thinner than normal (4 ± 0.79 cells thick for 5E1-injected brains versus 8.75 ± 1.28 cells thick for control brains), the PL was disorganized, and in some cases unrecognizable, and the IGL was undetectable in most cases.

To define the effects of blocking SHH *in vivo* on different cerebellar cell types, we tested for the presence of Purkinje neurons, Bergmann glia and granule neurons in 5E1- versus control-injected brains. Control brains showed the highly organized arrangement of Purkinje neurons expressing Calbindin (Fig. 8A) and SHH (Fig. 8D) forming the PL (9/9 examined embryos). 5E1-injected brains showed abnormal positioning of Calbindin⁺ Purkinje neurons, often found in clusters or in deep cerebellar regions (Fig. 8B,C; 11/13 examined embryos). Expression of SHH in 5E1-injected embryos was reduced or not detectable (Fig. 8H; 5/5 examined embryos). The morphology of Purkinje neurons as revealed by Calbindin labeling was also abnormal in 5E1-injected versus control brains (insets in Fig. 8A,B). Analyses of glial differentiation using BLBP (Fig. 8E) and GFAP (not shown) demonstrated that Bergmann radial glia were unaffected in control embryos (Fig. 8E; 4/4 examined embryos) but were reduced or absent in 5E1-injected affected brains (Fig. 8I; 5/5 examined embryos). In cases where only part of the cerebellum was affected, normal BLBP expression was detected in unaffected regions (not shown). Labeling Bergmann glia with anti-erbB4 antibodies (Rio et al., 1997) also showed a reduction in the number of glia in 5E1- (4/4 examined embryos) but not in control-injected (3/3 examined embryos) cerebella (not shown).

Analyses of granule neuron development showed that ZIC and TAG immunoreactivity were normal in control-injected embryos (Fig. 8F; 3/3 examined embryos) but were severely reduced or absent in 5E1-injected brains (Fig. 8J; 4/4 examined embryos), even in regions where Purkinje neurons formed a near-normal layer (not shown). Similarly, the expression of the IGL marker HNF-3 β and that of Nkx2.2 was normal in control embryos (Fig. 8G and not shown; 3/3 examined embryos for each marker) but strongly reduced or absent in 5E1-injected brains (Fig. 8K and not shown; 4/4 examined embryos for HNF-3 β and 3/3 embryos examined for Nkx2.2). Together,

these results show that inhibiting SHH signaling *in vivo* leads to abnormal or aborted development of Purkinje and granule neurons and Bergmann glia, results consistent with our *in vitro* studies.

DISCUSSION

Here we present evidence for a role of SHH in the development of the cerebellum, a dorsal CNS structure involved in motor and balance control, and provide a cellular basis for MBs. Our results show that, in the cerebellum, SHH is produced by Purkinje neurons (and transiently by mouse EGL cells), is a required mitogen for granule neurons and induces the differentiation of Bergmann glia. How these cells respond differently to SHH remains unclear although it could synergize with additional factors, such as FGFs (Ye et al., 1998), IGFs (e.g. Gao et al., 1991; D'Ercole et al., 1996), or Neuregulins (Rio et al., 1997) to elicit distinct responses. The proposed ability of SHH from Purkinje neurons to induce proliferation of granule cells illustrates how postmitotic neurons affect the development of neighboring precursors. Interestingly, Purkinje neurons regulate the development of granule neurons, which in turn modulate Purkinje activity. Postmitotic neurons have also been shown to induce the proliferation of astrocyte precursors (Fruttiger et al., 1996), and early-born postmitotic spinal motor neurons induce a fate change in later-born motor neurons (Sockanathan and Jessell, 1998).

Independent and simultaneous studies with complementary methods provide further support for a role of SHH as a mitogen for granule cell precursors, and suggest an antagonism between SHH and FGF (Wechsler-Reya and Scott, 1999). The differences in the level of proliferation of granule cell precursors driven by exogenous SHH could be due to the different activities of SHH batches, to the different age of precursors used, P1-3 versus P8, or the different methods of study. They plated purified cells and used brain slices whereas we allowed purified cells to form suspended aggregates and used cerebellar explants. Although we see similar effects on EGL development after hybridoma injections, we allowed for an interference with the formation of the Purkinje layer because we injected 100- to 1000-fold less cells at earlier times than in their study. In addition, we describe effects of SHH on glial differentiation *in vivo* and *in vitro*.

Our findings show that SHH regulates cerebellar development by acting directly on neighboring populations of neurons and glia. These results together with those of previous cellular studies showing that Purkinje cells regulate granule neuron proliferation and glial differentiation (e.g. Hatten and Heintz, 1995; Smeyne et al., 1995; Herrup and Kuemerle, 1997), suggest a molecular model for the coordinate regulation of cortical cerebellar development by SHH (Fig. 9). Committed EGL granule neuronal precursors require SHH signaling for proliferation, which at least in mice could be driven first by transient autocrine SHH signaling (Fig. 9A) and then by SHH from Purkinje neurons (Fig. 9D). This suggests a molecular basis for the known central role Purkinje neurons play in the development of granule neurons. SHH protein could be released from Purkinje cell bodies or dendrites, the latter representing a possible parallel with the inducing action of Hedgehog from fly retinal axons (Huang and Kunes, 1996).

Fig. 8. Blocking SHH signaling in vivo affects Purkinje and granule neurons and Bergmann glia.

(A-C) Sagittal sections of control hybridoma-injected (A) or 5E1-injected (B,C) cerebella showing the normal alignment of Purkinje neurons (PN) forming the Purkinje layer (PL) in the control cerebellum (A) and the abnormal positioning of Purkinje neurons in 5E1-injected cerebella (B,C). Counting the number of Calbindin+ Purkinje neurons was not possible due to their aggregation in clusters. (D,H) Expression of SHH in Purkinje neurons seen in myeloma-injected (D) or control hybridoma (not shown) cerebella is not detectable in 5E1-injected cerebella (H). (E,I) Expression of BLBP is similarly reduced in 5E1-injected cerebella (I) as compared with control hybridoma-injected cerebella (E). (F-K) Expression of the granule cell markers ZIC, TAG and HNF-3 β observed in control hybridoma-injected brains (F,G) was drastically diminished or absent in 5E1-injected brains (J,K). Note the presence of hybridoma cells growing in the cerebellum (arrows in H,J).

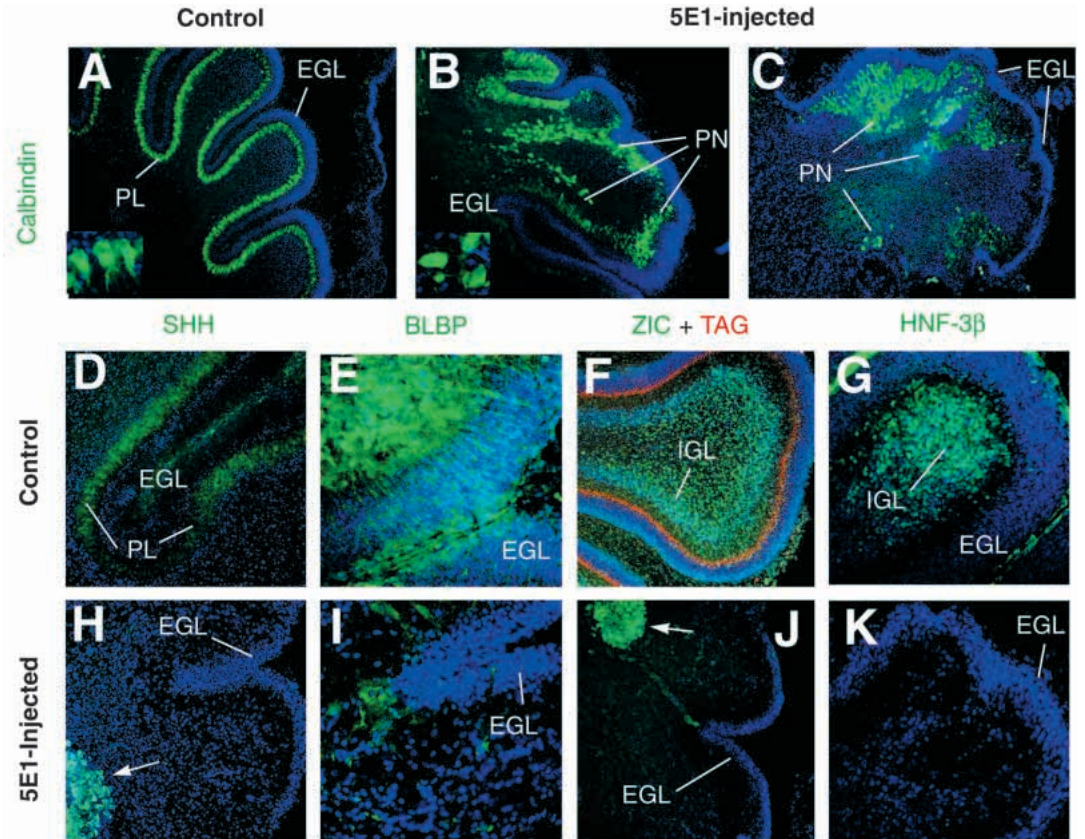
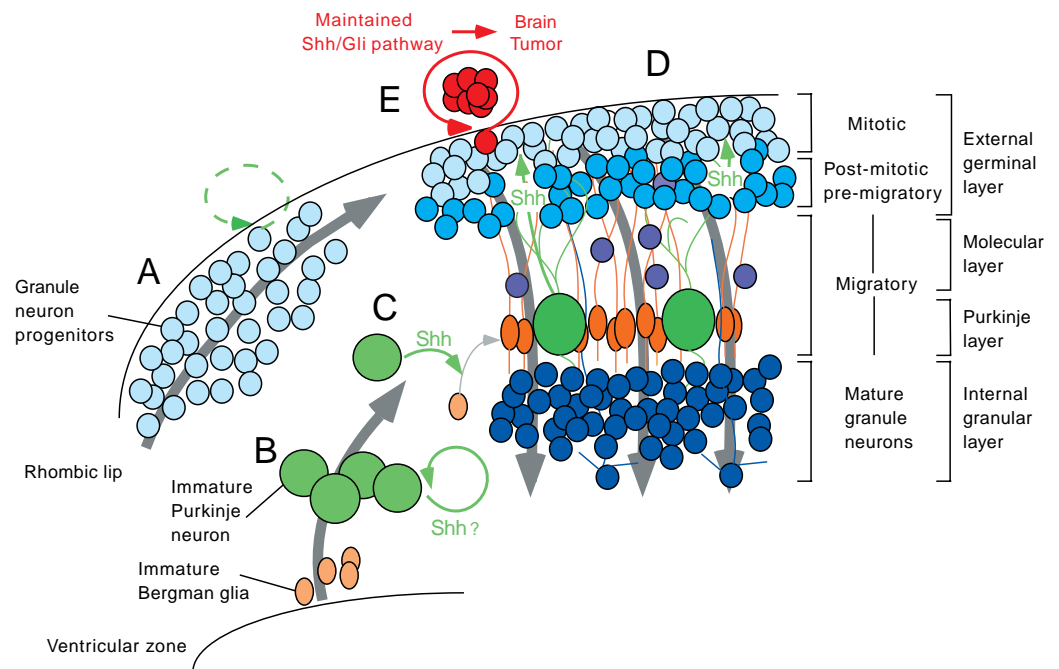


Fig. 9. Diagram of the role of SHH in cerebellar development.

(A-D) Granule neuronal precursors migrate tangentially from the rhombic lip and may use the Shh pathway in a transient autocrine manner in mice (dashed green arrow). (B) Purkinje neurons and later-born Bergmann glia derive from the ventricular zone and migrate towards the EGL. Purkinje neurons may use the Shh pathway in an autocrine manner. (C) SHH from Purkinje neurons induces Bergmann glia maturation. (D) In the later EGL, granule neuronal precursors proliferate in the outer zone utilizing SHH secreted from Purkinje neurons after expression of *Shh* in the EGL ceases. At the same time, mature glia send their extensions towards the inner EGL and these or other cortical cells may provide factors that promote the differentiation of granule neurons, antagonizing the effects of SHH. Granule cells then migrate on glial fibers across the molecular and Purkinje layers to form the IGL. (E) Maintained autocrine SHH signaling in EGL cells or failure to induce their differentiation may result in the development of cerebellar tumors.



We propose that the varying foliation patterns seen in different species derive from regionally and temporally regulated Shh signaling within the cerebellar cortex, inducing differential proliferation profiles in different areas and thus the appearance of distinct lobes. The earliest proliferation of granule neuron precursors soon after they leave the rhombic lip, however, may depend on dorsal factors. SHH is not expressed in the rhombic lip and its expression there would be predicted to prevent cerebellar development by ventralizing the dorsal neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ruiz i Altaba et al., 1995). Involvement of SHH in cerebellar granule cell development raises the question of whether it also regulates the development of granule neuron progenitors in the olfactory bulb and hippocampus, cells that share molecular and cellular properties with their cerebellar counterparts (e.g. Yang et al., 1996).

The importance of the Shh signaling pathway in cerebellar development is illustrated by the hypoplastic and disorganized cerebella of anti-SHH hybridoma-injected brains. In addition, defects in different SHH targets, such as HNF-3 β (Lee et al., 1997; Sasaki et al., 1997), is likely to result in more discrete abnormalities. In this context, we note that heterozygous HNF-3 β +/- knock-out mice are viable but show balance and motor defects (Weinstein et al., 1994; Ang and Rossant, 1994; and not shown), problems characteristic of deficient cerebellar function. Our findings that HNF-3 β is expressed by granule neurons and that it requires SHH for its expression, thus provide a possible cellular basis for the abnormal behavior of HNF-3 β +/- animals, further highlighting the critical role of SHH in this tissue.

SHH from Purkinje neurons also appears to direct the differentiation of Bergmann glia (Fig. 9B,C), consistent with the requirement of Purkinje neurons for Bergmann glial differentiation (Fischer et al., 1993; Sotelo et al., 1994). SHH could thus drive a regulatory circuit in which it induces both proliferation of the oEGL directly and the differentiation of iEGL cells into granule neurons indirectly through the intermediate action of factors from neighboring cells, possibly Bergmann glia induced to differentiate by SHH (Fig. 9D). Consistent with this, glia inhibit proliferation of co-cultured granule neuron precursors (Gao et al., 1991). Interestingly, Nkx2.2 expression domains appear to overlap those of the oligodendrocyte lineage marker *PDGFR α* both in the ventral spinal cord and in the cerebellum (Sun et al., 1998; Fruttiger et al., 1999). The reduction in the number or absence of Nkx2.2⁺ cells in anti-SHH hybridoma-injected brains could therefore indicate a more general involvement of SHH signaling in glial development than previously thought. Moreover, our results raise the possibility that there is a conserved patterning strategy driven by SHH, albeit with different topologies, and involving *HNF-3 β* , *Nkx2.2* and possibly *Pax* genes in the ventral spinal cord (e.g. Ericson et al., 1996) and cerebellum.

Purkinje neurons also appear to require intact SHH signaling for their proper development. This could be direct, acting in an autocrine fashion (Fig. 9B). Alternatively, SHH could act indirectly by inducing other cells to produce factors required by Purkinje neurons. Indeed, defects in Purkinje neuron migration and/or development are found in mice in which the EGL is defective (e.g. Ben-Arie et al., 1997). The abnormal arrangement of Purkinje neurons in brains expressing blocking

anti-SHH antibody is similar to the phenotype observed with an antibody blocking the function of Reelin (Miyata et al., 1997) as well as that of mice with abnormal Reelin signaling (Yuasa et al., 1993; Goldowitz et al., 1997; Yoneshima et al., 1997; Howell et al., 1997; reviewed in Curran and D'Arcangelo, 1998). One possibility is that SHH signaling from Purkinje neurons to EGL cells and Bergmann glia could be involved in the regulation of the expression of secreted factors in granule neurons, which in turn act to organize the positioning of Purkinje neurons.

Involvement of SHH in cerebellar development and disease is also suggested by the finding that distal inhibitors of cholesterol biosynthesis abolish Shh signaling (Incardona et al., 1998; Cooper et al., 1998), as SHH needs to be modified by cholesterol (Porter et al., 1996), and inhibit cerebellar development in rodents (Repetto et al., 1990; Dehart et al., 1997; Lanuoue et al., 1997). In humans, the Smith-Lemli-Opitz syndrome (SLOS; Gorlin et al., 1990; Kelly et al., 1996) arises from the failure to synthesize cholesterol (Salen et al., 1996; Farese and Herz, 1998), is partly similar to loss of SHH function (Belloni et al., 1996; Roessler et al., 1996; Chiang et al., 1996; Cooper et al., 1998) and SLOS patients display a hypoplastic cerebellum (e.g. Ness et al., 1997).

In contrast to the underdeveloped cerebellum of SHH-deficient individuals, cerebellar hyperproliferation appears to be driven by increased or maintained SHH signaling (Goodrich et al., 1997). Indeed, previous studies have suggested that the origin of MBs is the cerebellar EGL (e.g. Kozmik et al., 1995; Yokota et al., 1996). This and our results suggest that the inability to downregulate Shh signaling in EGL cells underlies the development of MBs. In addition, MBs could also arise from the absence of cortical factors that entice granule neurons to differentiate and antagonize the mitogenic effects of SHH. Anti-MB agents are thus likely to include factors that inhibit the function of the Shh signaling pathway, including that of Gli proteins, as well as factors that induce the differentiation of MB cells into neurons, which could die in the absence of appropriate trophic support.

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