Sonic hedgehog regulates adult neural progenitor proliferation *in vitro* and *in vivo*

Karen Lai[1], Brian K. Kaspar[2], Fred H. Gage[2] and David V. Schaffer[1]

[1] *Department of Chemical Engineering and The Helen Wills Neuroscience Institute, 201 Gilman Hall, University of California, Berkeley, California 94720-1462, USA*

[2] *Laboratory of Genetics, Salk Institute for Biological Studies, Box 85800, San Diego, California 92186-5800, USA*

Correspondence should be addressed to D.V.S. (schaffer@cchem.berkeley.edu)

Neural stem cells exist in the developing and adult nervous systems of all mammals, but the basic mechanisms that control their behavior are not yet well understood. Here, we investigated the role of Sonic hedgehog (Shh), a factor vital for neural development, in regulating adult hippocampal neural stem cells. We found high expression of the Shh receptor Patched in both the adult rat hippocampus and neural progenitor cells isolated from this region. In addition, Shh elicited a strong, dose-dependent proliferative response in progenitors *in vitro*. Furthermore, adeno-associated viral vector delivery of *shh* cDNA to the hippocampus elicited a 3.3-fold increase in cell proliferation. Finally, the pharmacological inhibitor of Shh signaling cyclopamine reduced hippocampal neural progenitor proliferation *in vivo*. This work identifies Shh as a regulator of adult hippocampal neural stem cells.

Within the subventricular zone[1–3] and hippocampal regions[4–6] of the adult mammalian brain, neural precursors continually proliferate and differentiate into the three major cell lineages of the central nervous system. Furthermore, cells isolated from regions where neurogenesis is not observed show the capacity for multipotent neural differentiation *in vitro* and *in vivo*, indicating the presence of quiescent neural stem cell populations[7,8]. Several fundamental questions about the biology of these cells require further investigation. Progress has been made in establishing that newly born neurons are functional[9], but further study will be needed to elucidate the biological purpose for their existence. Furthermore, the identities and characteristics of the stem cells that give rise to these functional neurons in different regions must be determined[10–12]. Finally, the environmental conditions and signaling molecules that regulate adult neural stem cell survival, proliferation, axonal guidance and differentiation must be fully investigated[13–16].

Sonic hedgehog (Shh) is a soluble signaling protein that was first discovered and analyzed for its ability to pattern cell differentiation in the neural tube and limb bud[17]. Shh activity has subsequently been found to be crucial in regulating numerous other processes in the developing nervous system, including midbrain and ventral forebrain neuronal differentiation and neuronal precursor proliferation[17–22]. Although there has been significant progress in understanding Shh regulation during development, little is known about its normal role in adults.

Here we investigated the possibility that Shh regulation of neural cell behavior continues from development into adulthood. We have determined that Shh is a potent mitogen for neural progenitor cells of the adult hippocampus. Rat hippocampal progenitors proliferated when cultured in Shh, and

clonal populations that expanded in Shh retained their multipotency. Furthermore, delivery of Shh to the hippocampus through the use of an adeno-associated viral (AAV) vector led to significant increases in cell proliferation *in vivo*. We also found that cyclopamine, a pharmacological inhibitor of Shh and a potential treatment for medulloblastomas involving mutations in Shh signaling[23], markedly reduced neural progenitor proliferation in the hippocampus. Finally, we posit that Shh may be transported to the hippocampus, where it regulates progenitor function. This work provides evidence that Shh is a regulator of adult neural progenitor proliferation.

RESULTS
Patched expression
To investigate the potential role for Shh in the regulation of adult neural progenitor cells, we immunohistochemically stained adult rat brain sections for Shh signaling components. The Shh receptor Patched (Ptc) was expressed in the hippocampal formation, particularly within the hilar region, and in pyramidal cells of CA1 through CA3 (**Fig. 1a**). Preincubation of the primary antibody with a blocking peptide eliminated all staining (data not shown). The commercially available antibodies against Shh or Smoothened (Smo), a cellular transducer of Shh signaling, did not yield specific staining. However, the Ptc immunohistochemistry is consistent with reported *in situ* hybridization results, which detected both *ptc* and *smo* mRNA in the adult dentate gyrus, and to a lesser extent in CA1–CA3 (ref. 24).

We next addressed whether Shh could regulate hippocampal neural progenitors, previously isolated and expanded in fibroblast growth factor 2 (FGF-2; ref. 8). All cells that expressed nestin, a marker for immature neural cells[25], also expressed Patched with
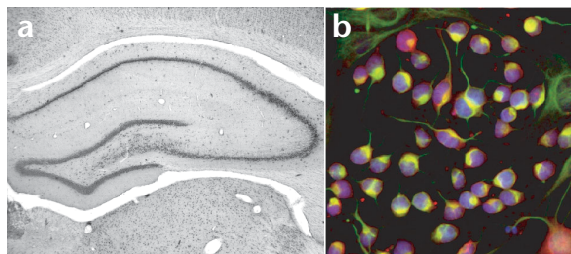
Fig. 1. Expression of Patched *in vitro* and *in vivo*. (**a**) Patched immunostaining in the adult rat hippocampus. (**b**) Progenitor cells were stained for Patched (red) and nestin (green), with nuclear counterstaining using DAPI (blue).

Fig. 2. Shh induction of neural progenitor cell proliferation *in vitro*. Cells were expanded at the concentrations and times indicated, and the final cell number per well is plotted. Each value represents the average of three points compared to the standard curve, with error bars representing standard deviations.

an apparent cell-surface localization (**Fig. 1b**). These results corroborate earlier work that used reverse transcription polymerase chain reaction (RT-PCR) to show that progenitor cell populations express Ptc and Smo[26]. To further confirm Ptc expression, we cloned rat Ptc and Smo by RT-PCR from progenitor cell total RNA. The full-length *ptc* cDNA sequence, which has not previously been reported, encodes a 1,434 amino acid protein with 99.1% identity to mouse and 95% identity to human Ptc protein (see **Supplementary Fig. 1** online).

Shh-dependent cell proliferation *in vitro*

To further investigate a potential role for Shh in regulating progenitor cell function, progenitor cells were cultured in recombinant Shh, and preliminary work indicated that it stimulated cell proliferation. To further characterize this effect, cells were incubated in a range of concentrations. Cultures in 5–50 nM Shh proliferated robustly throughout the 8-day experiment, expanding by a factor of 170 at the highest concentration. In contrast, cells at 0 or 1 nM Shh did not expand (**Fig. 2**). The cell doubling time was 44 hours at 50 nM, though saturation was not yet reached at this dosage, a result that is in agreement with a reported EC_{50} value of 18.5 nM for N-terminal Shh in cerebellar granule cell precursors[21]. By comparison, the doubling time in 20 ng/ml of FGF-2 was 28 hours (data not shown). The relatively high concentration needed for Shh may be due to the fact that recombinant Shh produced in bacteria is not as potent as the native, lipid-modified form. From these data, we conclude that Shh directly promotes progenitor proliferation *in vitro*.

Multipotency of clonal populations expanded in Shh

Although these results establish Shh as a mitogen for a population of cells derived from progenitors, it is unclear whether this factor expands multipotent cells or those that are already committed to a specific lineage. We therefore expanded individual cells into clonal populations for differentiation analysis. Individual cells were sorted into 96-well plates by flow cytometry, with approximately 60% cell viability just after sorting. FGF-2 requires an autocrine cofactor, a glycosylated form of cystatin C, for stimulating neural stem cell proliferation, particularly at low density where this factor becomes limiting[16]. To determine whether progenitors grown in Shh share a similar requirement, both fresh media and media previously conditioned for 24 hours by high-density cultures growing in Shh were used for the expansion of the clones. Out of 30 possible clones, no progenitors survived in the absence of recombinant Shh. However, individual cells cultured solely with 50 nM Shh, in either fresh or conditioned media, were able to survive and proliferate into clonal
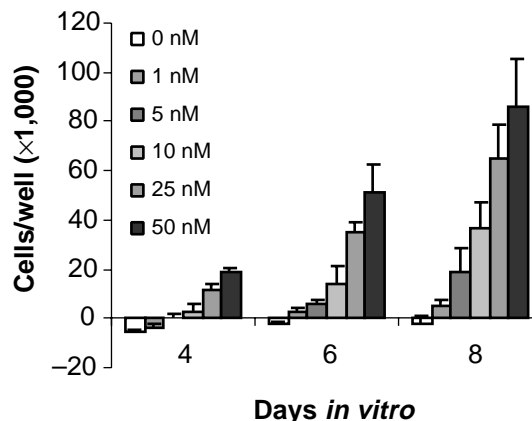
populations. From a total of 96 wells, 19 clones expanded and were stored for further study.

To assess multipotency, 7 of the 19 clonal populations were exposed to differentiating conditions and immunofluorescently stained for the presence of lineage-specific markers[27]. Nearly all (6 of 7) developed into neurons and glia, as indicated by the expression of the neuronal antigen βIII-tubulin and the astrocytic marker glial fibrillary acidic protein (GFAP; **Fig. 3a** and **b**). Parallel stains for the marker O4 indicated that the Shh-expanded cells were also capable of differentiating into an oligodendrocyte lineage (**Fig. 3c**). These results established that clones expanded in Shh plus fresh or conditioned media (**Fig. 3a–e**) maintained their multipotency and also indicated that Shh activity toward progenitor cells does not seem to require a cofactor.

Mitogenic effect of Shh *in vivo*

The expression of Ptc in the dentate gyrus of adult rats suggests that Shh may regulate progenitor proliferation *in vivo* as well as *in vitro*. To test this possibility, cDNA encoding the N-terminal active fragment of rat Shh was inserted into a recombinant AAV vector, which was produced at a titer of 4×10^{12} particles/ml. In contrast to adenoviral vectors, AAV vectors are gutted of all viral genes and lead to highly efficient gene delivery with essentially no toxicity, immunogenicity or other aberrant effects on cell function[28,29]. To confirm that cells infected with this virus produced Shh, human embryonic kidney (HEK-293) cells were infected with rAAV-Shh or rAAV-GFP virus containing green fluorescent protein (GFP) cDNA also at a titer of 4×10^{12} particles/ml. Subsequent western blotting analysis of cell lysate and media showed that the 20 kDa Shh was present only in cultures infected with the Shh virus (data not shown).

Next, rAAV-Shh, or rAAV-GFP as a control, was stereotaxically injected into the dentate gyrus of adult rats ($n = 8$). After a two-week period for viral genome processing[28,29], two animals were killed to confirm GFP and Shh expression. rAAV-GFP injection into the hippocampus resulted in high levels of transgene expression, confirming the high titer of the virus and the fact that the injection site was within the hilar region of the dentate gyrus (**Fig. 4a**). As commercially available Shh antibodies do not yield specific immunohistochemical staining[30], we used RT-PCR to
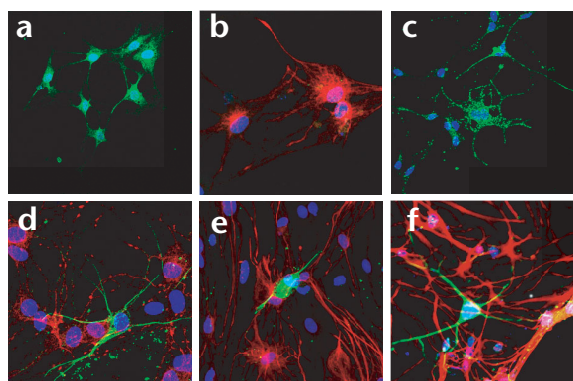
Fig. 3. Differentiation of clonal cell populations expanded in Shh. Cells from a differentiated clonal population were stained to detect (**a**) βIII-tubulin, a neuronal marker (green), (**b**) GFAP antibody, an astrocytic marker (red) and (**c**) O4, an oligodendrocytic marker (green). Nuclei were counterstained with TO-PRO3 (blue). Results from three additional individual clones were simultaneously stained to detect βIII-tubulin (green), GFAP (red) and TO-PRO3 (blue). (**d**) A clonal population expanded in Shh-conditioned media with 50 nM Shh. (**e, f**) Clonal populations expanded in fresh media with 50 nM Shh.

detect a viral message (**Fig. 4b**). With primers flanking the β-globin intron, amplification from single- or double-stranded viral genomic DNA generates a 1,400 base pair (bp) product encompassing the intron and the *shh* cDNA, whereas mRNA that has been processed to excise the intron yields a smaller 800 bp product. As indicated by the presence of an 800 bp band in lane 2, rAAV injection into the hippocampus yielded recombinant *shh* mRNA. In contrast, RT-PCR of tissue from the cerebellum of the same animal, as well as from the hippocampus and cerebellum of control animals injected with rAAV-GFP, did not yield a product. Also, PCR of mRNA that had not undergone reverse transcription did not generate a product in any samples. These results indicate that rAAV injection into the hippocampus leads to recombinant *shh* mRNA transcription.

The remaining animals ($n = 6$) were injected with the mitotic marker bromo-deoxyuridine (BrdU) for 8 days and killed on the ninth. Tissue sections (40 μm) were then stained for BrdU and NeuN by immunofluorescence and analyzed by fluorescence confocal microscopy. Both animal groups had significant numbers of cells that had undergone mitosis within the subgranular zone of the dentate gyrus, consistent with earlier results[4–6] (**Fig. 4d** and **e**). However, whereas rAAV-GFP animals had an average of 15.6 ± 3.0 BrdU-positive nuclei per section, animals injected with rAAV-Shh had 52.1 ± 9.1 cells, a robust 3.33-fold increase in cell proliferation ($P < 0.01$; **Fig. 4c**).

To determine the long-term fate of proliferating cells, a second group of animals ($n = 6$) was treated as described above, but were killed three weeks after the last BrdU injection. The average number of BrdU+ cells per section dropped significantly over this time period to 16.3 ± 4.5 in rAAV-Shh animals and to 8.9 ± 1.0 in AAV-GFP animals, and the 1.8-fold higher value in the Shh animals was statistically significant ($P < 0.05$). Tissue sections stained for BrdU and NeuN showed that a significant number of cells that divided during the course of the BrdU injection subsequently migrated into the granule cell layer and differentiated into NeuN+ neurons (**Fig. 5a** and **b**). The average number of BrdU+/NeuN+ cells per section was 7.1 ± 0.7 and 2.2 ± 0.7 for Shh and GFP, respectively, a three-fold difference ($P < 0.01$;

Fig. 5c). The fractions of BrdU+ cells that differentiated into neurons were 43% for AAV-Shh and 25% for AAV-GFP, although these percentages were not statistically different ($P = 0.063$). These results confirm that Shh is a mitogen for adult hippocampal neural progenitors *in vivo* as well as *in vitro*.

Inhibition of Shh
We next assessed whether inhibiting this potential endogenous Shh signaling in the hippocampus would reduce the proliferation of neural progenitor cells. Two micrograms of cyclopamine, a pharmacological inhibitor of Shh signaling[23], were complexed with the vehicle cyclodextrin (HBC) and injected into the hippocampus. HBC has been used extensively to increase the solubility of hydrophobic compounds for delivery to the brain and hippocampus, with no observed side effects[22,31]. After injection with cyclopamine or vehicle alone, followed by 3 days of BrdU injections, hippocampal sections adjacent to the injection site were analyzed. BrdU counts were high, probably owing to the twice-daily injections. Cyclopamine reduced the proliferation of neural progenitor cells in the dentate gyrus to half of that in animals injected with vehicle (**Fig. 6**). Furthermore, addition of 1 μg/ml cyclopamine to progenitor cells in culture reduced their proliferation in response to 50 nM Shh by more than nine-fold, as compared to the vehicle control (data not shown).

Shh protein, but not mRNA, has been detected in the hippocampus. However, Shh is expressed at high levels in the basal forebrain[24,30], which projects to the hippocampus via the fornix. Furthermore, anterograde axonal transport of both *Drosophila* Hedgehog (Hh) and Shh has been detected[30,32]. To address whether the anterograde transport of Shh via the fornix may regulate progenitor proliferation, BrdU uptake in the hippocampus was analyzed in animals receiving fimbria-fornix lesions. Progenitor proliferation was significantly reduced in lesioned animals, and the injection of cyclopamine into lesioned animals did not further reduce progenitor proliferation in these animals (**Fig. 6**).

DISCUSSION
In the present study, we have shown that Sonic hedgehog regulates the proliferation of progenitor cells of the adult rat hippocampus *in vitro* and *in vivo*. Although its numerous and important functions during development have been investigated, little is known about Shh's role in adults. We observed that the Shh receptor Patched is expressed in the dentate gyrus and Ammon's horn of the hippocampal formation of adult rats (**Fig. 1**), in agreement with *in situ* hybridization that showed high levels of Patched message in the granule cells, and, to a lesser extent, in the pyramidal cell layers[24]. In addition, progenitor cells isolated from the hippocampus, which apparently derive from the subgranular zone of the dentate gyrus[6], also express Patched. We also cloned and sequenced rat Patched from progenitor cell cultures, a sequence not previously reported in its entirety.

Although Shh signal transducers were clearly present in adult hippocampal progenitor cells, Shh activity could have a variety of potential effects in these cells. In different contexts during development, Shh regulates neural stem cell differentiation, survival and proliferation. In the spinal cord, Shh governs progenitor cell differentiation into floor plate cells, motor neurons and interneurons in a dose-dependent fashion[33]. It also regulates midbrain and forebrain dopaminergic and serotonergic neuronal differentiation[20,34]. In other contexts, however, Shh promotes differentiation into oligodendrocytes[35] and astroglia[36]. In addition to regulating differentiation, Shh is a mitogen for
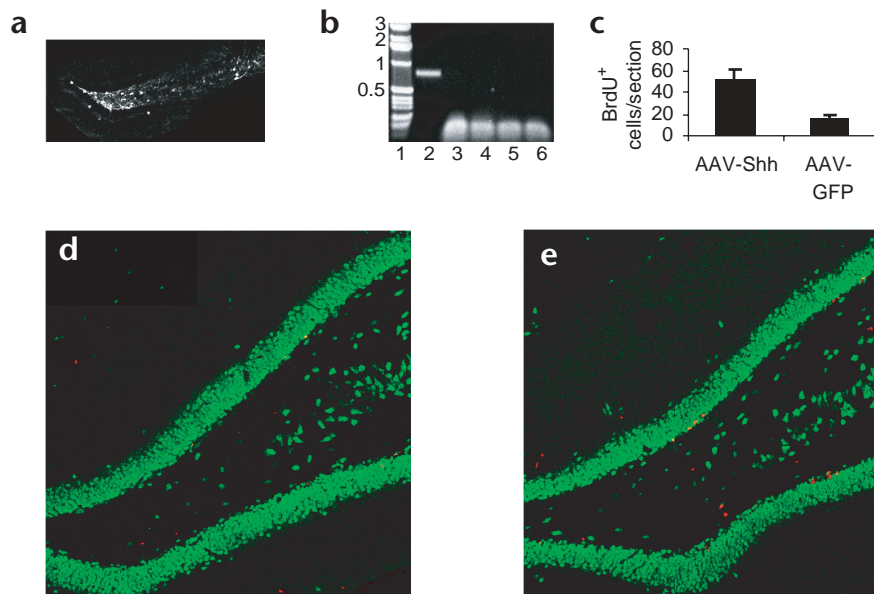
Fig. 4. Shh induction of progenitor proliferation *in vivo*. (**a**) Direct GFP fluorescence in the hilar region of the dentate gyrus after injection of rAAV-GFP. (**b**) RT-PCR analysis of rAAV-Shh transcription. Lane 1, DNA standard, with sizes indicated in kilobases; lane 2, hippocampal mRNA of an animal injected with rAAV-Shh; lane 3, hippocampal mRNA of an animal injected with rAAV-Shh, with no reverse transcription; lane 4, hippocampal mRNA of an animal injected with rAAV-GFP; lane 5, hippocampal mRNA of an animal injected with rAAV-GFP, with no reverse transcription; lane 6, cerebellar mRNA of an animal injected with rAAV-Shh. (**c**) The average number of cells in the hilus per section in which BrdU was detected in each animal group (total $n = 6$) is plotted. The asterisk indicates the values are significantly different ($P < 0.05$). Representative images of BrdU (red) and NeuN (green) staining within the dentate gyrus are shown for an animal injected with (**d**) rAAV-Shh or (**e**) rAAV-GFP.

cerebellar granule neurons and neocortical and spinal precursors during development[17,37].

Addition of recombinant Shh to adult hippocampal progenitor cells elicited potent time- and dose-dependent proliferation, whereas cells died in the absence of Shh (**Fig. 2**). The target of Shh activity was a multipotent neural progenitor cell, as clonal populations still had the capacity for differentiation into the three major CNS cell lineages (**Fig. 3**). This finding establishes Shh as the first known factor capable of clonal expansion of adult hippocampal neural progenitor cells in the absence of autocrine factors in conditioned media. Furthermore, adeno-associated viral vector delivery of *shh* cDNA to the rat hippocampus led to a greater than three-fold increase in the number of newly born cells in the dentate gyrus of adult rats (**Fig. 4**). Finally, progenitor cells that divided after rAAV-Shh injection later underwent differentiation into granule cell neurons (**Fig. 5**). There was a three-fold higher number of NeuN+/BrdU+ cells in the Shh animals as compared to the GFP controls; however, the fraction of cells that differentiated into neurons in each case was not statistically different, indicating that Shh does not bias cells toward a neuronal lineage.

Whereas the addition of exogenous Shh stimulated neural progenitor proliferation *in vitro* and *in vivo*, this result does not address the question of whether endogenous Shh is normally involved in regulating hippocampal progenitor function. Cyclopamine, a natural inhibitor of Shh signaling, has been used to elucidate Shh's role in a number of processes, including early brain expansion and medulloblastoma growth[22,23]. We found that the introduction of cyclopamine into the hippocampus repressed cell proliferation in the subgranular zone by a factor of two, indicating that endogenous Shh signaling is involved in adult progenitor expansion (**Fig. 6**). Furthermore, this finding reveals a potential side effect for the recently proposed use of Shh in the treatment of medulloblastoma[23].

Shh could induce progenitor proliferation by a number of intracellular mechanisms. Binding of Shh to Ptc releases this receptor's repression of Smo, which then transduces the signal by acting on the transcription factors of the Gli family. The transcriptionally activating Gli forms then upregulate Shh targets, including *ptc, gli1, gli2* and *shh* itself[17]. Shh signaling may directly regulate the cell cycle, as it can upregulate the expression of G1-phase cyclins of type D and E, and Patched can act directly on the G2-phase cyclin B[17]. Alternatively, Shh activity may activate other signaling systems that control cell proliferation. Gli1 upregulates platelet-derived growth factor receptor α (PDGFRα) expression to promote basal cell carcinoma expansion[38]. Moreover, *igf-2* is a known Shh transcriptional target, and Shh could therefore act upstream of this family of growth factors to induce cell proliferation[14,39].

Shh's proliferative effect in adult hippocampal neural stem cells is analogous to its regulation of cerebellar granule cell precursors during development, but with several distinctions. First, in contrast to the hippocampus, neurogenesis does not occur in the adult cerebellum. Second, Shh acts upon granule neuron precursors in the cerebellum, whereas adult hippocampal progenitors are multipotent. Further work is required, however, to identify the precise cellular target of Shh within the hippocampus[11]. Finally, although defects in Shh signaling have led to medulloblastomas, we did not find tumors in the hippocampus. This could indicate that in regions with ongoing adult neurogenesis, cell proliferation is balanced by signals that regulate differentiation and death.

We also probed the potential cellular source of Shh in the hippocampus. Shh is expressed in the dentate gyrus of E15 mice for an unknown function[40], but its transcripts are not present
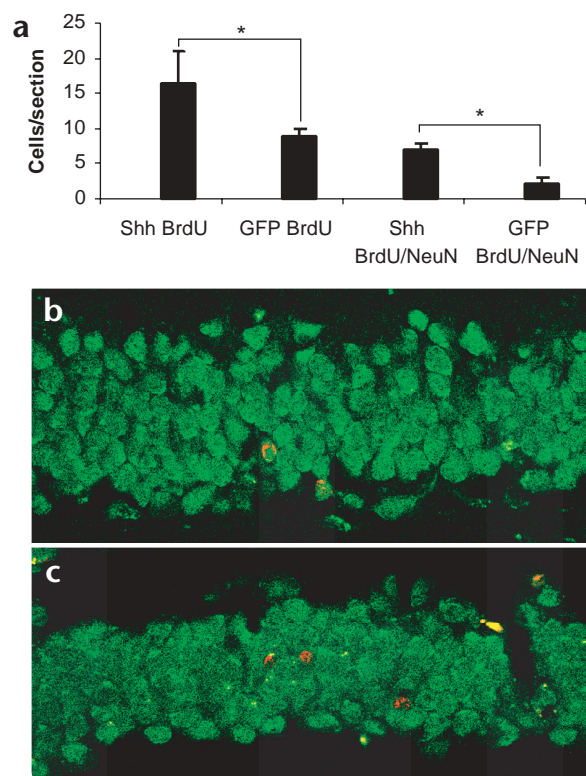
Fig. 5. Progenitor cell differentiation. (**a**) Quantification of the average number of cells in the dentate gyrus and hilar region per section that are BrdU[+] and BrdU[+]/NeuN[+] three weeks after the completion of BrdU injections (*$P < 0.05$). Representative images of the granule cell layer in animals injected with (**b**) AAV-Shh and (**c**) AAV-GFP, stained for the analysis of BrdU uptake (red) and differentiation into NeuN^{+} neurons (green).
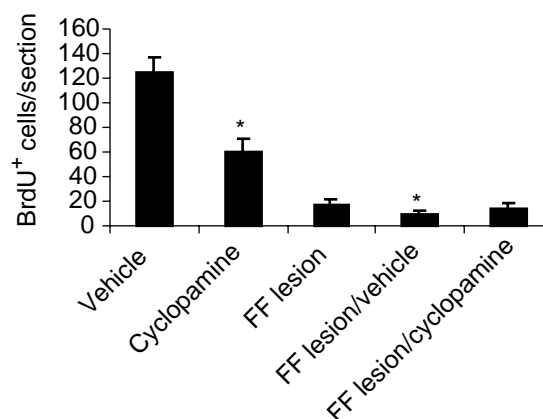

Fig. 6. Inhibition of Shh. BrdU uptake was analyzed in animals injected with cyclopamine or vehicle, in some cases after fimbria-fornix (FF) lesion. The average number of cells in the hilar region per section in which BrdU was detected under each condition ($n = 3$ animals each; *$P < 0.05$).

at detectable levels in the adult hippocampus[24]. In contrast, Shh is expressed at high levels in several structures of the adult basal forebrain[24] that are known to project to the dentate gyrus[41]. In addition, like the neurotrophins BDNF and NT-3, *Drosophila* Hh and Shh can undergo anterograde axonal transport along projections from the retina to the brain[30,32,42]. Finally, Shh protein is present in the adult hamster hippocampus[30]. These considerations collectively raise the intriguing possibility that the basal forebrain may regulate adult neurogenesis by transporting Shh to the hippocampus. In support of this possibility, we found that a lesion of the fimbria-fornix, which would block such Shh transport, significantly reduced neural progenitor proliferation, and that the introduction of cyclopamine into the hippocampus of lesioned animals did not further inhibit this proliferation (**Fig. 6**). Although this lesion disrupts both basal forebrain and other hippocampal inputs, these results support the hypothesis that Shh transport into the hippocampus regulates adult neural progenitor expansion.

Relatively few factors are known to regulate adult neural stem cell function. Conditions such as an enriched environment and exercise upregulate hippocampal neurogenesis[13,43]. At the molecular level, FGF-2 was first used to expand progenitors, and its required autocrine cofactor, cystatin C, was recently discovered[16]. In addition, peripheral administration of IGF-1 stimulates hippocampal progenitor proliferation[14], and heparin-binding EGF apparently mediates increased neurogenesis in response to

ischemia[44], whereas glucocorticoids downregulate neurogenesis[45]. By comparison, EGF and ephrins induce the proliferation of adult stem cells from the subventricular zone[3,46]. Whether Shh also regulates subventricular zone progenitor proliferation will require additional study.

The fact that multiple factors modulate neural stem and/or progenitor cell proliferation raises the possibility that they act in concert to regulate distinct steps in the progression of the cell to a terminally differentiated phenotype. For example, we propose a model in which FGF-2 is a general survival factor and mitogen for early, immature neural stem cells[8,16]. Additional factors such as Shh and IGF-1 may support the survival and proliferation of these cells and prepare them for differentiative instructions, such as neurotrophins for a neuronal fate or Notch for an astrocytic fate[27,47]. In addition to regulating sequential steps in the progenitor cell life cycle, these multiple factors could offer several points of either local or distant control over stem cell proliferation and neurogenesis. It is known that neurotrophins that undergo anterograde transport are released from presynaptic terminals in an activity-dependent manner to modulate synaptic properties[42]. In the basal forebrain, Shh appears to be expressed primarily by GABAergic neurons[24]. One possibility is that the activity-dependent synthesis or secretion of Shh into the hippocampus by these cells may be a mechanism for regulating neurogenesis, and thus modulates the processing of memories within these two structures.

In summary, we found that Shh, a signaling molecule that is well known for its control of numerous processes during development, regulates the proliferation of adult hippocampal neural stem cells *in vitro* and *in vivo*. To our knowledge, this represents the first known normal function of Shh in the adult nervous system and one of the few demonstrated functions of Shh in adult organisms[48]. Furthermore, the transport of signaling factors to neurogenic zones may represent a new mechanism for the control and regulation of adult neurogenesis. Finally, as few factors are known to directly stimulate neural progenitor proliferation in culture and *in vivo*, this finding of a proliferative effect of Shh in the adult nervous system may have therapeutic implications for regeneration of neural tissue in neurodegenerative disorders.

METHODS

Neural progenitor cell isolation and culture. Neural progenitors were isolated and cultured from the hippocampi of adult female Fischer 344 rats as previously described[8]. Cells were propagated on culture plates (Becton Dickinson, Franklin Lakes, New Jersey) that were coated with poly-ornithine (Sigma-Aldrich, St. Louis, Missouri) and mouse laminin (Invitrogen, Carlsbad, California) in HAMS F-12/DMEM media with N2 supplement and 20 ng/ml of FGF-2 (Invitrogen). Animal protocols were approved by the UCB Animal Care and Use Committee in accordance with NIH guidelines.

Recombinant Shh growth assay and clonal expansion. Recombinant rat Shh encompassing amino acids 25–198 was produced in *E. coli*. Progenitors were plated at a density of 500 cells/well in coated 96-well plates (Becton Dickinson). Recombinant Shh at a range of 0–50 nM was added to the cells, which were then incubated for a period of 8 d with an 80% media change every other day. Cell proliferation was assayed using the WST-1 reagent (Roche, Pleasanton, California) following the manufacturer's instructions and using a Bio-Tek Instruments spectrophotometer.

For clonal expansion, progenitor cultures were first expanded for 6 d in 1 µg/ml (50 nM) of Shh and then individually sorted into coated 96-well plates by flow cytometry (Becton Dickson). These single cells were then expanded in 1 µg/ml Shh in either fresh N2 medium or a 50:50 mixture of fresh and N2 + Shh media previously conditioned by high-density progenitor cultures. Surviving clones were expanded by a factor of 10^6 before cryopreservation. For differentiation studies, cells were plated at a density of 2,500 cells/cm^2 onto coated 8-well chamber slides (Becton Dickinson). After 24–48 h in Shh-supplemented media, cells were switched into 0.2 µM retinoic acid, 5 µM forskolin (Biomol, Plymouth Meeting, Pennsylvania) and 0.5% fetal bovine serum (BioWhittaker, Walkersville, Maryland) to induce cell differentiation[27] for 10–14 d.

Immunofluorescent staining. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich), dissolved in phosphate-buffered saline (PBS), and blocking and staining were performed in Tris-buffered saline containing 0.3% Triton X-100 and 5% donkey serum (Santa Cruz Biotechnology, Santa Cruz, California). The following primary antibodies were used: mouse anti-β-tubulin III (1:500; Sigma-Aldrich), guinea pig anti-glial fibrillary acidic protein (1:1,000; Advanced Immunochemical, Long Beach, California), mouse anti-O4 (1:2; gift from O. Boegler), mouse anti-nestin (1:1,000; Becton Dickinson) and goat anti-Patched (1:500; Santa Cruz Biotechnology). Detection of primary antibodies was performed with Alexa fluorochrome-conjugated secondary antibodies (Molecular Probes, Eugene, Oregon) at a dilution of 1:250. Nuclei were stained with the nuclear marker TO-PRO3 (Molecular Probes). Images were captured by fluorescent confocal microscopy (Leica Microsystems, Wetzlar, Germany). Sections of adult rat brains, which had been previously generated as described below, were stained for the presence of Patched using anti-Patched antibody (1:1,000; Santa Cruz) and biotinylated anti-goat IgG (Jackson ImmunoResearch, West Grove, Pennsylvania), with diaminobenzidine as a chromagen as described[13]. Images were analyzed on a Nikon Eclipse microscope (Nikon, Melville, New York).

Adeno-associated viral vector production. Recombinant AAV-2 using the CMV promoter to drive expression of the enhanced GFP (Becton Dickinson) or rat *shh* cDNA encoding the N-terminal active polypeptide with secretion signal peptide was produced. Virus was generated by calcium phosphate transient transfection of the vector plasmid and pAAV/Ad8 helper plasmid[49] into HEK-293 cells (ATCC, Manassas, Virginia), followed by infection with adenovirus dl312 (MOI 2.0). Purification and titering were conducted as previously described[28,29]. HEK-293 cells were infected with adeno-associated virus carrying the Shh cDNA (rAAV-Shh) at a multiplicity of infection of 10, and after two days, western blotting was performed on cell lysates and cell culture media to detect the presence of recombinant Shh protein using anti-Shh primary (Santa Cruz), anti-goat IgG HRP (Jackson) and ECL detection (Amersham-Pharmacia, Piscataway, New Jersey).

Animal surgeries and immunohistochemistry. Recombinant AAV was stereotaxically injected into the hippocampus (anteroposterior axis, –3.5; mediolateral axis, ±3.0; dorsoventral axis, –3.9 from skull, with nose bar at 3 mm up) of anesthetized female Fischer-344 rats (150–165 g). After two weeks, rats were administered a daily, intraperitoneal injection of BrdU (50 mg/kg, Sigma-Aldrich) for 8 d. Either the next day, or three weeks later, animals were perfused (4% paraformaldehyde), and the brains were excised, stored in fixative overnight, and transferred to 30% sucrose. Coronal sections (40 µm) were cut on a sliding microtome, and GFP fluorescence was visualized directly. Sections were processed for BrdU immunohistochemical staining as previously described[13] and stained using rat anti-BrdU (1:400; Accurate, Harlan Sera-Lab, Loughborough, England), biotinylated donkey anti-rat IgG (Jackson), and streptavidin-Cy3 (Jackson) in addition to mouse anti-NeuN (1:20; from R. J. Mullen, University of Utah) and FITC-labeled donkey anti-mouse IgG (Jackson). In some samples, staining was also conducted for GFAP (Advanced Immunochemical). Results were analyzed for statistical significance using the ANOVA test and are reported with standard deviations.

Animals received a lesion of the fibria-fornix as previously described[50]. Cyclopamine (Toronto Research Chemicals, Toronto, Canada) was dissolved in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma-Aldrich) in PBS. Two groups received a 2 µl injection of either cyclopamine or HBC vehicle into the hippocampus as described above. After 24 h, animals were injected with BrdU (50 mg/kg) every 12 h for the next 72 h.

RT-PCR. Animals ($n = 2$) received hippocampal rAAV-Shh or rAAV-GFP injections as described above. After two weeks, hippocampi were excised using RNase-free materials and reagents. Reverse transcription was performed as described previously[29]. The 5′ primer (GTGGATCCTGA-GAACTTCAG), homologous to the 5′ untranslated region of the rAAV-Shh transcript, and the 3′ primer (GCCGCCAGATTTGGCCGC-CACGGAGT), homologous to Shh, flank a human β-globin intron that is spliced from the mRNA. For Patched cloning, RNA from progenitor cells was subjected to RT-PCR using primers based on the known mouse sequence (GenBank #6679518). The products of three independent RT-PCR reactions were cloned and sequenced.

Note: Supplementary information is available on the Nature Neuroscience website.

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