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Control of Neuronal Precursor Proliferation in the Cerebellum by Sonic Hedgehog

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Summary

Cerebellar granule cells are the most abundant type of neuron in the brain, but the molecular mechanisms that control their generation are incompletely understood. We show that Sonic hedgehog (Shh), which is made by Purkinje cells, regulates the division of granule cell precursors (GCPs). Treatment of GCPs with Shh prevents differentiation and induces a potent, long-lasting proliferative response. This response can be inhibited by basic fibroblast growth factor or by activation of protein kinase A. Blocking Shh function in vivo dramatically reduces GCP proliferation. These findings provide insight into the mechanisms of normal growth and tumorigenesis in the cerebellum.

Introduction

The control of motor coordination by the cerebellum depends on precise numerical matching and synapse formation among at least five types of neurons (Altman and Bayer, 1997). By far the most abundant of these are the granule cells, glutamate-secreting neurons that regulate the output of the cerebellum by controlling the activity of Purkinje cells. The murine cerebellum contains about 10^8 granule cells, more than the number of neurons in the rest of the brain combined. The critical importance of these cells for normal cerebellar function is evident from mutant mice, in which loss of granule cells leads to severe ataxia (Kofuji et al., 1996; Hamre and Goldowitz, 1997; Mullen et al., 1997).

The process of granule cell differentiation has been studied in detail (Miale and Sidman, 1961; Fujita et al., 1966; Hatten and Heintz, 1995; Altman and Bayer, 1997). In contrast to most other neurons, which are born in the ventricular zone, granule cells are generated on the outside of the cerebellum, in a region known as the external germinal layer (EGL). For the first 2 to 3 weeks after birth, cells in the EGL undergo extensive proliferation to generate a large pool of granule cell precursors (GCPs). Developing GCPs then exit the cell cycle, extend axons (which will eventually synapse with Purkinje cell dendrites in the molecular layer), and migrate inward past the Purkinje cell bodies to their final destination, the internal granule layer (IGL).

While the steps in granule cell differentiation have been well characterized, the mechanisms that control the proliferation of GCPs remain a mystery. Cell ablation experiments (Smeyne et al., 1995) and studies of mutant mice (Sonmez and Herrup, 1984; Vogel et al., 1989; Mullen et al., 1997) indicate that granule cell growth and survival depend on Purkinje cells. In vitro studies suggest that granule cell survival and differentiation may be regulated by astrocytes (Bergmann glia), with which they come into contact during their inward migration (Hatten et al., 1988). Finally, the spontaneous proliferation of granule cells grown at high density in vitro has been seen as evidence that these cells regulate their own growth in an autocrine or paracrine manner (Gao et al., 1991). Thus, multiple cell–cell interactions may regulate the generation of granule cells, although the molecules that mediate these interactions are not known.

Sonic hedgehog (Shh) is a secreted factor that plays a role in patterning of many tissues, including the nervous system (Hammerschmidt et al., 1997). For example, during early neural development, Shh is secreted by the notochord and can induce the overlying cells in the neural tube to differentiate into floor plate (Echelard et al., 1993; Roelink et al., 1994). Floor plate cells, in turn, secrete Shh that induces ventrolateral cells in the spinal cord to become motor neurons at high concentrations and interneurons at lower concentrations (Roelink et al., 1995; Ericson et al., 1997). Shh also regulates the development of neurons in more rostral regions of the CNS, including the forebrain, midbrain, and retina (Ericson et al., 1995; Hynes et al., 1995; Wang et al., 1995; Dale et al., 1997; Jensen and Wallace, 1997; Ye et al., 1998).

A role for Shh in regulating cerebellar growth and development has been suggested by studies of *patched (ptc)*, a gene that encodes a Shh-binding protein that functions as an antagonist of Shh signaling (Ingham et al., 1991; Goodrich et al., 1996; Johnson and Scott, 1997). In humans, PTC mutations have been implicated in the etiology of sporadic medulloblastomas (cerebellar tumors) (Pietsch et al., 1997; Raffel et al., 1997; Vorechovsky et al., 1997) and have been shown to cause Basal Cell Nevus Syndrome (BCNS), which is associated with an increased frequency of medulloblastoma (Chidambaram et al., 1996; Hahn et al., 1996; Johnson et al., 1996). In addition, mice heterozygous for mutations in *ptc* develop medulloblastoma (Goodrich et al., 1997). Both human and murine tumors often consist of small, round cells on the outside of the cerebellum, features reminiscent of GCPs in the EGL.

Since mutations in *ptc* lead to abnormal growth of GCP-like cells, we hypothesized that Shh/Ptc signaling might regulate the production of GCPs during normal cerebellar development. To investigate this, we tested the effects of Shh on growth of cerebellar cells in vitro and examined the importance of Shh for GCP development in vivo.

Results

Expression of *shh*, *ptc*, and *gli* in the Developing Cerebellum

To determine whether Shh could play a role in regulating granule cell development, the expression of components of the Shh signaling pathway in the postnatal

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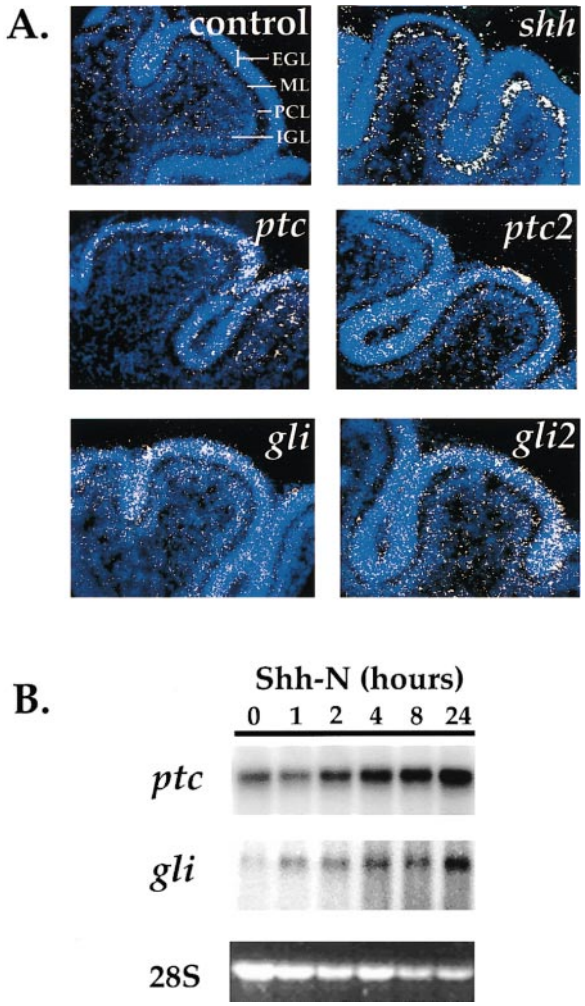

Figure 1. Shh Pathway Genes Are Expressed in the Developing Cerebellum

(A) In situ hybridization. Paraffin sections of cerebella from 4-day-old (P4) mice were hybridized with ^{35}S-labeled antisense riboprobes specific for *shh*, *ptc*, *ptc2*, *gli1*, or *gli2* or with a sense probe transcribed from the *ptc* vector (control). Slides were coated with photographic emulsion and incubated for 8 days before developing and counterstaining with DAPI to label nuclei. DAPI fluorescence (blue) and autoradiographic signal (white grains) were captured using a digital camera. The location of the external germinal layer (EGL), molecular layer (ML), Purkinje cell layer (PCL), and internal granule layer (IGL) are indicated on the first panel.

(B) Shh induction of *ptc* and *gli* expression. Cerebellar cells from P8 mice were cultured in serum-free medium containing 3 μg/ml Shh-N for the indicated amount of time. Total cytoplasmic RNA from each time point was purified, separated by electrophoresis, and transferred onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled cDNA probes specific for *ptc* or *gli1* and then exposed to a phosphorimager screen. RNA loading was determined based on ethidium bromide staining of 28S ribosomal RNA, as shown in the bottom panel.

cerebellum was analyzed by in situ hybridization. *shh* was expressed in the Purkinje cell layer (PCL) and the molecular layer (ML), which consists primarily of Purkinje cell dendrites (Figure 1A). In contrast, mRNAs encoding the *shh*-related genes *indian hedgehog* and *desert hedgehog* (Bitgood and McMahon, 1995) were not detected in the cerebellum at this stage. Transcripts for *ptc* and the related gene *ptc2* (Motoyama et al., 1998) were abundant in granule cell precursors in the EGL. Finally, mRNA for *gli1* and *gli2*, transcription factors involved in Shh signaling (Platt et al., 1997; Ding et al., 1998), was found in the EGL and in cells that had begun to migrate into the IGL. This expression pattern was confirmed by Northern analysis of cerebellar cells fractionated by Percoll gradient centrifugation and adherence to plastic (Hatten et al., 1988; Gao et al., 1991). This analysis revealed that small, nonadherent cells (granule cells and their precursors) contained high levels of *ptc* mRNA, while large, nonadherent cells (Purkinje cells and other neurons) contained low, but detectable levels of *shh* (data not shown). Together, these observations suggested that Shh might mediate an interaction between Purkinje cells and GCPs in the developing cerebellum.

To determine whether GCPs could respond to Shh stimulation, we tested the effects of Shh on transcription of *ptc* and *gli1*, which are induced by Shh in many tissues (Goodrich et al., 1996; Marigo et al., 1996; Platt et al., 1997). Cerebellar cells isolated from mice at postnatal day 8 (P8) were cultured in serum-free medium containing the biologically active N-terminal fragment of Shh (Shh-N) (Roelink et al., 1995). RNA was isolated from cells at various time points and analyzed by Northern blotting. Shh-N caused significant increases in transcription of *ptc* within 2–4 hr and of *gli1* within 1 hr (Figure 1B). The levels of these mRNAs continued to rise even after 24 hr in culture. These results indicated that the Shh signaling pathway was functional in cerebellar cells in culture.

Shh-Induced Proliferation of Cerebellar Cells in Culture

To test whether Shh regulates cerebellar cell growth, we cultured cells for 48 hr in the presence or absence of Shh-N and then pulsed them with bromodeoxyuridine (BrdU) to label proliferating cells. Cells were then stained with anti-BrdU antibodies and examined by fluorescence microscopy (Figure 2A). In control cultures, only a small percentage of cells (3%–4%) incorporated BrdU. In contrast, cultures treated with Shh-N for 48 hr showed a 10-fold increase in the number of BrdU-positive cells (to 30%–35%). Analysis of these cultures using the LIVE/DEAD assay (see Experimental Procedures) revealed no significant differences in viability between control cells and cells treated with Shh-N (data not shown), suggesting that Shh was not merely promoting cell survival. We concluded that Shh can induce proliferation of cerebellar cells in vitro.

Since the effects of Shh on cell fate have been observed to vary with concentration (Roelink et al., 1995; Ericson et al., 1997), we examined the dose of Shh-N necessary to induce proliferation of cerebellar cells. Cerebellar cells were cultured for 48 hr in medium containing various concentrations of Shh-N, pulsed with tritiated thymidine (^{3}H-Td), and harvested for quantitation of incorporated thymidine. Substantial increases in thymidine incorporation were observed (Figure 2B) when cerebellar cells were treated with concentrations of Shh-N as low as 0.1 μg/ml (6 nM). Increasing amounts

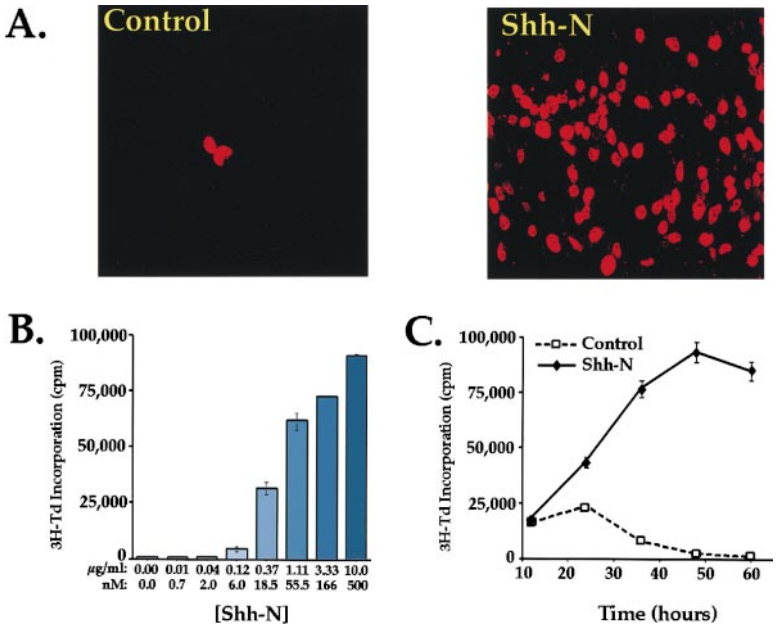

Figure 2. Shh Induces Proliferation of Granule Cell Precursors

(A) Shh induction of BrdU incorporation. Cells isolated from P8 cerebella were cultured on poly-D-lysine (PDL)-coated glass coverslips for 48 hr in the absence (control) or presence of 3 μg/ml Shh-N. Cells were pulsed with bromodeoxyuridine (BrdU) for an additional 12 hr, then fixed, treated with 2N HCl to unmask BrdU, and stained with biotinylated sheep anti-BrdU antibodies followed by rhodamine-streptavidin.

(B) Effects of concentration on Shh-induced proliferation. P8 cerebellar cells were cultured in PDL-coated 96-well plates containing the indicated concentrations of Shh-N. After 48 hr, cells were pulsed with ^{3}H-Td for 12 hr and then harvested for quantitation of incorporated radioactivity. Data points represent means of triplicate cultures ± SEM.

(C) Kinetics of proliferative response. P8 cerebellar cells were cultured for the indicated amount of time in medium containing no stimulus or 3 μg/ml Shh-N. Cells were pulsed with ^{3}H-Td for the last 12 hr of culture and then harvested and analyzed as described above.

of incorporation were seen with increasing doses of Shh. Since maximal responses were usually seen at 3 μg/ml (150 nM), this concentration was used for our subsequent experiments.

To determine the kinetics of the response to Shh, cerebellar cells were cultured in the presence or absence of Shh-N for 12–60 hr, pulsed with ^{3}H-Td, and assayed for ^{3}H-Td incorporation. In keeping with previous reports (Gao et al., 1991), we found that cells incubated at high density proliferate constitutively during the first 12–24 hr of culture (Figure 2C). During this period, exogenous Shh-N had little effect. When cells were cultured for longer periods of time (36–60 hr) in the absence of Shh, they exhibited a sharp decline in proliferation. In contrast, cells treated with Shh for 36–60 hr dramatically increased ^{3}H-Td incorporation, to more than 100-fold over background. The response to Shh-N peaked after 3 to 4 days, but cells grown in Shh-containing media for 14 days continued to show substantial thymidine incorporation at the end of the culture period (28,000 cpm in Shh-treated cultures versus 160 cpm in control cultures = 175-fold). Together, these studies indicated that Shh-N is a potent mitogen for cerebellar cells and can promote proliferation of some cells for long periods in culture.

Identification of Shh-Responsive Cells

During the early postnatal period in mice, GCPs are the major population of proliferating cells in the cerebellum. However, astrocytes and oligodendrocyte precursors also undergo division during this period (Hatten, 1985; Levi et al., 1987; Altman and Bayer, 1997). To identify the Shh-responsive cells in our cultures, we labeled Shh-treated cells with BrdU and then stained them with antibodies against markers of specific cell types. As shown in Figure 3A, BrdU-positive cells were negative for glial fibrillary acidic protein (GFAP) and O4, markers for astrocytes and oligodendrocyte precursors, respectively (Bignami et al., 1972; Sommer and Schachner, 1981), but positive for HNK-1, a cell-surface antigen present on GCPs in the proliferative zone of the EGL (Wernecke et al., 1985). This suggested that the Shh-responsive cells were GCPs. In support of this conclusion, we have observed that cerebellar cells isolated from 1- to 10-day-old mice proliferate significantly in response to Shh-N, while cells from older animals (in which the majority of GCPs have differentiated and the EGL has begun to disappear) are relatively unresponsive (data not shown).

To confirm that Shh could induce proliferation of GCPs, we tested the effects of Shh-N on cerebellar cell subpopulations purified by Percoll gradient centrifugation and adherence to plastic (Hatten et al., 1988; Gao et al., 1991). These experiments indicated that small, nonadherent cells (GCPs) exhibit a substantial proliferative response to Shh (94-fold stimulation), while large, adherent cells (glia) had little response (1.8-fold) (Figure 3B). We concluded that GCPs are the primary Shh-responsive cell population in the cultures and that the effect of Shh on GCPs is direct and does not require other cell types.

Although Shh-N could promote proliferation of GCPs in dissociated cell cultures, it was important to determine whether it could do so in the context of an intact cerebellar microenvironment. We therefore tested the effects of Shh-N on cerebellar slice cultures (Stoppini et al., 1991). Slices of P8 cerebellum were cultured for 48 hr in the presence or absence of Shh-N, labeled with BrdU, and then stained and examined by confocal microscopy. Control slices contained few BrdU-positive cells, while Shh-treated slices had a large number of labeled cells (Figure 4). Moreover, in control slices, BrdU-positive cells were found in the ML and IGL, suggesting that some granule cells in these cultures had exited the cell cycle and migrated inward during the labeling period. In contrast, the majority of BrdU-positive cells in Shh-treated slices remained in the outer EGL, where proliferating, premigratory GCPs normally

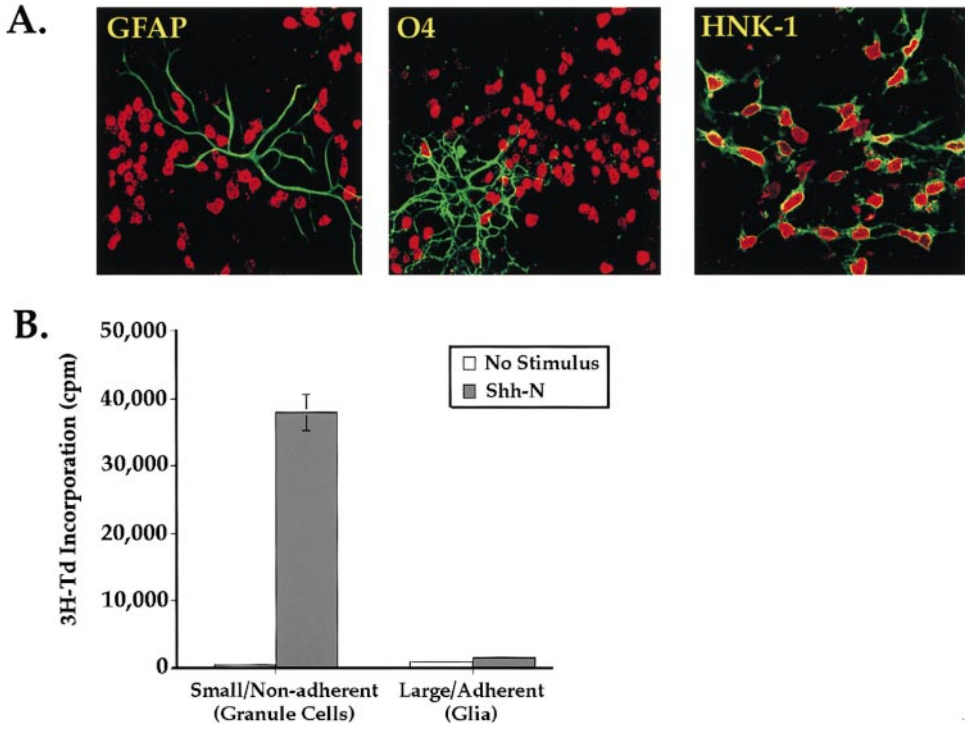

Figure 3. Shh-Responsive Cells Are Granule Cell Precursors

(A) Identification of proliferating cells. Cerebellar cells were stimulated with Shh-N for 48 hr, pulsed with BrdU for 12 hr, and then fixed and stained with antibodies specific for astrocytes (GFAP), oligodendrocytes (O4), or GCPs (HNK-1), followed by FITC-conjugated donkey-anti-mouse antibodies. Cells were then refixed, acid-treated, and stained with biotinylated sheep anti-BrdU antibodies and rhodamine-streptavidin to label proliferating cells. Staining of cell-specific markers (green) and BrdU (red) staining was visualized as described above.

(B) Proliferation of purified GCPs. Cells from P8 cerebella were centrifuged through a 35%-65% Percoll gradient to separate small and large cells and then plated on PDL-coated plastic to separate adherent and nonadherent cells. Small/nonadherent cells (granule cells) and large/adherent cells (glia) were cultured for 48 hr in medium containing no stimulus or Shh-N. Cells were then pulsed with ^{3}H-Td for 12 hr and harvested for quantitation of incorporated radioactivity.

reside. These studies suggested that in addition to promoting proliferation of GCPs, Shh-N could also inhibit migration of these cells toward the IGL.

Migration of granule cells is linked to withdrawal from the cell cycle and differentiation (Miale and Sidman, 1961; Fujita et al., 1966; Altman and Bayer, 1997). To determine the effects of Shh on granule cell differentiation, we treated cerebellar cells with Shh-N and stained them with antibodies specific for markers of neuronal differentiation. Immediately after dissociation, 80%–90% of the granule cells isolated from P8 cerebellum are undifferentiated (HNK-1-positive) GCPs (data not shown). Previous studies have shown that when GCPs are cultured for several days in serum-free medium, they undergo spontaneous differentiation (Lin and Bulleit, 1996). We too found that cerebellar cells cultured in the absence of Shh-N expressed high levels of NeuN, a nuclear antigen found in postmitotic granule cells (Mullen et al., 1992), and low levels of nonneuronal enolase (NNE), a marker of undifferentiated granule cells (Schmechel et al., 1980) (Figure 5). In contrast, the majority of cells treated with Shh-N were NeuN-negative and NNE-positive. Shh-responsive cells also maintained expression of HNK-1 (Figure 3A) and MATH-1 (data not shown), markers found exclusively in undifferentiated cells in the outermost EGL (Wernecke et al., 1985; Helms and Johnson, 1998). We therefore concluded that Shh-N promotes proliferation of GCPs and maintains them in an undifferentiated state.

Mitogenic Effects of Shh Compared to Other Growth Factors

A number of substances, including insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) have been reported to stimulate proliferation of GCPs (Gao et al., 1991; Tao et al., 1996; Ye et al., 1996; Lin and Bulleit, 1997). We compared the activity of Shh-N and these other factors using the thymidine incorporation assay. Optimal concentrations of IGF-1, EGF, and bFGF induced 2- to 6-fold increases in ^{3}H-Td incorporation over background, while Shh-N promoted more than 100-fold increases (Figure 6A). The low-level proliferation seen with EGF, IGF-1, and bFGF was not due to use of inadequate concentrations of these growth factors, since no increased proliferation was seen with doses ranging from 0.1 to 100 nM (data not shown). These data suggest that Shh-N is much more potent than previously described GCP mitogens.

Since IGF-1, EGF, and bFGF caused modest increases

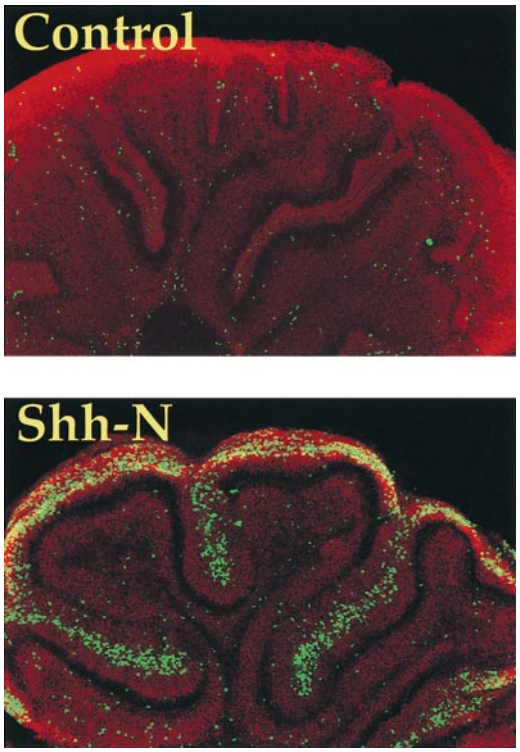

Figure 4. Shh Induces Proliferation of GCPs in Cerebellar Slice Cultures

Cerebella from P8 mice were cut into 300 μm slices using a vibratome. Slices were cultured on culture inserts in medium with no stimulus (control) or 3 μg/ml Shh-N. After 48 hr, slices were pulsed with BrdU for 16 hr, and then fixed, acid-treated, and stained with biotinylated sheep-anti-BrdU followed by FITC-streptavidin (green) and 7AAD (red) to label nuclei. Staining was visualized using confocal microscopy and digital photography.

in thymidine incorporation, we thought they might be able to synergize with Shh-N to enhance proliferation of GCPs. However, addition of any of these factors together with Shh-N did not cause an increased proliferative response (Figure 6B). Instead, bFGF suppressed the response to Shh-N by 90%, reducing proliferation to levels similar to those induced by bFGF alone. This reduced proliferation was accompanied by increased expression of the differentiation antigen NeuN (data not shown), consistent with previous reports that bFGF can promote granule cell differentiation (Hatten et al., 1988; Liu and Kaczmarek, 1998). Modest inhibition of the Shh-N response (10%–20%) was also observed with EGF and IGF-1. In all cases, cell viability was unaffected.

In some cell types, effects of hedgehog proteins are potently antagonized by activation of protein kinase A (PKA) (Fan et al., 1995; Epstein et al., 1996; Hammerschmidt et al., 1996). We treated cerebellar cells with forskolin, a reagent known to increase intracellular cAMP and thereby activate PKA. Forskolin on its own had no effect on proliferation of cerebellar cells (Figure 6A), but, like bFGF, it was a potent suppressor of Shh-induced proliferation, causing 85% inhibition (Figure 6B). Thus, activation of PKA can inhibit Shh-mediated responses in cerebellar cells.

Requirement of Shh for Proliferation of GCPs In Vivo

Exogenous Shh-N can promote proliferation of GCPs, but is Shh involved in regulating precursor proliferation in vivo? To block the effects of Shh during cerebellar development, we implanted hybridoma cells that secrete function-blocking anti-Shh antibodies (Ericson et al., 1996). The cells were injected into the brain on postnatal day four, and animals were maintained for 3 days before analysis. When hybridomas secreting a control antibody were injected, no perturbation of cerebellar development was observed on day seven. The EGL was eight to ten cells thick (Figure 7A) and contained a mitotic zone (TAG-1-negative) and a differentiating zone (TAG-1-positive) (Furley et al., 1990; Kuhar et al., 1993), as well as a normal Purkinje cell monolayer (Figure 7B), marked by expression of calbindin. In contrast, animals injected with anti-Shh-secreting hybridomas exhibited a dramatic reduction in the size of the EGL, down to a thickness of two to four cells (Figure 7A). TAG-1 staining was now seen close to the edge the EGL, indicating that there was little or no mitotic zone left (Figure 7B). The thickness of the TAG-1-positive differentiating zone was also somewhat reduced. However, Purkinje cells were arrayed in a normal monolayer and showed no alteration of dendritic morphology. Together, these studies suggested that Shh is required for expansion of the EGL during postnatal cerebellar development.

Discussion

Shh was originally identified as a factor that can induce the ventral neural tube to become floor plate and polarizes the developing limb bud (Echelard et al., 1993; Riddle et al., 1993). Since then, it has been shown to function in a variety of tissues, regulating differentiation of motor neurons, interneurons, and oligodendrocytes in the spinal cord (Roelink et al., 1995; Pringle et al., 1996; Ericson et al., 1997), specifying the fate of neurons in the midbrain and forebrain (Ericson et al., 1995; Hynes et al., 1995), and controlling the survival and differentiation of myogenic and chondrogenic cells in the somites (Borycki et al., 1998; Teillet et al., 1998). The majority of these functions occur during embryonic development and involve regulation of cell fate. Here, we show that Shh also functions later in development, to control the growth of GCPs in the cerebellum.

Shh Mediates an Interaction between Purkinje Cells and GCPs

A role for Shh signaling in cerebellar development was initially suggested by our observation that *ptc* knockout mice, in which Shh target genes are aberrantly activated, develop cerebellar tumors consisting of cells that resemble GCPs (Goodrich et al., 1997). To explore the importance of this pathway in granule cell development, we first examined expression of some of the components of this signaling pathway during postnatal cerebellar development. We observed that *shh* is made by Purkinje cells, while *ptc* and *gli* (and related genes) are expressed primarily in GCPs. This expression pattern

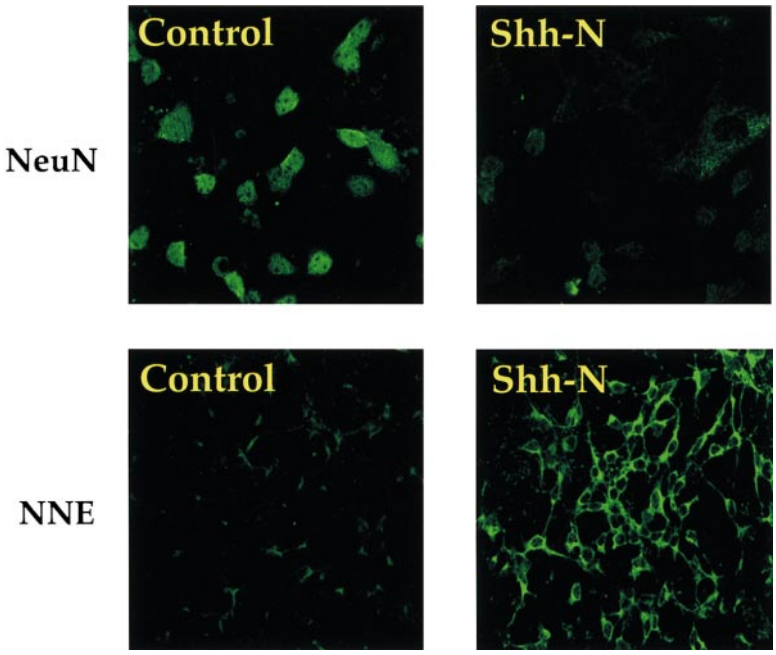

Figure 5. Shh Inhibits Granule Cell Differentiation

Cerebellar cells were cultured for 48 hr in the absence or presence of Shh-N and then fixed and stained with mouse anti-NeuN or sheep anti-nonneuronal enolase (NNE) antibodies followed by FITC-conjugated anti-mouse or anti-sheep secondary antibodies. Staining was visualized as described above.

suggested that Shh might mediate an interaction between Purkinje cells and GCPs during development.

Such an interaction has been postulated previously, based on studies of Lurcher mice, Staggerer mice, and transgenic mice that express diptheria toxin in Purkinje cells (Sonmez and Herrup, 1984; Vogel et al., 1989; Smeyne et al., 1995). In each of these cases, degeneration of Purkinje cells is followed by loss of GCPs and granule cells. Our studies suggest that this cell loss results, at least in part, from the loss of Purkinje cell-derived Shh. Expression of Shh has also been observed in Purkinje cells from adult mice (Traiffort et al., 1998). Since GCP proliferation ceases 2 to 3 weeks after birth, these findings suggest that Shh may have other functions in the cerebellum in addition to controlling granule cell generation.

Shh Induces Proliferation of GCPs

To examine the effects of Shh on developing granule cells, we added soluble Shh protein to dissociated cerebellar cells or to cerebellar slice cultures. The potent mitogenic effect that Shh exerts on cultured cerebellar cells can be seen as early as 24 hr after plating and persists for at least 2 weeks in culture. Moreover, the effect appears to be specific for GCPs, since proliferating cells are small and nonadherent, express high levels of HNK-1, and, in slice cultures, are found in the outermost region of the EGL.

Our analysis of the kinetics of the response to Shh revealed that cerebellar cells proliferate constitutively during the first day of culture and only begin to show significant responses to exogenous Shh thereafter. Constitutive proliferation of GCPs cultured at high density for short periods has been described previously (Gao et al., 1991) and has been suggested to be due to homotypic interactions between granule cells. We have observed that the constitutive proliferation is significantly reduced in cells isolated from animals treated with anti-Shh-secreting hybridomas and in cells exposed to anti-Shh antibodies in vitro (data not shown). Therefore, the constitutive proliferation of GCPs in vitro may also be mediated by Shh. Although it is possible that GCPs themselves make Shh, our detection of *shh* transcripts only in Purkinje cells suggests that the protein is more likely to have been secreted in vivo and carried over into culture. In any case, this protein has only a transient effect on GCPs because if additional Shh is not added, the cells stop proliferating within 24 hr.

Previous studies had indicated that EGF, IGF-1, and bFGF are mitogenic for GCPs (Gao et al., 1991; Ye et al., 1996; Tao et al., 1996; Lin and Bulleit, 1997). We found that the effects of these factors were relatively small (2- to 5-fold induction) compared to that of Shh (75- to 100-fold). On the other hand, while Shh appears to function primarily as a mitogen, IGF-1 has also been reported to promote granule cell survival (D'Mello et al., 1997; Dudek et al., 1997), and bFGF can stimulate granule cell differentiation and neurite outgrowth (Hatten et al., 1988; Saffell et al., 1997; Liu and Kaczmarek, 1998). Thus, while these factors may not synergize with Shh to promote proliferation in vitro, they probably cooperate to control GCP growth and development in vivo.

Termination of Shh-Induced Proliferation and Induction of Differentiation

Along with stimulating proliferation, Shh also appears to inhibit differentiation and migration of GCPs. Untreated GCPs undergo spontaneous differentiation in culture, while Shh-treated cells are maintained in an undifferentiated (HNK-1-positive, NNE-positive, NeuN-negative) state. Similarly, GCPs in control slice cultures are able to migrate inward, while those in Shh-treated cultures remain in the outer EGL. Shh-induced proliferation is therefore incompatible, at least in the short term, with

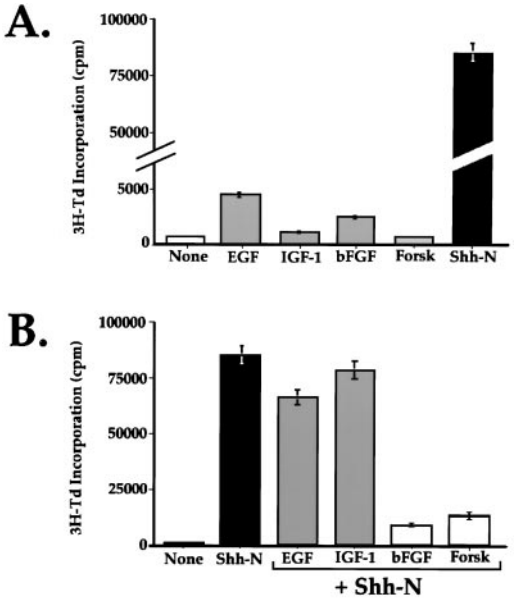

Figure 6. Shh-Induced Proliferation Is Inhibited by bFGF and Forskolin

(A) Response of cerebellar cells to other growth factors. Cerebellar cells were cultured in medium containing no stimulus (None), epidermal growth factor (EGF, 25 ng/ml), insulin-like growth factor-1 (IGF-1, 25 ng/ml), basic fibroblast growth factor (bFGF, 25 ng/ml), forskolin (Forsk, 4 μg/ml), or Shh-N (3 μg/ml). After 48 hr, cells were pulsed with ^{3}H-Td for 12 hr, harvested, and assayed for incorporated radioactivity. The lower part of the y axis (0–5000 cpm) is magnified to allow comparison of the responses to EGF, IGF-1, bFGF, and forskolin.

(B) Inhibition of Shh-induced proliferation by bFGF and forskolin. Cerebellar cells were cultured for 48 hr in medium with no stimulus, Shh-N alone, or Shh-N plus the indicated factors (concentrations and abbreviations described in [A]). Cells were pulsed with ^{3}H-Td for 12 hr and then harvested and assayed as described above.

further granule cell differentiation. The inhibition of differentiation by Shh is not absolute; when cells are exposed to Shh for more than 4 days, some of the GCPs in the culture begin to exit the cell cycle and differentiate. However, even after 2 weeks of culture in medium containing Shh, a significant amount of thymidine incorporation is observed, indicating that some GCPs can remain self-renewing precursors as long as they are exposed to Shh. The ability of Shh to inhibit differentiation of GCPs contrasts with its role in many other tissues, where it promotes cell fate determination and cellular differentiation.

The continued proliferation of cells in response to Shh raises the question of what normally stops GCP proliferation and allows cells to differentiate into mature granule cells. In this context, it is important to note that during their development GCPs migrate toward Purkinje cells — the major source of Shh — and thus should be exposed to higher and higher concentrations of Shh. Despite this, they stop dividing and differentiate by the time they reach the middle of the EGL. Thus, termination of the GCP proliferative response is probably not due to reduced exposure to Shh. Rather, it is likely to result from reduced responsiveness to Shh or conversion of a proliferative response into a differentiative one.

One factor that may contribute to exit of GCPs from the cell cycle is increased expression of the Shh-binding protein Ptc. Many studies suggest that Ptc is a negative regulator of signaling and target gene expression and that Shh functions by binding to Ptc and overcoming this repression (Goodrich et al., 1996). One of the target genes induced by Shh is *ptc* itself; this increased expression of a negative regulator is believed to limit the duration or increase the threshold of further responses (Chen and Struhl, 1996). Cerebellar cells stimulated with Shh-N increase transcription of *ptc*, and this could contribute to a dampening of their proliferative response. However, while Ptc levels increase substantially after 24 hr in culture, cerebellar cell proliferation does not peak until 3 to 4 days in culture and can still be seen 2 weeks later. Therefore, increased Ptc is probably not sufficient to terminate Shh responsiveness.

In light of our observation that forskolin inhibits the proliferative response of cerebellar cells to Shh-N, an alternative explanation for the termination of Shh-induced proliferation might be activation of PKA. One candidate for a physiologic activator of PKA in the cerebellum is pituitary adenylate cyclase-activating polypeptide (PACAP) (Basille et al., 1994; Arimura and Shioda, 1995; Gonzalez et al., 1996). PACAP is expressed at high levels in the developing cerebellum, and its receptors are expressed on GCPs in the EGL. In culture, PACAP promotes granule cell survival and neurite outgrowth in a PKA-dependent manner (Gonzalez et al., 1997; Vaudry et al., 1998). Thus, Shh-induced proliferation of GCPs may be modulated by PACAP.

Another important regulator of the response to Shh may be bFGF. In the developing cerebellum, bFGF is made by astrocytes and Purkinje cells (Hatten et al., 1988; Matsuda et al., 1994) and has been reported to stimulate proliferation as well as differentiation and neurite outgrowth in granule cells (Hatten et al., 1988; Tao et al., 1996; Saffell et al., 1997; Liu and Kaczmarek, 1998). Our observation that bFGF is a weak mitogen on its own, but a potent inhibitor of the response to Shh, suggests that the primary role of bFGF may be in GCP differentiation. Further studies on the precise timing and spatial localization of Shh, PACAP, bFGF, and other growth factors may shed light on the mechanisms by which these factors cooperate to orchestrate granule cell generation in vivo.

Shh Is Necessary for Granule Cell Generation In Vivo

The importance of Shh signaling for GCP proliferation in vivo was confirmed by our studies using anti-Shh hybridomas. Although control hybridomas had no detectable effect on EGL structure, anti-Shh-secreting hybridomas caused a severe reduction in the thickness of the EGL. This reduction in thickness was primarily due to a loss of the outer proliferative zone of the EGL, although some thinning of the postmitotic zone, marked by TAG-1 expression, was also observed. Importantly, anti-Shh hybridomas had no discernible effect on Purkinje cells.

The depletion of EGL cells by anti-Shh was observed after 3 days of exposure to antibodies in vivo. It would

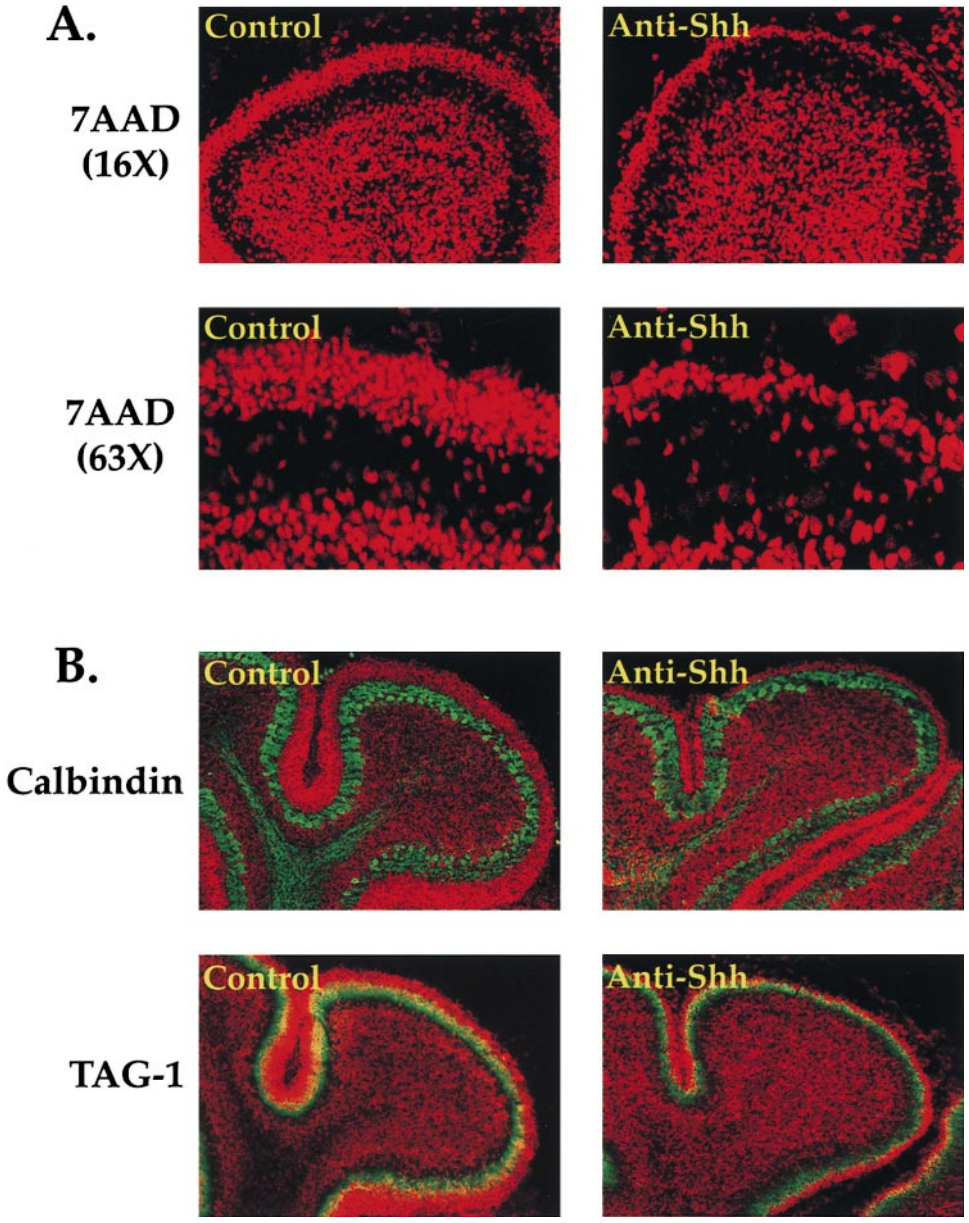

Figure 7. Shh Signaling Is Required for GCP Proliferation In Vivo

(A) Anti-Shh hybridomas cause thinning of the EGL. P4 mice were injected subpially (above the left frontal lobe) with hybridoma cells secreting anti-Shh antibodies (5E1 cells) or control antibodies of the same isotype (1B7 cells). After 3 days (P7), cerebella were harvested, frozen, and sectioned. Sections were stained with 7AAD and then examined by confocal microscopy. Low-magnification (16×) and high-magnification (63×) images of the same sections are shown in the top and bottom panels, respectively.

(B) Anti-Shh hybridomas do not affect Purkinje cells or postmitotic granule cells. Cerebellar sections from hybridoma-injected mice were stained with antibodies specific for Purkinje cells (calbindin) or postmitotic granule cells (TAG-1), followed by FITC-conjugated anti-mouse antibodies (green) and 7AAD (red). Sections were visualized as described above.

be interesting, of course, to determine the long-term effects of blocking Shh in vivo; for example, does the treatment result in long-term loss of granule cells, or are the cells able to recover? Unfortunately, it is difficult to test this using hybridoma injections because the rapid growth of hybridoma cells in vivo results in a massive tumor load within 4 to 5 days, and this begins to cause brain pathology unrelated to depletion of Shh.

An alternative approach to assessing the importance of Shh signaling in vivo is to examine the cerebella of mice in which the genes of the Shh signaling pathway have been disrupted or misexpressed. In fact, studies of mutant mice are consistent with a proliferative effect of Shh at much earlier stages of cerebellar development. For example, animals in which Shh target genes are ectopically activated — ptc$^{-/-}$ mutants (Goodrich et al., 1997) and mice in which *shh* and *gli* expression are controlled by heterologous promoters (Echelard et al.,

1993; Hynes et al., 1997) — show an expansion of tissue around the midbrain/hindbrain boundary. However, this expansion is accompanied by severe perturbation of neural tube structure and embryonic lethality, making it difficult to draw clear conclusions about the role of Shh in postnatal cerebellar development. Therefore, our experiments provide more definitive evidence that Shh is required for generation of granule cells in the EGL.

Role of the Shh Signaling Pathway in Growth and Tumorigenesis

In addition to the cerebellum, mitogenic effects of Shh have recently been described in retinal precursors (Jensen and Wallace, 1997) and in myoblasts (Duprez et al., 1998). In light of these effects, it is worth considering to what extent proliferation contributes to the function of Shh in other systems. For example, in tissues in which Shh has been shown to determine cell fate (Roelink et al., 1995; Ericson et al., 1996; Pringle et al., 1996; Borycki et al., 1998), it is possible that it actually functions to expand a pool of progenitor cells, which are then influenced by other signals to adopt a particular fate. However, the fact that distinct cell fates can be induced by different concentrations of Shh (Roelink et al., 1995) and the fact that Shh is required late in the differentiation process (Ericson et al., 1996) argue against a purely proliferative role of Shh. Thus, outside the cerebellum, Shh may control both proliferation and cell fate determination.

The discovery of Shh-induced growth of GCPs also contributes to our understanding of medulloblastomas in mice and humans with *ptc* mutations (Goodrich et al., 1997; Pietsch et al., 1997; Raffel et al., 1997; Vorechovsky et al., 1997). Medulloblastomas arise in only 15%–25% of *ptc* heterozygotes, suggesting that some other event is necessary for tumor formation. One possibility is loss of the second *ptc* allele, which could lead to more potent activation of Shh target gene expression. However, mutations in other genes might also be required for cell transformation and tumorigenesis. We have shown that Shh-induced proliferation can be terminated by other signals to which GCPs are exposed. It may be the loss of such signals or the ability to respond to them that allows a cell with abnormal Shh signaling to become a tumor. In this context, it is worth noting that bFGF has been observed to inhibit the growth of human medulloblastoma cells in vitro (Kenigsberg et al., 1997). Our studies set the stage for further investigations of the responsiveness of GCPs and tumor cells to such modulatory signals. We hope to shed light on the mechanisms of tumorigenesis in $ptc^{+/-}$ mice and, by implication, in human medulloblastoma patients.

Experimental Procedures

Animals

C57BL/6 × CBA F1 mice were bred and maintained in the animal facility at Stanford University.

In Situ Hybridization

Cerebella from 4-day-old (P4) mice were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (8 μm) were collected on slides and hybridized with ^{35}S-labeled antisense riboprobes (labeled using T3 or T7 polymerase from Pharmacia, Piscataway, NJ) specific for *shh*, *ptc*, *ptc2*, *gli1*, or *gli2* or with a sense probe transcribed from the *ptc* vector (control). Slides were coated with photographic emulsion and incubated for 8 days before being developed and counterstained with 6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR). Fluorescent (DAPI) and dark field (autoradiographic) signals were captured using a digital camera and merged using Photoshop software.

Northern Blotting

To examine induction of *ptc* and *gli* expression by Shh, cerebellar cells from P8 mice (isolated as described below) were cultured in serum-free medium containing 3 μg/ml Shh-N (a bacterially derived N-terminal fragment of Shh, kindly provided by Biogen, Cambridge, MA) for the times indicated. At the end of the culture, total cytoplasmic RNA was purified from each sample, separated by agarose gel electrophoresis, and transferred onto a nylon membrane. The membrane was hybridized in QuikHyb buffer (Stratagene, La Jolla, CA) containing ^{32}P-labeled cDNA probes specific for *ptc* or *gli1* (labeled by random priming) and then washed before exposure to a phosphorimager screen. Images were scanned using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and formatted using Photoshop software. RNA loading was determined based on ethidium bromide staining of 28S ribosomal RNA.

Cerebellar Cell Isolation and Fractionation

Cerebellar cells were purified using a modification of the procedures described by Baptista et al. (1994) and Meyer-Franke et al. (1995). Cerebella from P8 mice (unless otherwise indicated) were removed aseptically, cut into small pieces, and incubated at 37°C for 30 min in digestion buffer consisting of Dulbecco's PBS (DPBS, Life Technologies, Grand Island, NY) with 10 U/ml papain (Worthington, Lakewood, NJ), 200 μg/ml L-cysteine, and 250 U/ml DNase (Sigma, St. Louis, MO). The digestion buffer was then removed and replaced with DPBS containing 8 mg/ml soybean trypsin inhibitor (Boehringer Mannheim, Indianapolis, IN), 8 mg/ml bovine serum albumin (BSA, Sigma), and 250 U/ml DNase, and tissue was triturated using pipettes of decreasing bore size to obtain a single-cell suspension. Cells were centrifuged at room temperature and resuspended in PBS containing 200 μg/ml BSA (PBS/BSA). For cultures of mixed cerebellar cells, the cell suspension was passed through a cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove debris and then recentrifuged and resuspended in Neurobasal medium (Life Technologies) supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), and penicillin/streptomycin from Life Technologies, and thyroid hormone (T3, 0.038 μg/ml), insulin (5 μg/ml), N-acetyl cysteine (NAC, 60 μg/ml), apo-transferrin (100 μg/ml), bovine serum albumin (BSA, 100 μg/ml), progesterone (0.062 μg/ml), putrescine (16 μg/ml), and sodium selenite (0.04 μg/ml) from Sigma. This type of medium has been shown to support survival, growth, and differentiation of a variety of types of neurons (Brewer, 1995; Meyer-Franke et al., 1995). Cells were then transferred to culture dishes and incubated as described below.

For cultures enriched in granule cell precursors or astrocytes (Hatten et al., 1988; Baptista et al., 1994), the cell suspension in PBS/BSA was underlayed with a step gradient of 35% and 65% Percoll (Pharmacia) and centrifuged at high speed for 12 min at room temperature. Granule cells and precursors were harvested from the 35/65% interface, washed in PBS/BSA, and further purified by depleting adherent cells with two 30 min incubations on tissue culture dishes coated with 100 μg/ml poly-D-lysine (PDL, Sigma). Astrocytes were purified from cells above the 35% Percoll layer by adherence on PDL-coated plastic; strongly adherent cells (astrocytes) were removed using trypsin (Sigma).

^{3}H-Thymidine Incorporation Assay

Cerebellar cells isolated as described above were resuspended in serum-free medium (Neurobasal + supplements) and transferred to PDL-coated 96-well plates, at a density of 2×10^5 cells/well ($\sim 1 \times 10^6$ cells/cm^2). Stimuli were added immediately, and the cells were cultured for the indicated amount of time (48 hr, unless otherwise noted). Cells were pulsed with tritiated thymidine (methyl-^{3}H-Td, Amersham, Arlington Heights, IL) 12 hr before the end of the culture period. Following culture, cells were harvested onto filters using a

Wallac harvester (EG & G Wallac, Gaithersburg, MD), and the amount of incorporated radioactivity was quantitated by liquid scintillation spectrophotometry. For most experiments, Shh-N was used at a concentration of 3 μg/ml. Epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), and basic fibroblast growth factor (bFGF) were all from R & D Systems (Minneapolis, MN) and were used at a concentration of 25 ng/ml. Forskolin (Sigma) was used at 4 μg/ml. When cells were cultured for periods longer than 3 days, media was removed from cells every third day and replaced with fresh media containing the same stimuli.

Antibodies

Primary antibodies used for immunofluorescence analysis included 4D7/TAG-1 (Dodd et al., 1988; Furley et al., 1990) (hybridomas generated by Miyuki Yamamoto, and obtained from the Developmental Studies Hybridoma Bank [DSHB], developed under the auspices of the NICHD, and maintained by the University of Iowa, Iowa City, IA), HNK-1 (Abo and Balch, 1981) (hybridoma cells obtained from the American Type Culture Collection [ATCC], Rockville, MD), neuron-specific enolase (NSE; Zymed, South San Francisco, CA), nonneuronal enolase (NNE; Biotrend Chemikalien, Cologne, Germany), calbindin-D (Sigma), NeuN (Mullen et al., 1992) (Chemicon, Temecula, CA), glial fibrillary acidic protein (Bignami et al., 1972) (GFAP; Pharmingen, San Diego, CA), O4 (Sommer and Schachner, 1981) (provided by B. Barres, Stanford University), biotinylated sheep anti-BrdU (Biodesign International, Kennebunk, ME), and MATH-1 (Helms and Johnson, 1998) (provided by J. E. Johnson, University of Texas Southwestern Medical Center). Secondary antibodies included FITC-goat anti-mouse IgM (to detect TAG-1, HNK-1, and O4), donkey-anti-mouse IgG (to detect calbindin, NeuN, and GFAP), donkey anti-rabbit IgG (to detect NSE and MATH-1), donkey anti-sheep IgG (to detect NNE), and TRITC-conjugated streptavidin (to detect biotinylated anti-BrdU), all from Jackson ImmunoResearch (West Grove, PA). The DNA stain 7-aminoactinomycin D (7-AAD) was obtained from Molecular Probes.

Hybridomas used for in vivo blocking studies were 5E1 (anti-Shh) (Ericson et al., 1996), obtained from DSHB, and 1B7.11 (anti-2,4,6-trinitrophenyl), a control hybridoma that secretes antibodies of the same isotype, obtained from ATCC.

Immunofluorescence Analysis

Cerebellar cells were resuspended in serum-free medium and plated on PDL-coated glass coverslips in 24-well plates at a density of $0.5 - 1 \times 10^6$ cells/well. Cells were cultured for 48 hr and then fixed with 4% paraformaldehyde and incubated in blocking buffer (PBS with 2% BSA and 10% normal goat serum, and for intracellular staining, 0.1% Triton X-100 to permeabilize cells). Cells were stained with primary antibodies overnight at 4°C, washed, and incubated with fluorochrome-conjugated secondary antibodies for 4 hr at room temperature. Samples were mounted on slides using Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) and visualized using confocal microscopy and digital photography. Red and green images were merged and processed using Photoshop software.

For double-labeling of antigens and BrdU, cells were pulsed with 10 μM bromodeoxyuridine (BrdU, Boehringer Mannheim) for the final 12 hr of culture. At the end of culture, cells were fixed and stained with primary and secondary antibodies as described above. Cells were then postfixed with 4% paraformaldehyde and incubated with 2N hydrochloric acid (HCl) to denature DNA. The acid was neutralized with 0.1 M sodium tetraborate (pH 8.5), and cells were stained overnight with biotinylated sheep anti-BrdU antibodies. Samples were then washed, and anti-BrdU was detected with fluorochrome-conjugated streptavidin. Staining was visualized and processed as described above.

To determine cell viability, cells growing on coverslips were stained using the Live/Dead assay kit (Molecular Probes), a mixture of calcein AM and ethidium homodimer that labels live cells green and dead cells red. After staining cells according to manufacturer's instructions, cultures were photographed (four fields per condition) and counted.

Cerebellar Slice Cultures

Cerebellar slice cultures were performed using a modification of the procedure described by Stoppini et al. (1991). Briefly, cerebella were removed aseptically, embedded in 2% low–gelling temperature agarose (FMC Bioproducts, Rockland, ME), and cut into 300 μM thick parasaggital sections using a Vibratome (Ted Pella, Redding, CA). Slices were cultured on Millicel CM culture inserts (Millipore, Bedford, MA) in 6-well plates containing serum-free medium supplemented with 6 mg/ml glucose and 25 mM potassium chloride. After 48 hr, BrdU was added to the medium, and slices were cultured for 16 more hr. Slices were then fixed with 4% paraformaldehyde, treated with 2N HCl followed by 0.1M borate, and stained overnight (at 4°C) with biotinylated sheep-anti-BrdU. The next day, slices were washed and stained with FITC-streptavidin and 7AAD and mounted in Fluoromount G. Staining was visualized using confocal microscopy and digital photography, and red and green images were merged using Photoshop.

Intracerebral Injection of Hybridomas

To block the effects of Shh in vivo, hybridomas were injected into P4 mice as described in Barres et al. (1992). 5E1 (anti-Shh) or 1B7 (control) cells at log phase were washed and resuspended in serum-free medium containing 500 U/ml DNase, at a density of 3×10^5 cells/μl. Cells were taken up in a 10 μl Hamilton syringe, and each animal was injected subpially, above the left frontal lobe, with 3 μl (approximately 1×10^6 cells). Three days after injection, mice were sacrificed, and their cerebella were removed and fixed in 4% paraformaldehyde. Cerebella were then sunk in 25% sucrose, frozen in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA), and sectioned using a cryostat. Sections were postfixed with 4% paraformaldehyde, blocked in PBS containing 2% BSA, 10% NGS, and 0.1% Triton X-100, and stained overnight with primary antibodies (TAG-1, calbindin). The next day, sections were washed, incubated with fluoresceinated secondary antibodies and 7-AAD, and mounted with Fluoromount G. Samples were imaged and processed as described above.

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