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Sonic hedgehog controls stem cell behavior in the postnatal and adult brain

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Summary

Sonic hedgehog (Shh) signaling controls many aspects of ontogeny, orchestrating congruent growth and patterning. During brain development, Shh regulates early ventral patterning while later on it is critical for the regulation of precursor proliferation in the dorsal brain, namely in the neocortex, tectum and cerebellum. We have recently shown that Shh also controls the behavior of cells with stem cell properties in the mouse embryonic neocortex, and additional studies have implicated it in the control of cell proliferation in the adult ventral forebrain and in the hippocampus. However, it remains unclear whether it regulates adult stem cell lineages in an equivalent manner. Similarly, it is not known which cells respond to Shh signaling in stem cell niches. Here we demonstrate that Shh is required for cell proliferation in the mouse forebrain's subventricular zone (SVZ) stem cell niche and for the production of new olfactory interneurons in vivo. We identify two populations of $Gli1^{+}$ Shh signaling responding cells: $GFAP^{+}$ SVZ stem cells and $GFAP^{-}$ precursors. Consistently, we show that Shh regulates the self-renewal of neurosphere-forming stem cells and that it modulates proliferation of SVZ lineages by acting as a mitogen in cooperation with epidermal growth factor (EGF). Together, our data demonstrate a critical and conserved role of Shh signaling in the regulation of stem cell lineages in the adult mammalian brain, highlight the subventricular stem cell astrocytes and their more abundant derived precursors as in vivo targets of Shh signaling, and demonstrate the requirement for Shh signaling in postnatal and adult neurogenesis.

Keywords

Mouse; Stem cell; Brain; Hedgehog; Gli; Subventricular zone

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Introduction

Neurogenesis in the adult mammalian brain takes place in the subventricular zone (SVZ) of the lateral ventricular walls of the forebrain and in the subgranular layer of the dentate gyrus of the hippocampus (reviewed by Temple and Alvarez-Buylla, 1999; Taupin and Gage, 2002). In the adult SVZ, periventricular astrocytes (B cells) have been proposed to act as stem cells (Doetsch et al., 1999; Capela and Temple, 2002) and generate rapidly dividing transit amplifying (C) cells, which in turn generate migratory neuroblasts (A cells) which then join the rostral migratory stream to reach their final destination in the olfactory bulb where they differentiate into functional interneurons (Lois and Alvarez-Buylla, 1994; Doetsch et al., 1999; Alvarez-Buylla et al., 2002; Carleton et al., 2003).

The secreted factor Shh plays multiple roles in the formation of the CNS, including the regulation of early ventral pattern in the neural tube (Jessell and Sanes, 2000) and of later precursor proliferation in the dorsal brain (Dahmane and Ruiz i Altaba, 1999; Weschler-Reya and Scott, 1999; Wallace, 1999; Dahmane et al., 2001) (reviewed by Ruiz i Altaba et al., 2002). This and the finding that Shh regulates the behavior of cells with stem cell properties in the developing embryonic neocortex (Palma and Ruiz i Altaba, 2004) led us to investigate its possible involvement in the control of stem cell lineages in the postnatal and adult forebrain SVZ, the best studied stem cell niche in the adult mammalian brain (Alvarez-Buylla et al., 2002).

Here we show that SVZ cells express *Shh* and *Gli1* and that blockage of hedgehog (Hh) signaling in adult and perinatal mice results in diminished expression of *Gli1* and deficits in SVZ cell proliferation in vivo. Our data are consistent with the phenotype of conditional Shh signaling mutants (Machold et al., 2003) and with the increase in *Ptch1* expression observed after injection of Shh into the striatum (Charytoniuk et al., 2002). However, we further show that in vitro addition of Shh results in an increase in SVZ cell proliferation and in the number of cells with stem cell properties and resulting neurons, while blocking its function decreases their number. We provide evidence that Shh synergizes with EGF signaling in the modulation of SVZ cell proliferation. Moreover, cell-sorting and single-cell assays identify periventricular $GFAP^+$ astrocytes and $GFAP^-$ early precursors, but not ependymal cells or migrating neuroblasts, as $Gli1^+$ in vivo responders to endogenous Shh signaling. Our results thus show that Shh signaling is a critical mechanism for the maintenance of stem cell lineages and neurogenesis in the postnatal and adult brain. Together with the involvement of Shh signaling in the control of the behavior of stem cells in the embryonic neocortex (Palma and Ruiz i Altaba, 2004) and in the adult hippocampus (Lai et al., 2003; Machold et al., 2003), the present results demonstrate that Shh signaling regulates stem cell behavior in multiple brain niches throughout life. Moreover, we identify for the first time a cell type previously characterized as being a bona fide stem cell, the periventricular astrocyte (Doetsch et al., 1999), as an in vivo target of Shh signaling.

Materials and methods

Animals, dissections, cell-sorting and treatments

The SVZ of adult and postnatal mice were dissected and cultured as described (Lim et al., 2000; Lim and Alvarez-Buylla, 1999; Doetsch et al., 1999). For FACS, dissociated SVZ cells were prepared as previously described, and strained through a 40 μm nylon mesh (Beckton-Dickinson). All immunostaining incubations and washes were performed at 0–4°C; 1×10^6 SVZ cells were resuspended in 100 μl PBS-TR [PBS, 0.1% Tween-20 (Sigma), 100–200 units RNasin (Promega)] containing primary antibodies (biotinylated mCD24 antibody for ependymal (E) cells (Pharmingen, 1:10); rabbit GFAP antibody for B cells (DAKO, 1:100) and incubated for 15 minutes. Cells were centrifuged in a tabletop centrifuge (Beckman). Cells

were washed three times with 100 μl of PBS. Cells were then resuspended in 100 μl of PBS-TR containing streptavidin-Cy2 at 1:100 and anti-rabbit F(ab)2 at 1:25 (Jackson) and incubated for 10 minutes. Cells were washed three times with PBS as before and resuspended in PBS at 500,000 cells/ml. Immunostained cells were isolated on a FACSVantage (Beckton-Dickinson) cell-sorter. Neuroblasts (A cells) from postnatal animals were not sorted with antibodies; but were separated from type stem cell/transit amplifying precursor (B/C) cells by differential adhesion to poly-lysine treated plastic: type A cells do not stick to plastic, while B/C cells are tightly adherent. Anti-Shh (5E1) (Ericson et al., 1996) antibodies (used at 4 μg/ml) were purchased from the University of Iowa Hybridoma Bank. Recombinant octyl-modified Shh-N protein (used at 5 nM) was a kind gift from Curis.

SVZ neurospheres

To make SVZ neurospheres (Reynolds and Weiss, 1992; Doetsch et al., 1999) the lateral walls of the lateral ventricle of postnatal or adult mice were dissected, collected in PBS, and incubated in PBS containing 0.3% activated papain (Worthington Biochemical). After 30 min incubation, ovomucoid inhibitor (Worthington Biochemical) and DNAse (Sigma) were added in Neurobasal Medium (GIBCO) and the cells dissociated using a fire-polished pasteur pipette. After centrifugation, the cellular pellets were resuspended in neurosphere medium [Neurobasal Medium (GIBCO) supplemented with N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, 0.02 mg/ml insulin, antibiotics and 15 mM Hepes]. The cells were counted and plated in uncoated 25 ml culture flasks (1×10^6 cells per bottle) in the neurosphere medium containing 10 ng/ml epidermal growth factor (EGF; human recombinant, GIBCO). For passaging, neurospheres were centrifuged, triturated in 100 μl of medium with a P200 pipette and replated. For proliferation assays, neurospheres were plated at 4000 cells/well onto polyornithine/laminin-coated labtech chambers (Nunc) in the presence of 10 ng/ml EGF and grown for 1 week, unless otherwise indicated. For dose-response experiments, EGF was used at 1 ng/ml in combination with 5 and 0.5 nM Shh or 5 nM Shh with 5 and 0.5 μg/ml EGF. Clonal dilution assays were performed by plating at low cell densities documented to yield clonal cultures in one-third of conditioned media (Seaberg and van der Kooy, 2002).

RNA, RT-PCR and in situ hybridization

RNA was isolated from whole SVZ dissections or from purified cells (Lim et al., 2000). 5000–10,000 selected cells were collected directly into RNeasy lysis buffer. Total RNA was isolated with RNeasy columns and concentrated by vacuum centrifugation to 12 μl; 9 μl of total RNA was combined with 1 μl of 100 mM T7LD3′ primer, heated to 70°C for 5 minutes, then placed on ice. Forty units of RNasin, 4 μl of 5× first strand buffer, 2 μl of 0.1 M DTT, 1 μl of 20 μM SMART III oligo (Clontech), and 1 μl of 10 mM dNTPs were added, and tubes were incubated at 42°C for 5 minutes. Four-hundred units of Superscript II was then added, and reactions incubated for another 60 minutes at 42°C. Two units of RNaseH were added and tubes incubated at 37°C for 20 minutes; 3 μl of total RNA was used in parallel reactions without Superscript as RT-minus controls. PCR was then performed for *Gli1*, *Gli2*, *Gli3*, *Shh*, *Ptch1* and *Hprt* as previously described (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001). In situ hybridizations with anti-sense digoxygenin-labeled anti-*Shh* or anti-*Gli1* probes were performed on fresh-frozen or perfused sections (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001).

BrdU incorporation and immunohistochemical analyses

Incorporation of BrdU and immunochemical detection was performed as described (Lim et al., 2000; Dahmane et al., 2001) with anti-BrdU mouse monoclonal antibodies (Abs) (1:200; Roche). BrdU was added to cultures at 3 μM 16 hours prior to culture fixation except in the dose-response assays where 6-hour incubations were used. Neuronal phenotype was

determined by immunolabeling with anti-beta III tubulin TuJ1 antibodies (1:1000; Babco). Nuclei were counterstained with Hoechst 33258 (Molecular Probes).

Postnatal SVZ cell cultures

For the thymidine incorporation assay, 300,000 P3 SVZ cells were plated into uncoated wells into a 96-well plate in DMEM/F12/N2/B27/Gln/15 mM Hepes, pH 7.4 (Gibco) in the presence or absence of 5E1 or IgG (R&D systems) antibody at 4–5 μg/ml and cultured for a total of 44 hours. At this cell density, aggregates of SVZ cells form. At 27 hours, 2 μCi of [^{3}H]-thymidine was added to each well. Cells were collected onto glass filters with a Tomtec 96 cell harvester, and [^{3}H]-thymidine incorporation measured with a betaplate filter counter. Postnatal SVZ cultures for neurosphere growth were prepared as follows: 100,000 P7 SVZ cells were plated into 96-well plates in DMEM with 10% FCS for 3 days; at this point, cultures contain GFAP + and Tuj1+ cells. The cultures were then washed and the medium changed to DMEM/F12/N2 (Gibco). In DMEM/F12/N2, these cultures proliferate to produce type A cells. After incubation with or without Shh, cultures were then washed with PBS three times, dissociated with papain (Lim and Alvarez-Buylla, 1999), and 30,000 cells cultured in neurosphere medium in six-well plates.

In vivo and in vitro cyclopamine treatments

Cyclopamine (cyc, Toronto Research Biochemicals) was used at 1 mg/ml conjugated with 2-hydropropyl-β-cyclodextrin [HBC (Sigma)]; prepared as a 45% solution in PBS (van den Brink et al., 2001). Five- to ten-week-old inbred C57Bl6/j mice were injected intraperitoneally for one week with HBC alone as control or cyc at 10 mg/kg/day. The day following the last injection, the mice were pulsed for 2 hours with BrdU (20 mg/kg, IP injection). Immunofluorescence of cryostat sections was as described (Dahmane et al., 2001). The stainings were digitally recorded using a cooled CCD camera-equipped Axiophot (Zeiss) and the BrdU$^+$/DAPI$^+$ nuclei counted within the lateral wall of the lateral ventricles. For the in vivo cyc treatment followed by the preparation of neurospheres, P4 pups or adult animals were injected for 5 days with HBC alone or cyc at 10 mg/kg/day and neurospheres prepared as above from the SVZ of P9 or adult animals. Cyclopamine for in vitro use was dissolved in ethanol (10 mM) and used at 5 μM final concentration.

For measurements in the olfactory bulb, ten (2 months old) C57Bl6/j mice were injected intraperitoneally for one week with HBC alone as control or *cyc* at 10 mg/kg/day (Palma and Ruiz i Altaba, 2004) and were injected intraperitoneally with BrdU (15 mg/ml dissolved in 0.7 mM NaOH with 0.9% NaCl; Sigma) at 100 mg/kg body weight, four times, two hours apart on the day before the last HBC or *cyc* injection. Mice were perfused 30 days later, first with saline (0.9% NaCl) containing heparin (5 units/ml) at 37°C then with 4% PFA in 0.1 M PB at 4°C. Brains were harvested and post-fixed for 48 hours in 4% PFA at 4°C then cut in 40 μm coronal sections with a vibratome (Leica) and stored in 0.1 M PBS. Free-floating sections were washed in 0.1 M TBS with 0.1% Tween (TBST), pretreated in TBST with 0.7% H_2O_2 and 0.1% Triton X-100, then pretreated in 2 M HCl in TBS to denature DNA, blocked with rabbit serum (10% in TBST; Vector) and incubated overnight at 4°C with rat monoclonal anti-BrdU antibody (1:600 in blocking solution; Accurate Scientific). Detection was performed with rabbit anti-rat biotinylated secondary antibody (1:200; Vector) followed by ABC kit (Vector) and revelation with DAB (175 μg/ml; Sigma). Sections were mounted on slides and counterstained with cresyl violet acetate (0.5%; Sigma). One in three sections, 120 μm apart, were selected for BrdU quantitation. An area of interest comprising the granule cell, internal plexiform and mitral cell layers was measured by computer-assisted microscopy (MicroBrightField). The number of BrdU-positive nuclei in this area of interest was related to its sectional volume to obtain a density per millimeter cubed.

SVZ slice preparation and cell harvesting

C57/Bl6j mice (5–6 weeks old) were anesthetized by isoflurane inhalation before decapitation. The brain was rapidly removed and immediately placed in a 4°C normal artificial cerebrospinal fluid (ACSF) solution. The ACSF contained (mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 10 D-glucose with pH=7.3 when bubbled with 95% O_2–5% CO_2. Coronal slices (150–200 μm) were cut with a vibrating microslicer (Leica), kept in normal oxygenated ACSF at 34°C for about 20 min, and then stored at 20°C before the experiment. For cell harvesting, the slices were placed under a microscope in a chamber superfused with oxygenated ACSF warmed at 37°C. Cells close to the lateral ventricle were optically identified. Patch clamp pipettes filled with 6 μl RNase free intracellular solution (in mM: 100 K-gluconate, 20 KCl, 10 HEPES, 4 ATP, 0.3 GTP, 10 phosphocreatine) were used to capture the cells. The seal of the pipette was monitored using a patch-clamp amplifier (Axoclamp 2B, Axon Instruments). The cell was then harvested from the tissue by applying negative pressure to the pipette and removing it from the tissue. The cell and pipette content were then expelled into a reaction tube.

Primer design and multiplex single-cell RT-PCR

Primer design and single-cell RT-PCR were performed based on methods described previously (Dulac, 1998; Toledo-Rodriguez et al., 2004; Wang et al., 2002). In brief, primers were designed using MacVector. Possible interactions between primers were then tested using Amplify 2.1. In addition, primers were subjected to a nucleotide database search to check for sequence specificity. To perform the reverse transcription, 5 pmol of each antisense primer, 0.3 μl (40 U/μl) recombinant RNasin ribonuclease inhibitor (Promega, Madison/WI, USA), 1 μl 6.15% NP-40, and RNase-free water were added to the cell content (final volume 12.3 μl) and incubated for 3 minutes at 65°C. The tube was put on ice and the content spinned down. For a final volume of 20 μl, 0.4 μl ribonuclease inhibitor, 4 μl X5 First-Strand Buffer, 2 μl 0.1 M DTT (both Invitrogen), and 1 μl (10 mM) dNTP mix were added. The mixture was incubated for 5 minutes at room temperature, placed at 42°C and 0.3 μl (200 U/μl) SuperScript II RNase H^- reverse transcriptase (Invitrogen) were added. After incubation for 1 hour at 42°C and 10 minutes at 65°C, the cDNA containing mix was aliquoted into 2×10 μl. One aliquot was kept at 4°C for a maximum of three days until use, the other one was stored at –20°C. The amplification and analysis of the single cell cDNA was performed in two distinct steps. For the first step, the ten cDNAs of interest were amplified in a single tube containing (in a final volume of 50 μl): 10 μl RT product, 0.2 mM dNTPs (Promega), 0.1 μM of each primer, 1× PCR buffer, 1× solution Q, and 1.25 U HotStarTaq DNA polymerase (all Qiagen). As positive control 1 ng of whole brain total RNA was subjected to reverse transcription and 1/10 of the product was used for the first round of cDNA amplification; the negative control contained water. The first amplification consisted of 10 minutes hot start at 95°C followed by 28 cycles of (40 seconds 94°C, 40 seconds at 56°C, 1 minute at 72°C), 10 minutes at 72°C. The PCR was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany). The second round of PCR consisted of 3 minutes at 95°C followed by 35 cycles of (40 seconds at 95°C, 40 seconds at 56°C, 1 minute at 72°C), 10 minutes at 72°C. The amplification was performed in a total volume of 20 μl containing water, 2 μl first PCR product, 0.2 mM dNTPs, 1 M Betaine, 1 μM of corresponding primer pair, 1× ThermoPol Reaction buffer, and 1 U Taq DNA polymerase (both New England BioLabs). After amplification, the PCR products were analyzed on 1.5% agarose gels. Used primers were as follows: GAPDHsense: 5′-TGACATCAAGAAGGTGGTGAAGC-3′ and GAPDHantisense: 5′-CCCTGTTGCTGTAGCCGTATTC-3′, amplifying a 203 bp fragment. GFAPsense: 5′-ATCCGCTCAGGTCATCTTACCC-3′ and GFAPantisense: 5′-TGTCTGCTCAATGTCTTCCCTACC-3′, amplifying a 287 bp fragment. Gli1sense: 5′-CAGCCTCTGTTTTCACATCATCC-3′ and Gli1antisense: 5′-CGGTTTCTTCCCTCCCACAAC-3′, amplifying a 215 bp fragment.

Results

Expression of Shhand Gli1 in the mouse postnatal and adult SVZ

To test whether Shh could have a role in SVZ neurogenesis in the adult brain, we first analyzed the expression of the *Shh* and *Gli1* genes, the latter being a consistent target and reliable marker of Shh signaling (Lee et al., 1997; Hynes et al., 1997; Bai et al., 2001). Both *Shh* and *Gli1* were detected by in situ hybridization at low levels in the lateral wall of the forebrain ventricles (Fig. 1A-H) where neurogenesis occurs. In this region expression was strongest ventrally. Expression was also found in the medial wall in a ventral domain (Fig. 1A,B,F,G), but it was absent from most of the medial wall of the lateral ventricle in more dorsal areas. Other brain regions around the SVZ were devoid of staining indicating the specificity of the hybridization (Fig. 1E). *Gli1* expression was very low or absent in cells immediately adjacent to the ventricle, presumably the ependymal cells (Fig. 1D,H). Expression of these genes in the adult striatum and septum was detected at much lower levels in scattered cells (Fig. 1A,B). Other sites of *Shh* and *Gli1* expression in the perinatal and adult brain were observed, and have been documented elsewhere (Dahmane et al., 2001; Traiffort et al., 2001; Lai et al., 2003; Machold et al., 2003). Sense probes gave no signal (not shown).

Gli1$^+$/ *GFAP*$^+$ cells are present in the SVZ stem cell niche

To attempt to identify the cells that express *Shh* and those that respond to it, dissociated and sorted postnatal (Lim and Alvarez-Buylla, 1999) and adult SVZ cells were used to perform RT-PCR assays testing for the expression of genes involved in Shh signaling (Fig. 2A). Like *Gli1*, the transcription of the gene encoding the Shh receptor *Ptch1* is Shh responsive. In contrast, *Gli2* activation by Shh is context dependent, whereas *Shh*/*Gli1* and *Gli3* often have an antagonistic relationship (e.g. Ruiz i Altaba, 1998). In the postnatal SVZ, all tested components are expressed. However, in neuroblasts (A cells), purified by differential adhesiveness to polylysine-coated plates, only expression of *Ptch1* and low levels of *Gli2* but not *Shh, Gli1* or *Gli3* were detected. The GFAP$^+$ fraction enriched in B and C cells [the GFAP$^+$ SVZ astrocytes (B cells) and the transit amplifying cells (C cells) that contaminate the pool] expressed *Shh, Gli2, Gli3, Ptch1,* and low levels of *Gli1* (not shown). In the adult, sorted mCD24$^+$ ependymal (E) cells expressed low levels of *Gli2* and *Ptch1*, but not *Gli1*, whereas the fraction containing B and C cells (labeled as B in Fig. 2), expressed high levels of *Gli1*, *Gli2* and *Ptch1*. We did not detect expression of *Gli3* in sorted adult GFAP$^+$ or mCD24$^+$ cells. To extend these findings, we analyzed gene expression in postnatal P7 SVZ neurospheres, stem cell-derived colonies, grown in standard media containing EGF. Cells in neurospheres express *Shh, Gli1, Gli2, Gli3* and *Ptch1* (Fig. 2C), further indicating that Shh signaling is active in SVZ stem cell lineages.

Since Shh normally acts through both Gli1 and Gli2, and both *Gli1* and *Ptch1* are responsive to Shh (reviewed in Ruiz i Altaba et al., 2002), the data raises the possibility that adult SVZ stem (B) cells and/or early progenitor (C) cells respond to Shh signaling as they express *Gli1*, *Gli2* and *Ptch1*. While E cells express *Gli2* and *Ptch1,* their lack of *Gli1* expression suggests that they may not respond to Shh signaling. The absence of *Shh* expression in adult isolated B or E pools suggests that either other cells express it or that rare messages were lost during cell sorting and cDNA amplification, as *Shh* mRNA is indeed found in the adult and postnatal SVZ (Fig. 2A,B).

In order to confirm that B cells do indeed express *Gli1*, we performed single cell multiplex RT-PCR from single randomly collected SVZ cells with a patch-clamp pipette after recording their position (Fig. 3A). This method allows analysis of the expression of several genes at a single cell resolution. We found that 32.3% and 27.7% out of 65 sampled cells expressed *GFAP* and *Gli1*, respectively (Fig. 3B,C). Importantly, 17% of the cells co-expressed both

genes, confirming that one population of *GFAP*$^+$ cells, known to be stem cells (Doetsch et al., 1999), express *Gli1* (Fig. 3C). Not all *GFAP*$^+$ cells expressed *Gli1*, possibly reflecting the choice of non-SVZ astrocytes in some cases, the existence of two subpopulations of GFAP$^+$ SVZ astrocytes or the activation by Shh of only a fraction of all SVZ B cell astrocytes at any one point. Moreover, many *Gli1*$^+$ cells did not express *GFAP*, suggesting that more abundant precursors (C cells) also respond, or may have recently responded, to Shh signaling as purified A or E cells do not express *Gli1* (Fig. 2).

Inhibition of Shh signaling in vivo decreases cell proliferation in the intact SVZ

To investigate the requirement of Shh signaling in vivo we have used systemic injections of cyclopamine. Cyclopamine is a plant alkaloid that selectively inhibits Shh signaling (Incardona et al., 1998; Cooper et al., 1998). This drug was injected intraperitoneally, daily, into mice after its conjugation with cyclodextrin as carrier. This method does not injure the brain, a common unavoidable result of direct intracerebral injections. Injections of carrier alone were used as control. A 2-hour pulse of BrdU to label proliferating SVZ precursors was given before tissue harvesting ~12–24 hours after the last injection. Cyclopamine treatment of C57black adult mice for 7 days resulted in a marked decrease of BrdU incorporation in the SVZ as compared with the level of BrdU labeling in control injected siblings (Fig. 4A-D,G). Cyclopamine did not appear to affect the persistence or overall abundance of Nestin$^+$ or GFAP$^+$ cells (Fig. 4E,F). A decrease in BrdU incorporation, however, was accompanied by a decrease in *Gli1* and *Gli3* expression in tissue isolated ~4–6 hours following the last injection (Fig. 4H), indicating a correlation between lower *Gli1* expression levels, as a marker of a decrease in Shh pathway activity, and lower SVZ cell proliferation.

In order to study the consequences of reducing SVZ cell proliferation, we monitored changes in the number of olfactory bulb interneurons. We performed a 7 days' treatment with cyclopamine or vehicle alone followed by a pulse of BrdU during the treatment (see Fig. 5A). Animals were then killed 30 days later, which is the time necessary to obtain a fully functional interneuron (Carleton et al., 2003). As expected, reducing the proliferation in the SVZ led to a reduction of the number of BrdU$^+$ cells in the olfactory bulb granule cell layer (Fig. 5B-F), showing that no compensatory mechanisms were affecting the amount of newborn cells surviving. An overall 15% reduction (n=5 mice for each group) was significantly observed after 30 days along the entire olfactory bulb (ANOVA, F=16.97, P<0.0001 and post-hoc Newman-Keuls test P<0.0002). Treatments at higher doses and/or for longer periods might result in greater neuronal deficits.

Shh is a mitogen for SVZ precursors increasing the number of new neurons

To further test the role of Shh on SVZ cells, we plated dissociated postnatal day (P) 5 SVZ cells on a quiescent astrocytic monolayer in the absence of exogenous growth factors. These conditions recapitulate the large production of neurons observed in vivo from SVZ progenitors (Lim and Alvarez-Buylla, 1999; Lim et al., 2001). Addition of Shh (5 nM) doubled the number of BrdU$^+$ cells after five days (Fig. 6A) in these cultures. The requirement of Shh for SVZ cell proliferation in vitro was tested by making aggregates of dissociated postnatal SVZ cells in the absence of the astrocytic monolayer, and treating them with anti-Shh monoclonal antibody (4 μg/ml) (Ericson et al., 1996) in the presence of [^{3}H]-thymidine. Addition of anti-Shh antibody decreased proliferation by ~30% after two days, as compared to sibling cultures treated with an isotype-matched unrelated antibody at the same concentration (Fig. 6B).

We next tested the effects of Shh on the formation of neurons in SVZ cells plated onto astrocyte monolayers in a defined, serum-free medium. SVZ progenitors proliferate to form colonies of TuJ1$^+$ neuroblast (A) cells. In this assay, addition of Shh increased the number of newly born TuJ1$^+$ neurons, three-fold after 3 days and ten-fold after 7 days (Fig. 6C). Shh does not appear

to increase neurogenesis by acting directly upon neuroblasts: Shh treatment of purified A cells did not increase their numbers as compared to controls (Fig. 6D).

The differences between control and Shh-treated samples are likely to reflect a cumulative effect of Shh on neurogenesis, as seen by the number of $TuJ1^+/BrdU^+$ cells (Fig. 6E,F), derived from a direct effect on early progenitors (B or C cells). Since Shh is added only at the beginning of the culture period, the observed increases in proliferation and neurogenesis after several days in vitro could be explained by an initial amplification of these early progenitors. A transient increase in stem/precursor cells by acute Shh treatment would explain the later increase in production of neuroblasts derived from such expanded progenitor pools.

Exogenous Shh increases the number of SVZ neurospheres

To directly test for an effect of Shh on SVZ early progenitors, postnatal SVZ cell cultures (see Materials and methods) were grown with or without Shh (5 nM) for four days. Cells were then dissociated and washed, and equal numbers of cells cultured without additional Shh in the presence of EGF (10 ng/ml). This latter treatment induces the formation of neurospheres, floating colonies derived from single cells with stem cell properties exhibiting self-renewal and multipotentiality (Reynolds and Weiss, 1992). After 1 week in culture, the number of neurospheres was counted. Shh treatment was found to increase the number of neurospheres 2.5-fold over that obtained in untreated control samples (Fig. 7A,B).

Shh is a mitogen for adult SVZ cells able to form neurospheres

Recent data analyzing *Smo* and *Shh* null mice did not resolve whether SHH acts as a mitogen for adult SVZ cells (Machold et al., 2003). Here we have tested this possibility directly by treating adult SVZ neurospheres with different levels of Shh and diminished doses of EGF. Such modification derives from the observation that Shh and EGF synergize in the control of proliferation of neocortical cells with stem cell properties (Palma and Ruiz i Altaba, 2004). Indeed, in cultures of postnatal SVZ cells, Shh alone was unable to sustain neurosphere growth and addition of Shh to neurosphere media containing standard saturating doses of EGF (10 ng/ml) was ineffective in increasing the number of neurospheres formed (not shown), consistent with previous data using embryonic or adult progenitors (Machold et al., 2003; Palma and Ruiz i Altaba, 2004).

At lower EGF concentrations, however, Shh had a synergistic effect on the proliferation of neurosphere cells. Adult SVZ neurospheres were prepared by standard methods using 10 ng/ml EGF and passaged twice. Such neurospheres were then plated on an adhesive substrate in the presence of 1 ng/ml EGF plus 5 or 0.5 nM recombinant Shh. Analyses of BrdU incorporation after 48 hours showed enhanced proliferation with 5 versus 0.5 nM Shh (~2.5-fold; Fig. 7C). Conversely, adult neurospheres plated on adhesive substrate in the presence of 5 nM Shh plus 5 or 0.5 nl/ml EGF resulted in enhanced proliferation with 5 versus 0.5 ng/ml EGF (~2-fold; Fig. 7C). Interestingly, the percentage of BrdU incorporation was higher with 5 nM Shh plus 1 ng/ml EGF than with 5 nM Shh plus 5 ng/ml EGF. This difference suggests that higher doses of EGF negate any proliferative effects of Shh, explaining the lack of effect of Shh with full media containing 10 ng/ml EGF (not shown) (Machold et al., 2003;Palma and Ruiz i Altaba, 2004). Because Shh on its own, without EGF, is unable to sustain growth of neurospheres (not shown) (Machold et al., 2003;Palma and Ruiz i Altaba, 2004), our results suggest that Shh acts as a mitogenic cofactor when other growth factors are present. The lower synergistic effect of 5 ng/ml versus 1 ng/ml of EGF with Shh suggests that such synergism occurs within a limited concentration range, paralleling the effects we described with embryonic neocortical neurospheres (Palma and Ruiz i Altaba, 2004).

Inhibition of Shh signaling decreases SVZ cell proliferation and the number of neurosphereforming stem cells

To directly test for the requirement of endogenous Shh signaling on adult SVZ cell proliferation and self-renewal using the neurosphere assay, we have utilized cyclopamine on floating neurosphere cultures. Treatment of adult SVZ neurospheres with cyclopamine (5 μM) led to an inhibition of proliferation as measured by BrdU incorporation (Fig. 7D), showing that adult SVZ progenitors require Hh signaling for normal proliferation. In addition, cyclopamine treatment also decreased the number of neurospheres obtained in cloning assays (Fig. 7E), indicating that Hh signaling controls the number of neurosphere-forming adult SVZ cells. The lower relative decrease in clone number versus BrdU incorporation (Fig. 7D,E), together with the in vivo data, suggests a differential effect on two targets: *GFAP*$^{-}$/*Gli1*$^{+}$ C cells (Fig. 3), and *GFAP*$^{+}$/*Gli1*$^{+}$ B stem cells (Fig. 3) as the former account for the bulk of BrdU^{+} cells while the latter can form neurospheres in clonogenic assays.

Discussion

The results presented here demonstrate an involvement of Shh signaling in the regulation of SVZ stem cells, leading to sustained neurogenesis, in the postnatal and adult mouse brain. Taken together, the gene expression analyses and the in vitro and in vivo experiments indicate that Shh signaling is critical for the modulation of the number of cells with stem cell properties, for the proliferation of early precursors and consequently for the production of new neurons.

Our in vivo single cell analyses indicate that Shh acts directly on GFAP^{+} periventricular astrocytes (B cells) and more abundant GFAP^{-} early precursors (C cells). In vitro results are consistent with this conclusion, although responsiveness to Shh, present in stem (B) cells, could also be induced in precursors in vitro. Indeed, transit amplifying precursors (C cells) can give rise to neurospheres in vitro under the influence of EGF, which may induce them to display stem cell properties (Doetsch et al., 2002). Ependymal (E) cells, which were also proposed to behave as stem cells (Johansson et al., 1999) (see Doetsch et al., 1999; Capela and Temple, 2002), and migrating neuroblasts (A cells) expressed *Gli2* and *Ptch1*, but not *Gli1*, suggesting that these cells do not show the canonical response to Shh if they respond at all. Consistently, A cells did not increase their proliferation in vitro in response to Shh.

In the developing cerebral cortex, Shh acts in cooperation with EGF but Shh on its own is not sufficient to alter neurosphere size or number (Palma and Ruiz i Altaba, 2004). We show here that Shh similarly has a proliferative effect on SVZ neurospheres in cooperation with low doses of EGF, demonstrating the conserved action of Shh as a mitogen that synergizes with EGF. The selective expression of EGFR in C cells (Doetsch et al., 2002), and thus the ability to respond to EGF, provides additional evidence that Shh and EGF synergize in the proliferation of these early precursors. Moreover, the inhibition of adult cell proliferation and neurosphere formation by cyclopamine further proves the requirement of Shh signaling in adult stem cell lineages.

In contrast to our results in the SVZ, Shh is sufficient to induce proliferation of hippocampal precursors (Lai et al., 1993). Such a difference could suggest the endogenous production of cofactors, such as EGF, by hippocampal cells at sufficient quantities in vitro. In addition here, and in contrast to other data (Machold et al., 2003), we show that Shh is sufficient to increase the number of neurospheres derived from SVZ cultures grown over quiescent astrocytes, indicating that in this case, such cultures may also produce sufficient levels of EGF or other cofactors. This difference might relate to the method used: it is possible that the astrocytes in the feeder layer produce enough cofactors but at low enough levels for Shh to act, whereas saturating levels of EGF mask the effects of Shh (Palma and Ruiz i Altaba, 2004).

Interestingly, our present data show that Shh and EGF synergize maximally within a narrow concentration range, in a manner similar to that we described in the embryonic neocortex (Palma and Ruiz i Altaba, 2004). It is therefore possible that in both cases, the range of effective Shh and EGF concentrations determines the neurogenic niche where stem cells exist and where the population of early precursors can expand.

The finding that *Shh* mRNA is detected in the walls of the lateral ventricles supports the idea that this molecule contributes to the definition of stem cell niches in the adult brain. However, since we have not yet been able to detect expression of Shh in sorted SVZ cell populations or in isolated single cells (not shown), it remains unclear which cells are the signaling cells. One can therefore not reject the possibility that Shh may be produced at a distance and transported through axonal terminals or dendritic arbors that reach the SVZ from afar. This possibility is also suggested, in part, by the finding that dopaminergic neurons in the ventral midbrain control SVZ cell proliferation at a distance (Höglinger et al., 2004). Whether these cells exert their action through the secretion of Shh remains to be determined. A similar scenario has been proposed for the control of cell proliferation in the hippocampal stem cell niche, where Shh has been proposed to be transported from the septum to the subgranular layer (Lai et al., 1993). However, we note that there are cells that express *Shh* in the hilus (Dahmane et al., 2001), which could also exert a local effect. Shh has also been shown to be axonally transported in the fly visual system (Huang and Kunes, 1996), and it is possible that it is secreted from Purkinje cell dendrites to affect external germinal layer cell proliferation in the cerebellum (Dahmane and Ruiz i Altaba, 1999; Weschler-Reya and Scott, 1999; Wallace, 1999).

How Shh signaling is integrated with other niche factors, such as APP (Caillé et al., 2004), is not yet clear. For instance, BMP and Shh signaling show an antagonistic relationship in the neural tube patterning (Liem et al., 1995). In the postnatal brain SVZ, BMP signaling inhibits neurogenesis and promotes glial differentiation: ependymal cells secrete the BMP antagonist Noggin, thereby creating a favorable microenvironment for neurogenesis to occur (Lim et al., 2000). It is possible that in the postnatal and adult SVZ, as in the embryonic neural tube, BMP and Shh signaling act antagonistically, and inhibition of the former is necessary for the latter to act.

Our results showing that Shh is a niche factor that regulates the number of SVZ cells with stem cell properties and neurogenesis, parallel those in other CNS regions: *Shh* is expressed in the septum and the hilus of the hippocampus (Dahmane et al., 2001; Lai et al., 2003; Machold et al., 2003), it regulates cell proliferation in the subgranular layer (Lai et al., 2003; Machold et al., 2003), and is also involved in the control of stem cell behavior in the developing neocortex (Palma and Ruiz i Altaba, 2004). Shh-Gli signaling may thus be a general mechanism for the regulation of the number of stem cells and the number of precursors derived from primary progenitors. Moreover, it is interesting to propose that the control of the production of new adult neurons, from stem cell astrocytes in the SVZ and hippocampus (Doetsch et al., 1999; Seri et al., 2001), is largely regulated by already existing cells, located nearby or at a distance, through the action of Shh (see also Dahmane et al., 2001), providing a mechanism for the homeostatic regulation of neuronal number and perhaps a mechanism for response to injury and disease.

Finally, our findings also suggest a method for manipulating stem cell lineages for the generation of new neurons through the regulation of Shh signaling. Such a method may help develop new strategies for the treatment of neurodegenerative diseases, such as Parkinson's disease (reviewed by McKay et al., 2004), by expanding stem/precursor cell population in vitro, prior to reintroduction in vivo or by the activation of dormant endogenous stem cell activity in vivo.

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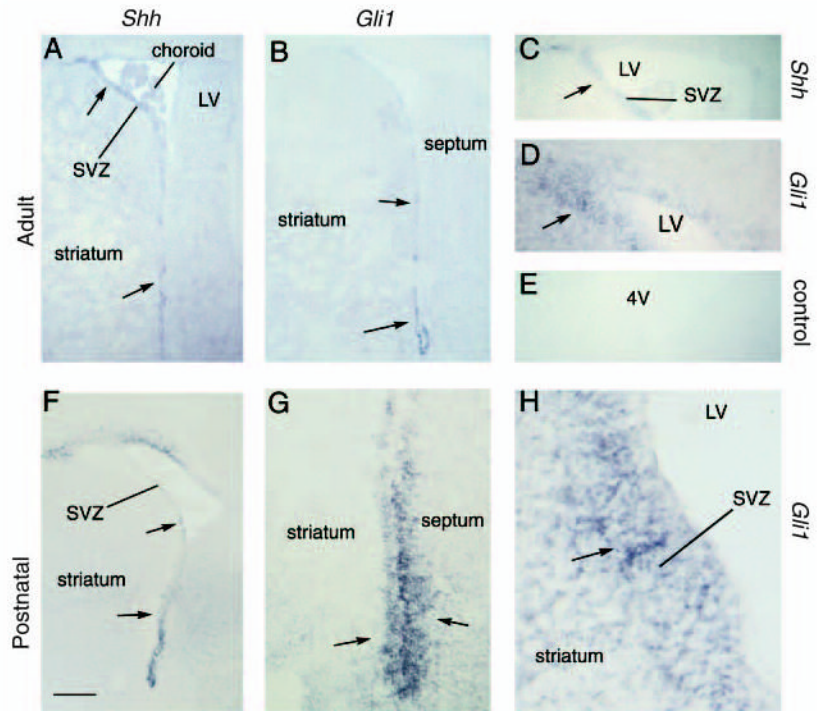

Fig. 1.

Gli1 and *Shh* gene expression in the SVZ. (A,C) Expression of *Shh* mRNA in the lateral wall of the lateral ventricles (LV) of adult mice. At high magnification, *Shh* expression is clearly detected in SVZ cells (C). (B,D,F-H) Expression of *Gli1* mRNA in the lateral wall of the lateral ventricle of adult (B,D) and postnatal (P3; G,H) mice. (E) Control section showing lack of hybridization of *Shh* antisense RNA probes in the fourth ventricle (4V) of an adult mouse. Sense probe controls gave no signal. (A-H) in situ hybridizations on cross sections. Arrows point to sites of expression. Dorsal is to the top. Scale bar in F: 400 μm for A,F; 200 μm for C-E; G, 150 μm; H, 30 μm.

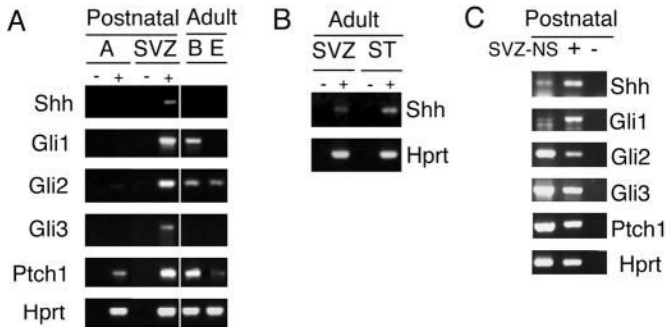

Fig. 2.

Gene expression analyses in cell populations in the postnatal and adult SVZ. (A) RT-PCR analyses of postnatal (P5) and adult sorted cells. Postnatal whole SVZ is also shown as control. (B) RT-PCR analyses of *Shh* expression in the SVZ and adjacent striatum (ST) from the same animal. Note that *Shh* is expressed in the adult SVZ but it is not detected in either B or E sorted cells (see text). All samples were tested with (+) or without (−) reverse transcriptase to control for any possible signal resulting from contaminating genomic DNA. (C) RT-PCR analyses of gene expression in P7 SVZ neurospheres (SVZ-NS). As positive control (+), RNA from a P7 brain was used. As a negative control P7 SVZ RNA without reverse transcriptase was used (−). As control for RT-PCR, all genes were found to be expressed in dissected but non-cell-sorted SVZ pieces. As control for RNA recovery and amounts of cDNA, the levels of the housekeeping gene *Hprt* were measured.

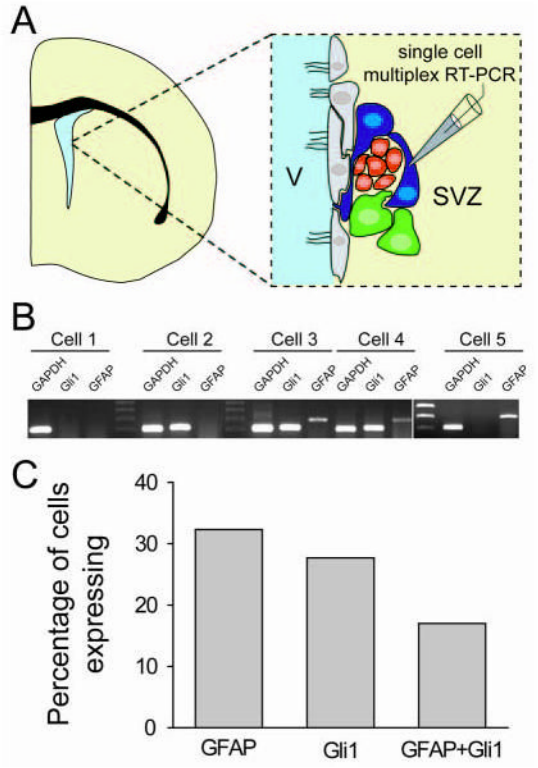

Fig. 3.

Gene expression in single SVZ cells. (A) Schematic representation of the experimental procedure. Individual SVZ cells were randomly harvested with a patch-clamp pipette from fresh living slices, and were used for single cell multiplex RT-PCR. (B) Examples of RT-PCR assays showing gene expression in single SVZ cells. (C) Summary graph showing the expression of *GFAP*, *Gli1* or both in single cells, shown as percentage of the total number of collected cells (*n*=65).

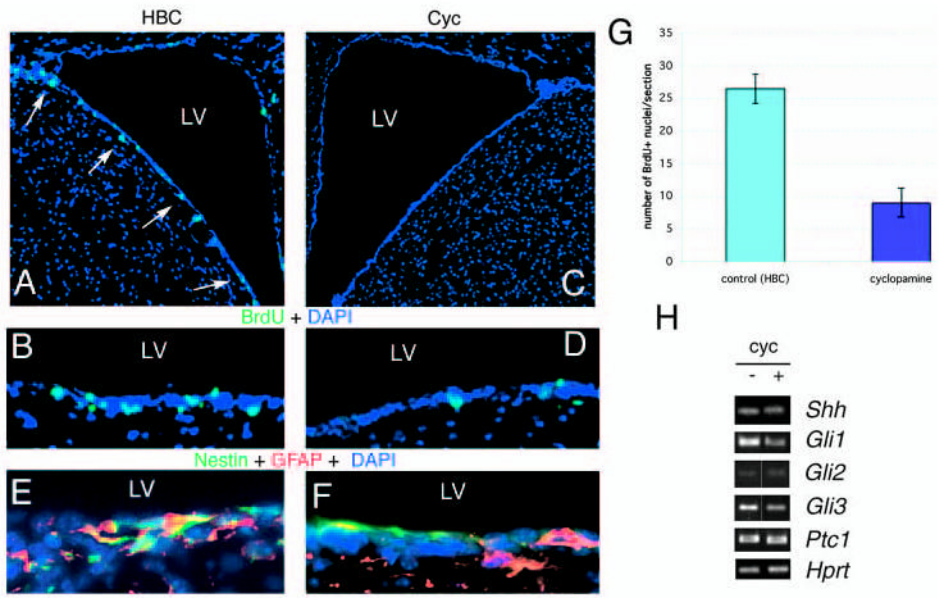

Fig. 4.

Cyclopamine inhibits SVZ cell proliferation in vivo. (A,B,E) Cross section through the forebrain SVZ of an adult mouse showing normal BrdU incorporation (arrows in A,B) or the expression of GFAP and Nestin (E) following one week's injection of HBC carrier (cyclodextrin) alone. Animals were perfused ~12–24 hours after the last injection. (C,D,F) Decrease of $BrdU^+$ cells in adult mice treated with cyclopamine for one week (C,D) does not lead to the loss of $Nestin^+$ or $GFAP^+$ cells (F). (G) Quantification of the number of $BrdU^+$ cells in the SVZ of control and cyclopamine-treated adult mice. Counts are averaged and shown per section. Error bars=s.e.m., n=13 for control HBC-injected mice and n=18 for cyclopamine-injected mice in four independent experiments pooled together. Out of 18 cyclopamine-injected mice, three animals did not respond, five animals decreased the number of $BrdU^+$ cells by ~50%, and ten animals reduced incorporation by ~100%. No reduction was observed in the HBC-injected mice. (H) RT-PCR of fresh SVZ tissue from adult control or cyclopamine-treated mice, dissected 4 hours after the last injection. *Hprt* levels are used as loading controls.

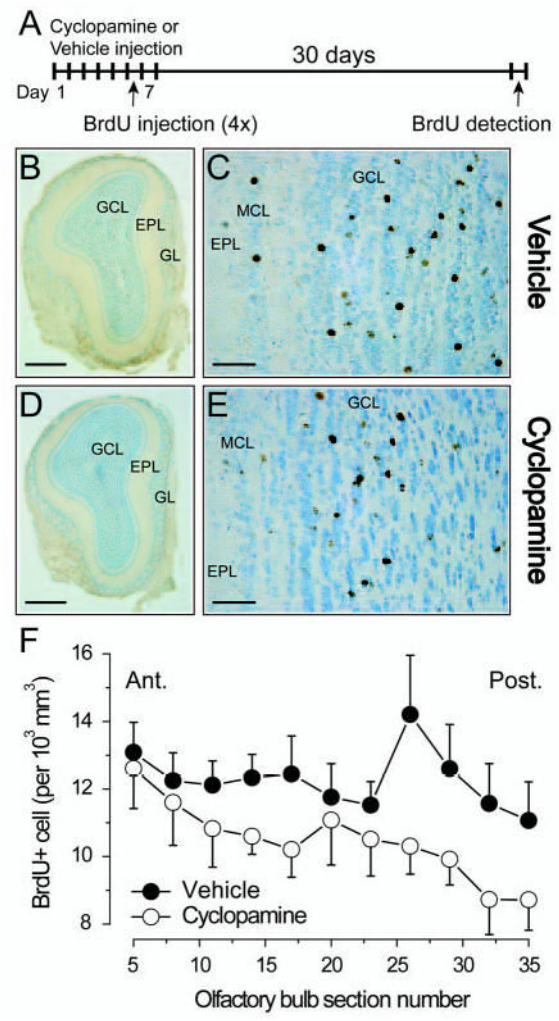

Fig. 5.

Reduced number of newborn interneurons in the adult olfactory bulb after cyclopamine treatment. (A) Experimental procedure. BrdU injections are done during cyclopamine or vehicle treatment. One month post-injection, the number of newborn neurons is quantified after BrdU staining. (B,C,D,E) Photographs of the BrdU staining in the olfactory bulb of vehicle- (B,C) or cyclopamine- (D,E) treated mice. (F) The number of $BrdU^+$ cells in the olfactory bulb of cyclopamine-treated mice (open circle, *n*=5) is significantly reduced in comparison to vehicle-treated mice (filled circle, *n*=5) along the antero-posterior axis. Error bars=s.e.m.

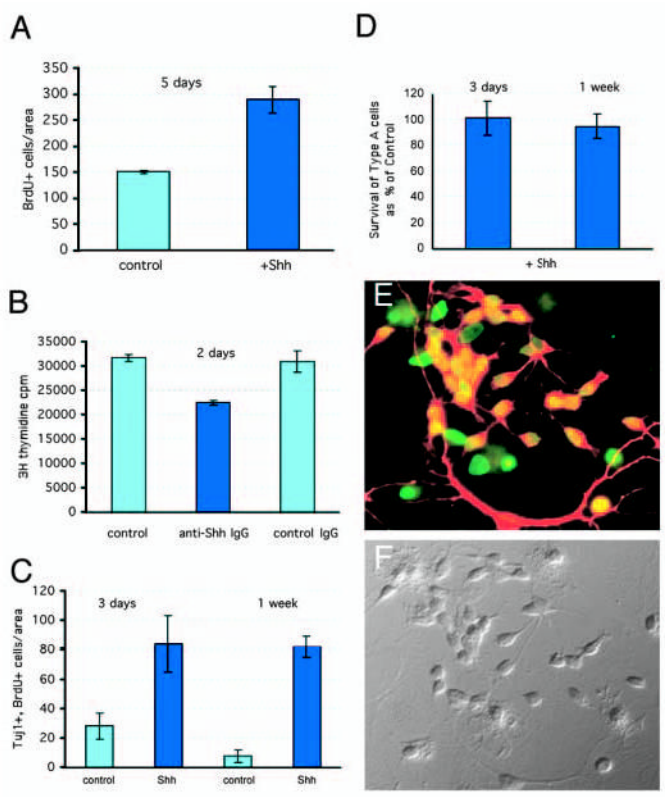

Fig. 6.

Shh signaling regulates SVZ proliferation and neurogenesis. (A) Quantification of the effects of Shh on the proliferation of dissociated P5 SVZ cells plated on a quiescent astrocytic monolayer. BrdU incorporation was quantified by immunofluorescence. Under these conditions, SVZ precursors proliferate and generate new neurons, as they normally do in vivo. (B) Quantification of the effects of blocking anti-Shh monoclonal antibody (5E1) on the proliferation of P5 SVZ cells after dissociation and reaggregation. Cell proliferation was measured by radioactive thymidine incorporation. (C) Quantification of the effect of Shh on neurogenesis in dissociated adult SVZ cells plated on an astrocytic monolayer. Generation of new neurons was measured by co-labeling with Tuj1, identifying neurons, and anti-BrdU antibodies, identifying cells that replicated after BrdU addition. (D) Quantification of the effects of Shh on isolated type A SVZ neuroblasts. Type A cells were purified from P5 mice and cultured with or without Shh. At 3 and 7 days, the number of Tuj1+ cells in Shh-treated cultures were compared to control cultures. Error bars=s.e.m. (E) Immunocytochemistry of a 7-day SVZ cell culture on an astrocytic monolayer showing the labeling of neurons with Tuj1 (red) and recently divided cells with anti-BrdU (green) antibodies. Note the large number of doubly labeled (yellow) cells representing newly born neurons. (F) Nomarski optics image of the same panel shown in E.

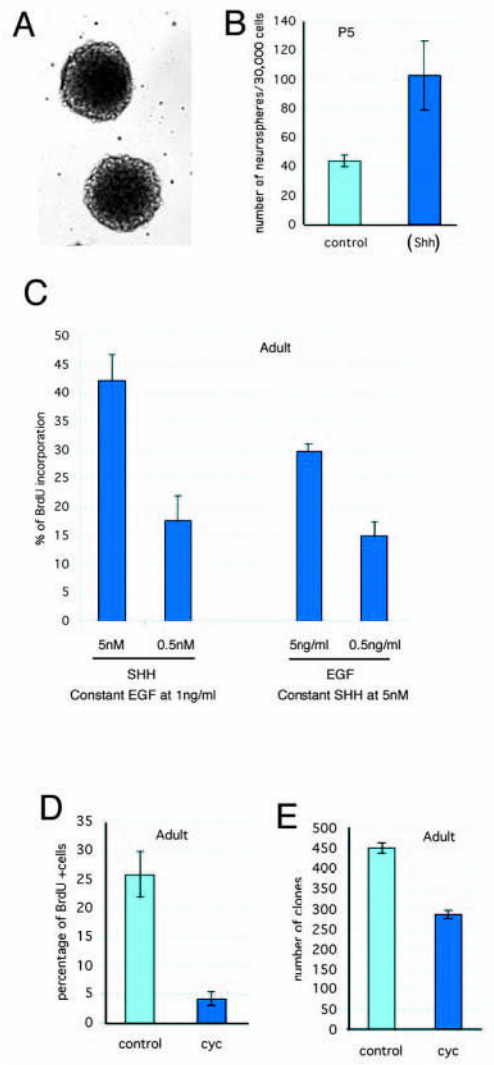

Fig. 7.

Shh regulates proliferation and neurosphere formation in cooperation with EGF. (A) Image of neurospheres from adult SVZ cultures. (B) Quantification of the number of primary neurospheres formed from cultures of SVZ cells previously grown on astrocytic monolayers with or without exogenous Shh (5 nM). Shh was not added to the neurosphere cultures. (C) Synergism of Shh and EGF on neurosphere proliferation. The assay was done with a constant dose of EGF at 1 ng/ml, and varying doses of Shh at 5 or 0.5 nM (left) or with a constant does of Shh at 5 nM and varying doses of EGF at 5 and 0.5 ng/ml (right). Treatments were for 48 hours. (D,E) Quantification of proliferation as measured by the percentage of $BrdU^+$ cells (D) and the number of clones obtained in cloning assays (E) in adult SVZ neurospheres treated with cyclopamine (5 μM) or treated with an equal dose of ethanol used as carrier for in vitro work. In all cases, error bars indicate s.e.m. of triplicate cultures.