Noggin Expands Neural Stem Cells in the Adult Hippocampus

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Abstract

New neurons are added to the adult hippocampus throughout life and contribute to cognitive functions including learning and memory. It remains unclear whether ongoing neurogenesis arises from self-renewing neural stem cells (NSC) or from multipotential progenitor cells that cannot self-renew in the hippocampus. This is largely based on observations that neural precursors derived from the subventricular zone (SVZ) can be passaged long-term whereas hippocampal subgranular zone (SGZ) precursors are rapidly depleted by passaging. We demonstrate here that high levels of BMP signaling occur in hippocampal but not SVZ precursors *in vitro*, and blocking BMP signaling with Noggin is sufficient to foster hippocampal cell self-renewal, proliferation, and multipotentiality using single cell clonal analysis. Moreover, NSC maintenance requires continual Noggin exposure, which implicates BMPs as crucial requlators of NSC aging. *In vivo*, Noggin is expressed in the adult dentate gyrus and limits BMP signaling in proliferative cells of the SGZ. Transgenic Noggin overexpression in the SGZ increases multiple precursor cell populations, but proportionally increases the glial fibrillary acidic protein (GFAP)$^+$ cell population at the expense of other precursors, suggesting that Noggin acts on NSCs *in vivo*. To confirm this, we used a dual thymidine analog paradigm to repeatedly label slowly dividing cells over a long duration. We find that small populations of label-retaining cells exist in the SGZ and that Noggin overexpression increases their numbers. Thus, we propose that the adult hippocampus contains a population of NSCs, which can be expanded both *in vitro* and *in vivo* by blocking BMP signaling.

Keywords

hippocampus; progenitor; bone morphogenetic protein; neurogenesis; development; aging

Introduction

Neurogenesis persists throughout life in the adult brain in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) as well as in the subventricular zone (SVZ) adjacent to the

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lateral ventricle (Altman and Das, 1965; Kaplan and Hinds, 1977). Neuron turnover in the granule cell layer (GCL) of the adult DG maintains normal hippocampal function, is correlated with hippocampus-dependent learning, and may be involved in depression and epilepsy (Parent et al., 1997; Gould et al., 1999; Shors et al., 2001; van Praag et al., 2002; Santarelli et al., 2003; Raber et al., 2004). The SVZ contains self-renewing neural stem cells (NSCs) (Reynolds and Weiss, 1992; Morshead et al., 1994), and early studies hypothesized that self-renewing NSCs are also present in the adult hippocampus (Palmer et al., 1997). However, more recent studies have concluded that the adult hippocampus contains neurogenic progenitor cells (NPCs), but not stem cells, since hippocampal precursors appear to lack the ability to self-renew (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Meanwhile, the adjacent posterior lateral ventricle (pLV) contains NSCs, and it has been proposed that these are the cells that maintain continual neurogenesis in the SGZ (Bull and Bartlett, 2005).

Neural stem and progenitor cells reside in microenvironmental niches that are modified by environmental stimuli to regulate multiple aspects of precursor development (Hagg, 2005; Ma et al., 2005). For example, bone morphogenetic proteins (BMPs) are negative regulators of the adult SVZ and juvenile SGZ neurogenic niches and limit precursor number, proliferation, and neuronal fate specification (Lim et al., 2000; Bonaguidi et al., 2005). BMPs exert their biological effects by binding to type I (BMPRIa and BMPRIb) and type II (BMPRII) receptor subunits that are organized with minor modifications of the prototypical TGFβ subclass of serine-threonine kinase receptors (For review, see (Derynck and Zhang, 2003)). Binding of BMPs to their receptors leads to phosphorylation of SMAD1, SMAD5, and SMAD8 (SMAD1/5/8), which translocate to the nucleus and activate transcription. BMP actions are regulated *in vivo* by proteins such as Noggin, follistatin, chordin, and neurogenesin that antagonize BMP signaling by directly binding BMPs and blocking ligand activity.

In previous studies we found that neural precursor cells endogenously produce BMPs, which promote neural precursor exit from cell cycle and loss of precursor characteristics (Bonaguidi et al., 2005). This suggested that prior attempts to demonstrate the presence of a hippocampal stem cell *in vitro* might have been confounded by the endogenous production of BMPs. Here, we demonstrate that hippocampal cells cultured in the presence of the BMP inhibitor Noggin are able to self-renew and to generate neurons. We further establish *in vivo* that the adult SGZ contains a cell population with neural stem cell characteristics, and that BMP signaling regulates their numbers within the neurogenic niche.

Materials and Methods

Animals

The generation of the neuron specific enolase (NSE)-Noggin and NSE-BMP4 transgenic mice is described elsewhere (Gomes et al., 2003; Guha et al., 2004). FVB and C57/BL6 male mice were purchased from Jackson Laboratories. Adult mice were used between 2 and 4 months of age. All mice were housed in a facility with a 14 hr light/10 hr dark cycle and allowed free access to food and water. Experiments were conducted according to protocols approved by IACUC and Northwestern CCM.

Thymidine Analogs

BrdU (Sigma) was given via four i.p. injections of 50 mg/kg daily every 3 hrs for 3 consecutive days followed by cardiac perfusion 3 hrs after the final injection (Cao et al., 2004). In a separate set of experiments, 1.15 mg/ml CldU and 0.85 mg/ml IdU (Sigma) were provided in drinking water with 2.5% sucrose. IdU was administered for 14 consecutive

days, followed by water for 7 d, CldU for 14 d, water again for 7 d, and the animals were sacrificed (Bauer and Patterson, 2006). Wild type and transgenic animals consumed similar amounts of drinking water. For EdU (Invitrogen) labeling of hippocampal neurospheres, Noggin induced neurospheres were cultured with or without Noggin for 5 days before enzymetically dissociated into single cells. 24hrs later, cells were incubated with EdU for 4 hours then processed for EdU detection according to the kit manufacturer's protocol (Click-IT™ Flow Cytometry Assay Kit, Invitrogen). Following staining, cells were analyzed by flow cytometry based on 488 (EdU^+) and 633 (Propidium Iodide$^+$) fluorescence positivity and percentage of labeled cells were quantified (Cyan ADB, Dakocytomation).

TERT RT-PCR

Total RNA was collected using the RNAqueous-4PCR kit (Ambion) from passage 3–4 Noggin induced hippocampal neurospheres after being cultured with or without Noggin for 5 days. 1μg of RNA was used for generating cDNA using the Thermoscript reverse transcriptase and Oligo-dT primers (Invitrogen), and 2μl of cDNA was used per PCR reaction. PCR was performed with the SybrGreen master mix (Applied Biosystems) and Realplex^2 Mastercycler (Eppendorf) using following cycling parameters: 95°C, 15sec; 58°C, 60sec for 40 cycles. Primers against the mouse TERT (5′-GAC ATG GAG AAC AAG CTG TTT GC-3′; 5′-ACA GGG AAG TTC ACC ACT GTC-3′) were designed according to published sequences (Cai et al., 2002) and purchased from IDT techonologies. PCR product was analyzed on a 2.0% agarose gel and imaged with Gel Doc XR (Biorad).

Immunochemistry of tissue sections

Adult mice were perfused with saline, then 4% paraformaldehyde (PFA). Brains were harvested, post-fixed in 4% PFA, dehydrated with 30% sucrose in PBS, and embedded into OCT. 10–20 μm saggital sections were processed for antigen retrieval using 10mM sodium citrate, pH 7.1 at 95°C for 20 min and cooled for 30 min (p-SMAD, ID3, and SOX2, and dual-thymidine experiments). Sections were blocked with 10% goat serum for 1 hour. Primary antibodies diluted in PBS containing 1% BSA and 0.25% Triton X-100 were applied overnight at 4°C. Antibodies used include BrdU (mouse IgG2a 1:1000; Millipore), CldU (rat monoclonal anti-BrdU 1:250; Accurate Biosciences), IdU (mouse IgG1 anti-BrdU 1:500; BD Biosciencse), GFAP (mouse IgG1, 1:400; Sigma; and rabbit, 1:500; Dako), PSA-NCAM (mouse IgM, 1:500; Millipore), phospho-SMAD1/5/8 (rabbit, 1:100, Cell Signaling Technologies), ID3 (rabbit, 1:100, Santa Cruz), SOX2 (rabbit, 1:100, Millipore), Noggin (rat, 3 μm/ml, Regeneron), and green fluorescent protein (rabbit, 1:500, Molecular Probes). Primary antibodies were visualized with appropriate mouse or rabbit Alexa-Fluor-350/488/594/647 secondary antibodies (Molecular Probes). Nuclei were counterstained with Hoechst 33342 (Sigma). Negative staining controls lacked application of the primary antibodies. Sections stained for BrdU, CldU, and IdU were pretreated with 2N HCl for 30–45 min and neutralized with Borax, pH 8.5, for 10 min before blocking. Cells were visualized using confocal laser microscopy (LSM 510 META, Zeiss) and counted as previously described (Bonaguidi et al., 2005).

Primary neurosphere cultures

Adult neurospheres were isolated according to published methodologies (Bull and Bartlett, 2005). Adult (2–4 months of age, 3–4 per group) male C57/BL6 mice were killed by cervical dislocation and their brains immediately removed. A 2-mm-thick coronal slice was cut between −0.70 and −2.70 mm, relative to bregma, according to an adult mouse brain atlas (Paxinos and Franklin, 2001). Hippocampal (Hip) and posterior SVZ (pSVZ) areas were dissected (see Fig. 1) from the caudal side and carefully trimmed of all white matter. Tissue samples were minced with scissors and enzymatically digested with 0.1% trypsin-EDTA (Sigma) for 7 min at 37°C. The digestion was quenched with 0.014% w/v trypsin

inhibitor (type I-S from soybean; Sigma) in Hank's Buffered Saline Solution (HBSS). After centrifugation, the pellet was resuspended in 1ml serum-free medium (SFM) and mechanically triturated until smooth. The cells were filtered through a 70 µm cell sieve (BD Biosciences), centrifuged, resuspended in 100 µl SFM, and viable cells were counted on a hemocytometer using trypan blue (Sigma). Hippocampal and pSVZ cells were plated at a density of 7000 cells/cm^2 in non-vacuum treated 24-well plates (BD Biosciences) with 1ml SFM per well. SFM consisted of DMEM:F12 medium, supplemented with N2, B27, 100 U/ ml penicillin/streptomycin, glutamine (all from Gibco), and 2 µg/ml heparin (Sigma). The following growth factors were also included: 20 ng/ml human recombinant EGF, 10 ng/ml mouse recombinant FGF-2 (both from BD Biosciences), and where denoted 250 ng/ml Noggin (R&D Systems). Primary hippocampal cells were incubated for 8 d, and pSVZ cells for 7 d, in humidified 5% CO2 to permit primary neurosphere formation. The neurospheres were then counted and collected for either passaging or immunocytochemistry.

Neurosphere passaging and differentiation

Neurospheres were dissociated as previously described (Bonaguidi et al., 2005). Spheres were harvested after 4–5 days, cells were counted and plated for self-renewal at a density of 1×10^4 cells/cm^2 in SFM plus EGF, FGF, and where denoted Noggin. The cell number per sphere was calculated by dividing the cell number by sphere number. Secondary sphere formation was performed in 96-well plates as previously described (Bonaguidi 2005). Spheres were plated for differentiation at passage 11. For passage 11 expansions, cells were plated at low density (1×10^3 cells/cm^2) in EGF, FGF, and Noggin. Once cells had grown for 5 days, spheres were plated in SFM (without heparin) onto poly-D-lysine-coated (PDL, Sigma, 20 µg/ml for >1 hour) coverslips within 24-well culture plates, and allowed to differentiate until flattened and adherent for 5–7 d in humidified 5% CO$_2$. This was achieved by plating with 1 ng/ml FGF for the first day and removal of FGF thereafter.. The differentiated neurospheres were then fixed with 4% formaldehyde (Sigma) in 0.1 M PBS at room temperature for 15 min. After washing with PBS, cells were double stained for the neuronal pan-neurofilament (NF, rabbit IgG, 1:4 Zymed) and the 'astrocytic' marker GFAP with Hoechst counter stain or triple stained with the neuronal marker MAP2 (mouse IgG1, 1:200, Millipore), GFAP (Rabbit IgG, 1:1000, DAKO), and oligodendrocyte marker O4 (mouse IgM, 1:200, Millipore).

Single Cell Clonal Analysis

Passage 8 hippocampal neurospheres generated in the presence of Noggin were dissociated, and sorted by flow cytometry on the basis of size and granularity at the density of 1 cell per well. Cells were collected in non-adherent 96-well plates containing growth media with or without Noggin (4 plates each). Cultures were refed with growth factors (and Noggin if needed) every 3–4 days and assayed for neurosphere formation after 14 days *in vitro*. Cell clusters greater than 50µm in diameter were counted as neurospheres. To test self-renewal, individual neurospheres were picked using a microscope, dissociated and plated in proliferation conditions including Noggin for several passages. To assess multipotentially, passage 11 individual clonally derived neurospheres were differentiated as intact spheres under conditions described above and processed for immunocytochemistry of lineage markers as described.

Immunochemistry of primary hippocampal neurospheres

Primary neurospheres were processed in suspension. Spheres were fixed with 4% formaldehyde in PBS for 30min at room temperature, blocked with 10% goat serum for 1 hr, and primary antibodies applied overnight at 4°C. Primary antibodies were visualized using Alexa-350, -488 and -594 secondary antibodies (Molecular Probes) applied for 5 hrs at room temperature. Adherent cultures were processed and all images acquired as previously

described (Bonaguidi et al., 2005). Primary antibodies were used at the same concentrations as described above.

Morphometric and statistical evaluation

P-SMAD and ID3 quantization was performed using MetaMorph software version 6.3r5 (Molecular Devices). *In* vivo, confocal z-stacks (0.8 μm optical sections of 10 μm tissue) acquired at 100x were flattened into 2.5 μm optical sections using LSM 510 Image Examiner (Zeiss) and imported into MetaMorph. Individual cells were picked in random, traced in the GCL and SGZ, and measured for signal intensity. Average intensity in GCL cells was normalized to 1. *In vitro*, 1 μm optical sections were acquired through whole neurospheres and imported into MetaMorph. For *in vivo* proliferation studies, Hoechst, BrdU, GFAP, and PSA-NCAM were acquired using 4-channel confocal microscopy. GFAP and PSA-NCAM were pseudo-colored red for easier visualization using Image Examiner software. All images were imported into Photoshop CS (Adobe) to prepare figures. Statistical evaluations for 2-group tests (unpaired, 2-tailed, student's t-test) and 3-group (ANOVA, with Bonferroni post-hoc test) were performed using Prism software (GraphPad).

Results

Noggin allows for self-renewal and multipotential differentiation of hippocampal cells

Adult neural precursors proliferate *in vitro* in the presence of EGF and/or FGF to form floating cell clusters termed neurospheres (Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1996). Neural stem cells (NSC) are distinguishable from neural progenitor cells (NPCs) by the ability to self-renew, i.e. expand continually (Bull and Bartlett, 2005). Glial fibrillary acidic protein (GFAP)-expressing cells from the anterior SVZ (aSVZ) and posterior SVZ (pSVZ), but not the hippocampus, give rise to neurospheres that continually expand (Gritti et al., 1999; Bull and Bartlett, 2005). We prepared adult EGF and FGF-responsive neurosphere cultures to investigate the effects of BMP inhibition on hippocampal and pSVZ cell self-renewal. Cells were isolated from the dorsal part of the hippocampus (Hip) or the lateral wall of the lateral ventricle (pSVZ) and cultured through multiple passages (Fig. 1F). As previously reported (Bull and Bartlett, 2005), neurospheres isolated from the hippocampus failed to expand when cultured in EGF and FGF alone (Fig. 1A,C). However, Noggin addition allowed hippocampal cultures to expand for 10 passages, the longest time studied, with more than a 1000-fold increase in cell number (Fig. 1A,D). SVZ-derived neurospheres cultured without Noggin expanded 7 times faster than Noggin-treated hippocampal neurospheres (pSVZ: 16.9x, Hip: 2.35x), but Noggin addition to pSVZ cultures did not further increase the expansion in cell numbers (Fig. 1B).

Neural stem cells possess the ability to self-renew and generate both neurons and glia. However, adult hippocampal stem/progenitor cells cultured in the presence of EGF and FGF alone predominately generate only glia, and inclusion of additional factors such as BDNF is necessary to facilitate neuronal differentiation *in vitro* (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Since we found that endogenous BMP signaling is responsible for limiting self-renewal by cultured adult hippocampal precursors, we asked whether it is also responsible for limiting neuronal lineage commitment. After 10 passages in the presence of Noggin, hippocampal neurospheres were differentiated as intact spheres for 5–7 days. The majority of neurospheres contained both neurons (neurofilament immunoreactive cells) and astrocytes (GFAP immunoreactive cells with mature morphology and loss of precursor characteristics (Bonaguidi et al., 2005) (67±5%; Fig. 1E). Further, addition of BMP4 during differentiation significantly reduced neuronal differentiation (13±3%, P=0.010; Fig. 1E).

To address the multi-lineage potential of these putative hippocampal NSCs in more detail, we asked whether clonally-derived neurospheres from a single cell are capable of generating cell types of all three neural lineages. Dissociated passage 8 hippocampal neurospheres maintained in the presence of Noggin were sorted by flow cytometry at one cell per well density into 96-well plates (Fig. 2A, arrow), and cultured with or without Noggin for 14 days. For quantification, cell clusters greater than 50μm in diameter were counted as neurospheres (Fig. 2B–C). Interestingly, we found that even after extended passages *in vitro*, hippocampal neurosphere formation remained dependent on Noggin, as the percentage of sphere forming clones was greater with Noggin than without (4.23% vs. 0.26%). Additionally, single-cell derived neurospheres were able to expand in cell number throughout subsequent passages (Fig. 2D), suggesting that these spheres possess the ability to self-renew. More importantly, differentiation of clonally derived neurospheres as intact spheres on PDL coated coverslips produced all three neural lineage cell types: neurons (Fig. 2F,I), astrocytes (Fig. 2G,I), and oligodendrocytes (Fig. 2H,I). These findings provide further evidence that the Noggin-promoted cell types in neurosphere forming conditions exhibit neural stem cell characteristics.

To further define effects of BMP signaling on self-renewal and expansion, we counted the number of spheres as well as the number of cells in each sphere. Noggin addition to primary hippocampal neurospheres had no effect on either the number of spheres that formed ($0.04 \pm 0.01\%$ vs. $0.05 \pm 0.02\%$; Fig. 3A) or the number of cells per sphere (206 ± 72 vs. 257 ± 171; Fig. 3B). However addition of Noggin to secondary hippocampal neurospheres significantly increased both sphere number ($0.45 \pm 0.08\%$ vs. $1.23 \pm 0.11\%$, P=0.0072; Fig. 3C) and cells per sphere (193 ± 74 vs. 1135 ± 269, P=0.025; Fig. 3D). By contrast, addition of Noggin to either primary or secondary pSVZ neurospheres had no effect on sphere number or on the number of cells per sphere (Figure 3).

In addition to the quantification of primary and secondary neurosphere cell numbers, we next examined the effect of Noggin on the cell cycle entry of hippocampal precursors. After 5 days with or without Noggin, hippocampal neurospheres were dissociated and labeled with the thymadine analog EdU for 4 hours. The percentage of EdU labeled cells were subsequently analyzed by fluorescent activated cell sorting (FACS). We found that cells grown in the presence of Noggin contained higher percentages of EdU labeling ($24.20 \pm 2.58\%$) compared to cells lacking Noggin exposure ($9.90 \pm 0.16\%$, P=0.029; Fig. 3E), suggesting that inhibition of BMP signaling promotes proliferation of hippocampal NSCs. The maintenance of proliferative precursors is also supported by the expression of telomerase reverse transcriptase (TERT) in the Noggin promoted hippocampal neurospheres (Fig. 3F). TERT is an essential component of telomerase activity, a well established hallmark of stem cells that is also expressed by neural precursors (Meeker and Coffey, 1997; Cai et al., 2002; Caporaso et al., 2003). We found the level of TERT in Noggin induced neurospheres was comparable to that found in the embryonic stem cell (ES) control, but we failed to detect TERT expression in hippocampal precursors grown without Noggin under the same experimental conditions. These findings indicate that BMP inhibition promotes self-renewal, proliferation, and multipotential differentiation of adult hippocampal precursor cells *in vitro* but does not alter self-renewal or expansion of cultured adult pSVZ precursor cells.

Precursors in hippocampal, but not pSVZ, neurospheres possess BMP signaling in vitro

During BMP signaling, SMAD proteins 1, 5, and 8 are phosphorylated and translocate with SMAD 4 to the nucleus. SMAD proteins act as transcription factors in the nucleus and also induce the synthesis of ID (inhibitor of differentiation or inhibitor of DNA binding) proteins 1–4. Therefore, the presence of phosphorylated-SMAD1/5/8 in the nucleus (np-SMAD) or induction of ID proteins are indicators of BMP signaling within a cell and act as key

molecules in regulating biological responses to BMPs (Miyazono and Miyazawa, 2002). First, we examined the cellular localization of p-SMAD in primary hippocampal and SVZ neurospheres to determine whether endogenous BMP signaling occurs in the cultured cells. Hippocampal neurospheres had strong np-SMAD staining in most cells, which was reduced by culturing in the presence of Noggin (88.2±2.2% vs. 31.5±3.6%, P=0.0005; Fig. 4A–B). In contrast, neurospheres from the pSVZ had np-SMAD staining in only a few cells (3.5±0.8%, P=0.0005; Fig. 4C), and the staining was observed predominantly in the cytoplasm rather than the nucleus (Fig. 4D). Next, we performed immunocytochemistry to investigate levels of ID3 staining in primary hippocampal and SVZ neurospheres. Similar to p-SMAD observations, ID3 staining was dramatically lower in primary neurospheres from the SVZ compared to hippocampal neurospheres (1.00±0.03 vs. 0.28±0.07, P=0.0002; Fig. 4F–G). Therefore, BMP signaling seems to occur largely in adult hippocampal precursors but not adult pSVZ precursors *in vitro*.

Noggin inhibits BMP signaling in SGZ precursor cells *in vivo* to amplify their numbers

We next determined whether Noggin is expressed in the SGZ *in vivo* using immunocytochemistry (Fig. 5 and supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We observed that Noggin is expressed in the GCL, SGZ, and hilus, indicating that it is appropriately localized to be involved in regulation of hippocampal neurogenesis (Fig. 5A–C) as has been previously reported at the RNA level (Fan et al., 2003; Chmielnicki et al., 2004). Neurogenesis in the adult SGZ originates from GFAP-expressing multipotential precursor cells in the SGZ and SVZ (Doetsch et al., 1997; Doetsch et al., 1999; Seri et al., 2001; Garcia et al., 2004). Activation of relatively quiescent GFAP$^+$ precursors gives rise to a rapidly dividing progenitor pool (tAP: transient amplifying progenitors). Amplifying progenitors commit to PSA-NCAM$^+$ migratory precursors that differentiate into immature neurons, mature in the GCL, and integrate into the hippocampus circuitry (for review see (Ma et al., 2005; Ming and Song, 2005)). We next sought to determine the relationship between proliferation and BMP signaling in the SGZ by examining BrdU incorporation and its relationship to (i) np-SMAD staining and (ii) BMP signaling target gene: ID3. BrdU$^-$ cells in the GCL display strong np-SMAD staining whereas BrdU$^+$ cells, and specifically BrdU$^+$GFAP$^+$ cells, demonstrate reduced np-SMAD (BrdU$^-$: 1.00±0.02, BrdU$^+$: 0.71±0.01, BrdU$^+$GFAP$^+$: 0.58±0.04, P=0.009 Fig. 5D–H). Further, ID3 is only expressed in a small population of BrdU$^+$ cells in the SGZ (22.1±1.0%, Fig. 5I–K). Thus, reduced BMP signaling is associated with proliferation in the hippocampal DG *in vivo*.

We then examined whether altering levels of Noggin expression influence hippocampal neurogenesis. To alter levels of Noggin expression we utilized transgenic mice in which the neuron specific enolase promoter drives expression of Noggin and a GFP cassette (NSE-Noggin) (Guha et al 2004; Bonaguidi et al, 2005). Transgene expression begins in the hippocampus of these animals when the GCL differentiates during postnatal development and continues into adulthood (Guha et al., 2004; Bonaguidi et al., 2005; Li and Pleasure, 2005). In addition, when cells in the granule cell layer overexpress Noggin, Noggin seems to be secreted unidirectionally towards the hilus to regulate precursors in the SGZ (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). We next sought to confirm that BMP signaling is altered in SGZ precursors by assessing ID3 expression in NSE-Noggin, wild type, and NSE-BMP4 overexpression mice. Compared to wild-type animals, the percentage of BrdU$^+$ cells that expressed ID3 were significantly decreased in Noggin animals (22.1±1.0% vs. 11.5±0.6%, P=0.0026) and increased in BMP4 animals (22.1±1.0% vs. 40.2±3.0%, P=0.0026; Fig. 5I–K). Therefore, adult SGZ precursors respond to changes in BMP levels as assessed by ID3 within our transgenic system.

To specifically test whether the level of BMP signaling determines the rate of cell proliferation in the adult SGZ precursor populations, we examined BrdU incorporation in the NSE-Noggin mice. This approach allowed examination of proliferation of the major SGZ precursor populations: GFAP$^+$ precursors – putative stem cells - (BrdU$^+$GFAP$^+$), putative transient amplifying cells (BrdU$^+$, GFAP$^-$ and PSA-NCAM$^-$), and proliferative migratory precursors cells (BrdU$^+$PSA-NCAM$^+$) (Ming and Song, 2005). Noggin overexpression significantly increased the number of overall proliferative precursors (481±18 vs. 778±61 cells/mm^2, P=0.007; Fig. 6A–B,E), proliferative GFAP$^+$ cells (104±4 vs. 229±28 cells/mm^2, P=0.010; Fig. 6A–B,E), proliferative putative transient amplifying progenitor cells (271±12 vs. 381±15 cells/mm^2, P=0.004; Fig. 6A–E), and proliferative migratory precursor cells (107±6 vs. 168±23 cells/mm^2, P=0.048; Fig. 6C–E). This increase in the number of BrdU$^+$ cells in each precursor population could reflect either enhanced proliferation of each cell type or increased proliferation of the GFAP$^+$ population that then generated increased numbers of the other proliferative precursor pools. To distinguish between these two possibilities, we examined the proportion of proliferative cells in each precursor population. Noggin did not change the proportion of proliferative migratory precursors (22.1±1.2% vs. 23.1±0.8%, P=0.382; Fig. 6F). However, the proliferative GFAP$^+$ cell fraction in the transgenic animals was significantly increased (21.5±0.2% vs. 29.2±1.5%, P=0.006; Fig. 6F) at the expense other precursor cells (56.4±1.4% vs. 47.6±1.4%, P=0.010; Fig. 6F). These results suggest that the effects of Noggin on proliferation in the SGZ occur primarily on the GFAP$^+$ putative stem cells that then, in turn, generate more proliferative precursor cells.

Noggin increases slowly dividing stem-like cell numbers in the SGZ

NSCs are considered to be a relatively quiescent cell population with long cell cycle duration. This property distinguishes stem cells from more proliferative precursor pools including transient amplifying progenitors (Doetsch et al., 1999; Seri et al., 2001). NSCs may be isolated *in situ* according to their retention of exogenous thymidine analogs after long-term survival (Morshead et al., 1994; Morshead et al., 1998). To confirm the presence of stem cells in the adult SGZ and investigate the role of Noggin on putative NSC number we performed a dual thymidine analog experiment. Wild-type and NSE-Noggin mice were given the nucleoside analog IdU in their drinking water for 2 weeks as a first marker for replicating cells. After a period of 1 week in the absence of label, the animals were administered the second nucleoside analog CldU for an additional 2 weeks followed again by the absence of label for 1 week (Fig. 7A). During the initial labeling period with IdU, both putative NSCs and proliferative progenitors will become labeled. During the 4-week period after IdU administration, this label becomes diluted in rapidly dividing cells. Two classes of cells could retain the label: slowly dividing NSCs or cells that have ceased to divide (by differentiation or cell death). To remove the latter possibility, CldU is administered as its incorporation is expected in all dividing cells, including putative NSCs. Animals were then sacrificed 1 week post CldU to allow transient amplifying cells to migrate away from the SGZ, primarily into the GCL. This strategy allows the subset of long-term label retaining cells with stem cells tendencies to be labeled a second time (Maslov et al., 2004; Bauer and Patterson, 2006).

We first tested the efficacy of the paradigm by first performing control experiments. Animals administered IdU and CldU were processed singularly with the IdU (BD, anti-BrdU) or CldU (Accurate, anti-BrdU) antibodies and both appropriate secondary antibodies. The opposite channel was devoid of staining demonstrating that we could distinguish between the two labeling periods. Also, IdU$^+$ cells were located farther within the GCL than CldU$^+$ cells, which is consistent with labeling two temporally distinct precursor populations (supplemental Fig. 2A–H, available at www.jneurosci.org as supplemental material). Next,

we evaluated differentiated tissue for the absence of dual thymidine analog cells. In the adult, neural precursors in the SVZ migrate through the rostral migratory stream (RMS) and differentiate in the olfactory bulb (OB). High numbers of $CldU^+$ or IdU^+ cells were present in the OB. However, we did not observe any cellular co-localization of CldU and IdU, confirming the absence of putative NSCs in the OB (supplemental Fig. 2I–L, available at www.jneurosci.org as supplemental material, (Ming and Song, 2005)). We then investigated whether wild type animals possessed long-term dual thymidine-labeled cells in the SGZ. Dual labeled cells were clearly discernible from single labeled cells in the SGZ (Fig. 7B), and quite low in number (17.7 ± 3.4 $CldU^+$ IdU^+ cells/mm^2). In addition, some double-labeled cells co-localized with precursor makers Sox2 and GFAP, which confirms precursor identity of dual thymidine label retaining cells (M.A.B, unpublished). Finally, we assessed the numbers of $CldU^+$ IdU^+ cells in the NSE-Noggin animals. Noggin overexpression significantly increased the number of label retaining cells in the SGZ compared to wild-type controls ($29.7\pm2.1\%$, P=0.040). Thus, this study suggests that neural stem cells are present in the SGZ and their numbers are expanded by Noggin overexpression.

Discussion

This work provides *in vitro* and *in vivo* evidence that the adult hippocampus contains NSCs, which may be revealed through BMP inhibition. In single cell and low density cultures, high levels of BMP signaling occur endogenously in neurospheres derived from hippocampal precursors. Inhibiting BMP signaling with Noggin is sufficient to foster long-term hippocampal cell self-renewal, increased proliferation, and multipotential differentation. In the SGZ, BMP signaling in precursors is limited by Noggin production. Overexpression of Noggin expands the overall precursor pool by increasing NSC numbers, which were assessed as GFAP-expressing and relatively quiescent cell populations.

This study, in part, investigated whether a local stem cell or non-self renewing progenitor is the source of constitutive neurogenesis in the adult hippocampus. The uncertainty largely originates from differences in methodologies for harvesting and growing precursors *in vitro* (summarized by (Babu et al., 2007)). Contentions have arisen from more recent studies using neurosphere cultures (Bull and Bartlett, 2005; Walker et al., 2008) that hippocampal monolayer preparations of earlier studies (Gage et al., 1995; Palmer et al., 1997) may be contaminated by stem cells from the adjacent SVZ. Our findings directly recapitulates the finding by Bull and Bartlett (2005) that hippocampal precursors do not self-renew as neurospheres when cultured in EGF and FGF alone, and both studies support similar observations by Seaberg and van der Kooy (2002). However, the self-renewing cells that were revealed in our hippocampal cultures by Noggin treatment likely do not represent contamination with cells from the pSVZ since (i) hippocampal precursors and pSVZ precursors expand with different growth kinetics; (ii) endogenous BMP signaling differs between the precursor populations (Fig. 1,3) and (iii) pSVZ precursors expand without the need for Noggin whereas the hippocampal precursors required Noggin for expansion.

Likewise, another potential source of NSCs may have originated from subcortical white matter. Although surrounding white matter was carefully removed during our hippocampal dissection, we can not claim with 100% certainty whether $NG2^+$ progenitors remain in our cultures. $NG2^+$ white matter oligodendrocyte progenitor cells (OPCs) form neurospheres and undergo multilineage differentiation *in vitro* (Belachew et al., 2003; Nunes et al., 2003; Aguirre et al., 2004). However, unlike our Noggin preparations, these $NG2^+$ cells lack long-term self-renewal and TERT expression (Fig. 1–3, (Nunes et al., 2003)). While it remains possible that noggin 'reprograms' OPCs, prior studies have demonstrated that applying BMP4 or a function blocking antibody against noggin actually increases OPC plasticity (Kondo and Raff, 2000; Hampton et al., 2007). Ultimately, we believe that SGZ cells, and

not SVZ or OPC, were the origin of expanding neurospheres in our cultures. Meanwhile, a recent report found that cultured hippocampal but not pSVZ NSC expand in response to depolarization promoted by KCl addition (Walker et al., 2008). Therefore, while it remains to be determined whether the cultures from earlier monolayer and later neurosphere studies have similar cell sources, means to distinguish between SVZ and hippocampal origin are becoming more prevalent. Nonetheless, this and other recent studies now conclude that the adult hippocampus contains NSC populations as revealed by long term culture under modified conditions (Babu et al., 2007; Walker et al., 2008).

However, the *in vivo* identities of cells capable of undergoing long-term self-renewal *in vitro* or *in vivo* still require clarification since a few cell sources have been suggested. SVZ precursors tagged *in vivo* by a GFAP-promoter driven virus or transgene are capable of neurosphere formation and propagation. Meanwhile, GFAP-expressing cells are considered relatively quiescent, remain after removing rapidly dividing cells and contribute to neurogenesis *in vivo* and *in vitro* (Doetsch et al., 1999; Seri et al., 2001; Garcia et al., 2004; Seri et al., 2004). Taken together, GFAP$^+$ radial glia-like cells in the SVZ and SGZ have been postulated as one source of NSC. Recently, Nonradial Sox2$^+$ GFAP$^-$ non-quiescent cells were hypothesized as multipotent and self-renewing cells in the SGZ that give rise to the radial cells (Suh et al., 2007). To identify putative NSCs *in vivo*, we administered thymidine analogs for 14 days each with 7 days water after each exposure (Fig. 7). This additional exposure length allowed for further dilution of the analog acquired by rapidly dividing precursors in case the estimated cell cycle time for NSC in the SGZ is longer than those in the SVZ (Morshead et al., 1998). Interestingly, ~50% of dual-labeled cells were positive for SOX2 and a smaller percentage for GFAP in wild-type and NSE-Noggin animals (M.A.B., unpublished observations). Since we did not have a tag to identify the morphology of SOX2$^+$ cells, we were unable to confirm if SOX2$^+$ cells contain a radial process. Therefore, it seems plausible that the label-retaining cells are a heterogeneous population, which may contain subpopulations of the two aforementioned cells types and possibly other postulated (Aguirre et al., 2004; Yamashima et al., 2004) or unidentified ones. Once definitive markers of NSCs become available, NSC identity and relatively quiescence may be elucidated as has been demonstrated in other stem cell niches (Kiel et al., 2005).

This study also demonstrates that removal of adult hippocampal precursors from niche components alters their capacity to self-renew. Similarly, hematopoietic stem cells (HSCs) rapidly lose the ability to self-renew when placed in culture and they do not regain it when they are subsequently used in reconstitution assays *in vivo*. However, isolation and direct cell transplantation of HSCs into lethally irradiated animal rebuilds the entire immune system of the first and subsequent hosts (Lemischka et al., 1986; Spangrude et al., 1988; Osawa et al., 1996). Thus, HSC culture conditions do not properly recapitulate the niche in which the cell normally resides and leads to loss of HSC potential. We show here that this is also relevant for studying precursors from the adult hippocampus. Precursors removed from their niche do not self-renew *in vitro* unless BMP signaling is inhibited (Fig. 1–3). Importantly, this inhibition must be continually maintained to allow repeated self-renewal, otherwise the stem cell population is lost. This observation implicates BMP signaling as a crucial regulator of neurogenesis decline during hippocampal aging (Kuhn et al., 1996).

Additionally, cell fate choices are altered through removal from the niche. SGZ precursors *in vivo* preferentially commit to neurons instead of glia, but hippocampal precursors *in vitro* preferentially differentiate into astrocytes (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005; Lie et al., 2005). We established that endogenous BMP signaling is responsible, at least in part, for the bias of cultured hippocampal precursors towards astrocytic differentiation since Noggin addition enhanced neuronal lineage commitment

(Fig. 1–2). It is likely that critical niche components that promote neurogenesis and/or inhibit gliogenesis are missing within current NSC culture assays (Hagg, 2005; Ma et al., 2005). As the roles of extrinsic factors in regulating self-renewal, proliferation, fate commitment, and differentiation are elucidated, *in vitro* assays may better reflect their *in vivo* counterparts.

Further, we demonstrate that BMP signaling is a key niche regulator of NSC in the adult hippocampus. BMP4 and Noggin are both expressed in the SGZ and GCL *in vivo* in a scattered non-uniform distribution (Fig. 5; (Fan et al., 2003)). BMPs are produced by precursors themselves, which likely regulate proliferation in an autocrine/paracrine manner (Fig. 3–4). However, BMP signaling in SGZ proliferative cells is reduced relative to non-proliferative cells in the GCL (Fig. 5). Noggin is produced by cells in the GCL and is in close juxtaposition to cells with different levels of BMP signaling suggesting that the adult SGZ neurogenic niche occurs in focal locations as opposed to the entire GCL/hilus border. Since BMPs regulate proliferation, this hypothesis is reinforced by the observations that proliferative precursors are located in the SGZ as focal clusters even after a long (12- or 14-day) period of daily BrdU exposure (Fig. 7, (Kempermann et al., 1998)). Also, largely non-proliferative areas of CA1 and CA3 have higher levels of np-Smad1/5/8 than dentate (M.A.B., unpublished observations, (Colak et al., 2008)). This suggests a model in which the generation of neuronal precursors that synthesize BMP4 locally in the SGZ increases to the point at which the effects of Noggin are negated and proliferation ceases. Such a feedback mechanism could provide a way of regulating neurogenesis by either increasing Noggin synthesis or decreasing BMP synthesis in response to environmental cues (K.T.G., unpublished observations).

The robust effects of Noggin overexpression in the hippocampus (this study and (Bonaguidi et al., 2005)) suggest that BMP signaling is a key regulator of neurogenesis intensity. BMP signaling regulates neural stem cell number, proliferation, and cell fate commitment in the adult dentate and SVZ (Fig. 6–7, (Lim et al., 2000)). It is therefore a good candidate for molecular interventions to define the functional roles of adult neurogenesis in the young and aged hippocampus. Since neurogenesis is correlated with hippocampus-dependent learning, and may be involved in depression and epilepsy (Parent et al., 1997; Gould et al., 1999; Shors et al., 2001; van Praag et al., 2002; Santarelli et al., 2003; Raber et al., 2004), pharmacologic manipulation of BMP signaling in the dentate gyrus may potentially exert profound effects on hippocampal function and behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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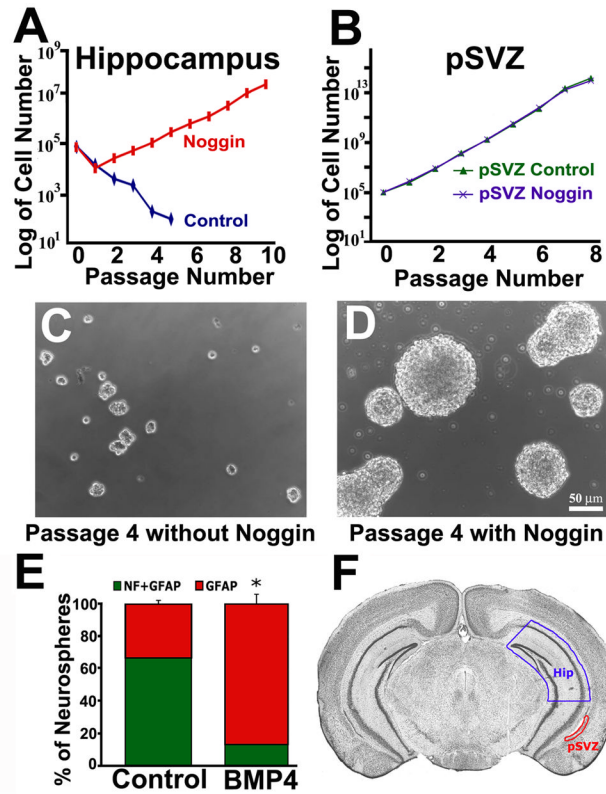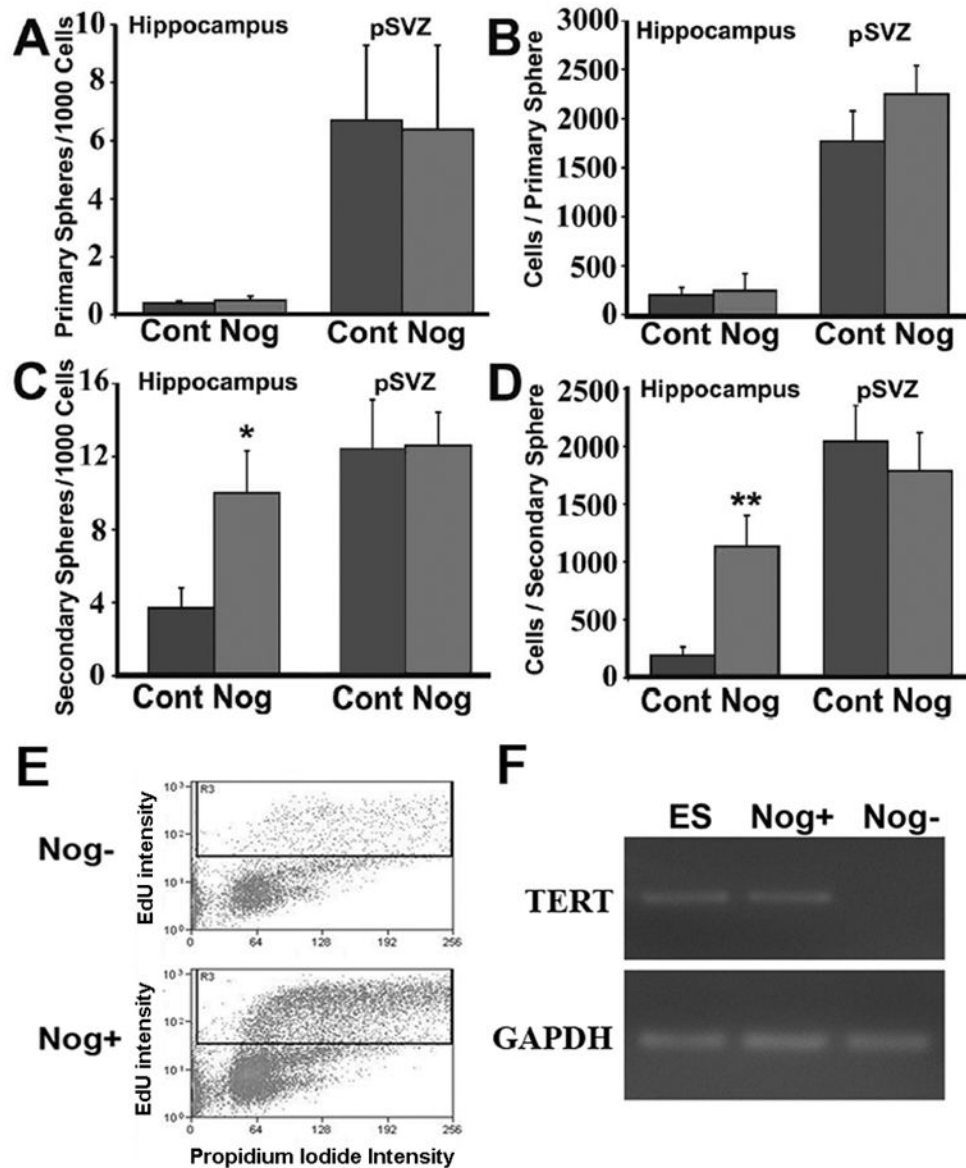
Figure 1.
Noggin allows for hippocampal cell self-renewal and multipotential differentiation. (F) 2 mm thick coronal sections were prepared from adult brain and placed caudal side up. The dorsal hippocampus (Hip) was isolated followed by the posterior subventricular zone (pSVZ) region. (A, C) Neurosphere cultures derived from the hippocampus fail to expand cell numbers in the presence of EGF and FGF through multiple passaging. (A, D) Hippocampal cultures grown with Noggin addition expand for over 10 passages. (B) Neurospheres derived from pSVZ expand at a faster rate than do hippocampal neurospheres with Noggin addition. Noggin addition to pSVZ cultures does not affect expansion cell numbers. (E) Passage 11 hippocampal spheres expanded in Noggin were plated for differentiation with or without BMP addition. The number of spheres possessing both neurons (pan-neurofilament (NF) immunofluorescence) and glia (glial fibrillary acidic protein (GFAP) immunofluorescence) or glia only was counted. While most neurospheres expanded with Noggin generate both neurons and glia, BMP addition during differentiation inhibits neuronal differentiation and promotes more glia-only spheres. Error bars (in E) represent mean ± SEM of three experiments. *P=0.01 by unpaired Student's t-test. (A–D) Hip (n=4). pSVZ (n=4). Panel (F) is modified from Paxinos and Franklin (2001), with permission.
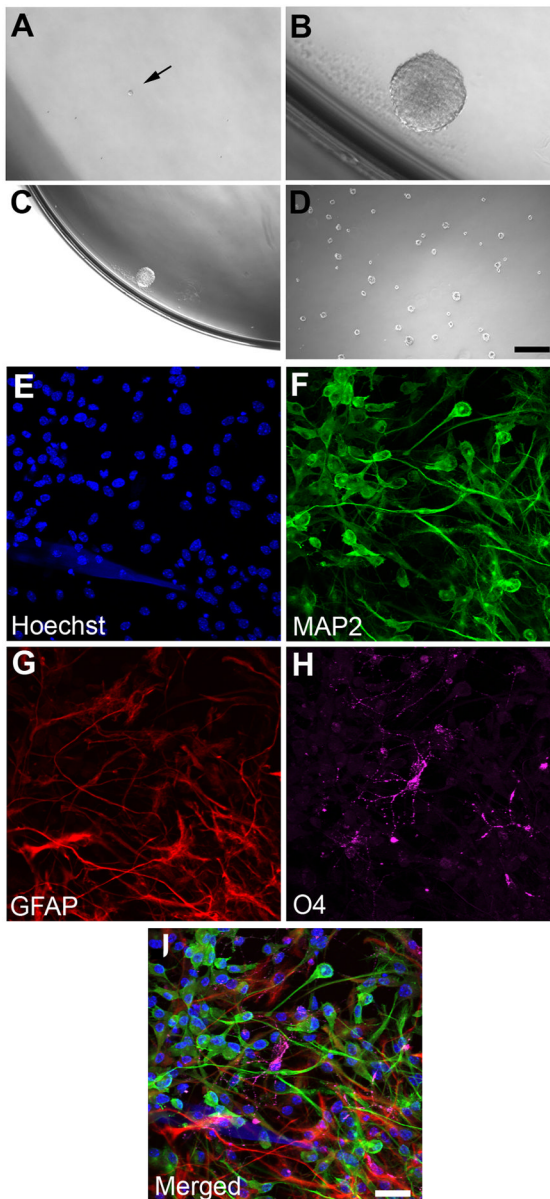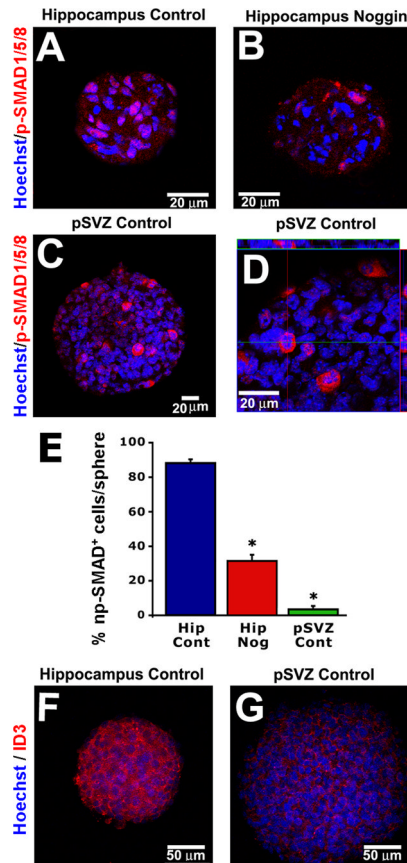
Figure 2.
Single-cell derived, Noggin promoted hippocampal progenitor gives rise to multiple neural lineages. (A) Representative single cell (arrow) after sorting by flow cytometry based on size and granularity. (B) Representative neurosphere cultured in the presence Noggin after 14 day. Only cell clusters greater than 50µm were considered as neurospheres. (C, D) Expansion of a Noggin induced neurosphere. (C) Single neurosphere after 14 days prior to dissociation; (D) additional neurospheres were observed 3 days after the second passage. Percentages of single cell clones that form neurospheres are higher in the presence of Noggin (4.43%) compared to Noggin withdrawal (0.26%). (E–I) A single clonally derived neurosphere is capable of generating MAP2+ neurons (F), GFAP+ astrocytes (G), and O4+ oligodendrocytes (H) after 7 days *in vitro* under differentiation conditions. Scale bar: 50µm in A and B; 200µm in C and D; 25µm in E–I.
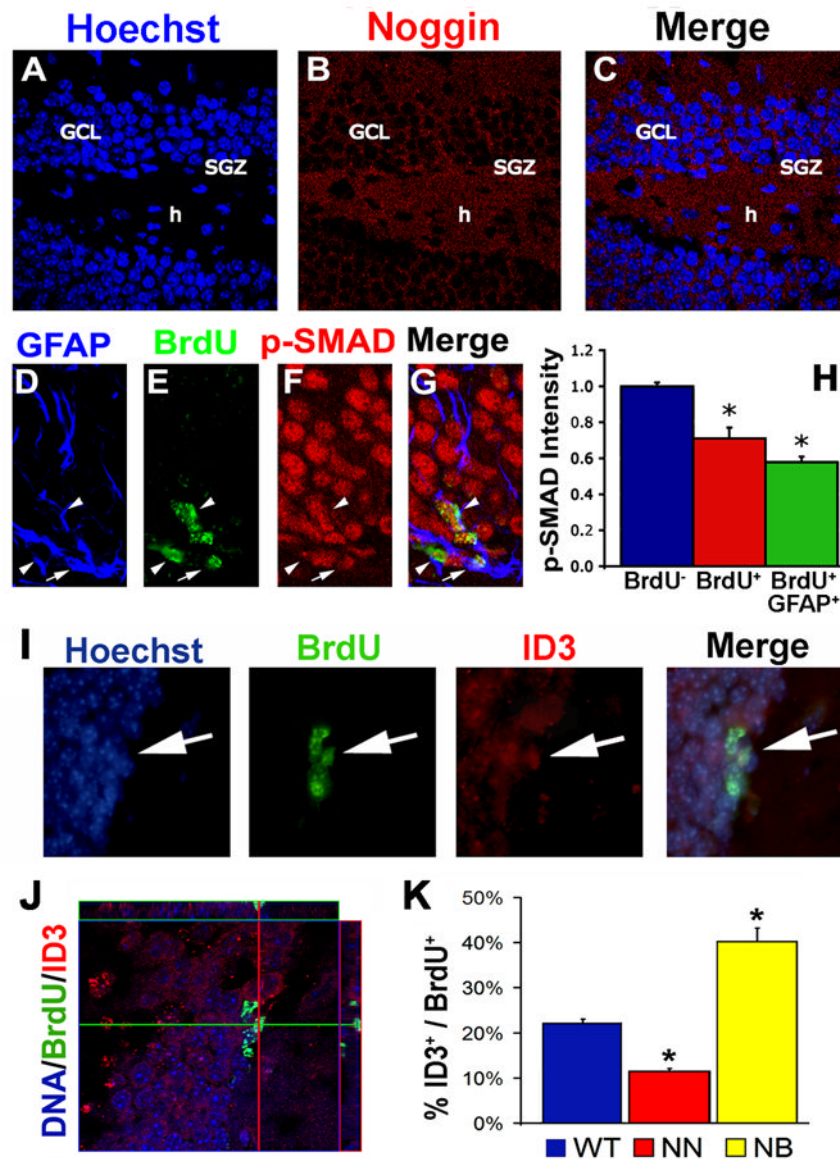
Figure 3.
Noggin maintains stem cell characteristics in neurospheres. (A, B) Primary spheres derived from the pSVZ are more numerous and contain more cells than do spheres derived from the Hippocampus. Noggin does not affect the number of primary neurospheres or the number of cells per primary neurosphere derived from both the pSVZ and Hippocampus. (C, D) Noggin addition significantly increases the number of secondary hippocampal neurospheres as well as the number of cells per neurosphere. However Noggin does not affect either the number of secondary pSVZ neurospheres or the number of cells per pSVZ neurosphere. (E) Noggin increases the number of progenitors in cell cycle. 4 hour labeling experiments with the thymadine analog EdU (10μM) and FACS analysis reveals a higher percentage of labeled cells in Noggin exposed neurospheres (24.20±2.58%) compared to cells with Noggin withdrawal for 5 days (9.90±0.16%; n=3). (F) Representative RT-PCR analysis of TERT expression in mouse embryonic stem cells (ES), Noggin induced hippocampal neurospheres cultured with (Nog+) or without (Nog-) Noggin for 5 days (n=3). TERT expression is

detected in the Noggin induced hippocampal neurospheres. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. Cont: control; Nog: Noggin. Error bars represent mean ± SEM, Hip (n=4). pSVZ (n=4). *P< 0.01, **P< 0.03 by unpaired Student's t-test.
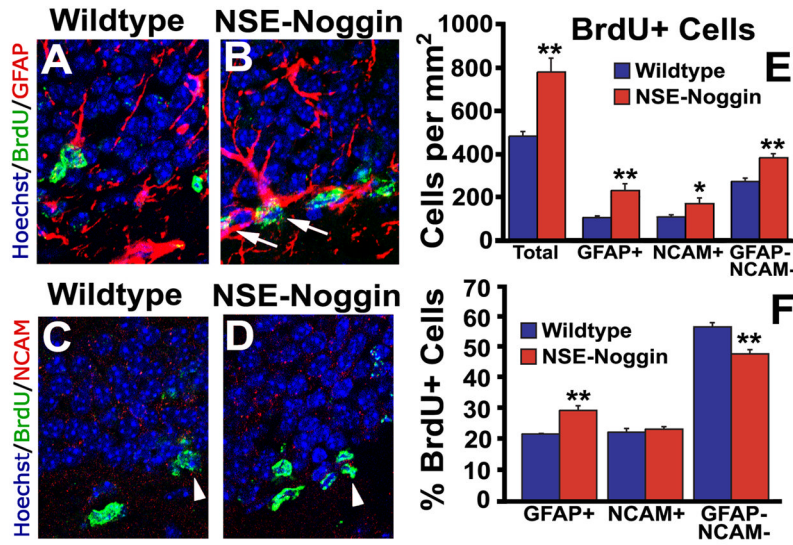
Figure 4.
Strong BMP signaling occurs in hippocampal progenitors, but not pSVZ progenitors, *in vitro*. (A) Primary hippocampal neurospheres grown in EGF and FGF for 8 days exhibit prominent nuclear phospho-SMAD1/5/8 (np-SMAD). (B) Noggin addition reduces np-SMAD staining in hippocampal neurospheres. (C) pSVZ primary neurospheres do not display strong np-SMAD staining. (D) pSVZ spheres do have p-SMAD staining in the cytoplasm of some cells, but not the nucleus as demonstrated by confocal orthogonal views (E) Quantification of cell percentage per sphere that exhibit np-Smad. (F–G) Primary hippocampal neurospheres also have significantly higher staining intensity for the BMP target gene ID3 compared to pSVZ primary neurospheres (1.00 ± 0.03 vs. 0.28 ± 0.07, $P=0.0002$). Error bars represent mean $\pm$ SEM of multiple spheres. *$P<0.001$ by ANOVA with the Bonferroni post hoc test, **$P<0.0005$ by unpaired student's t-test.
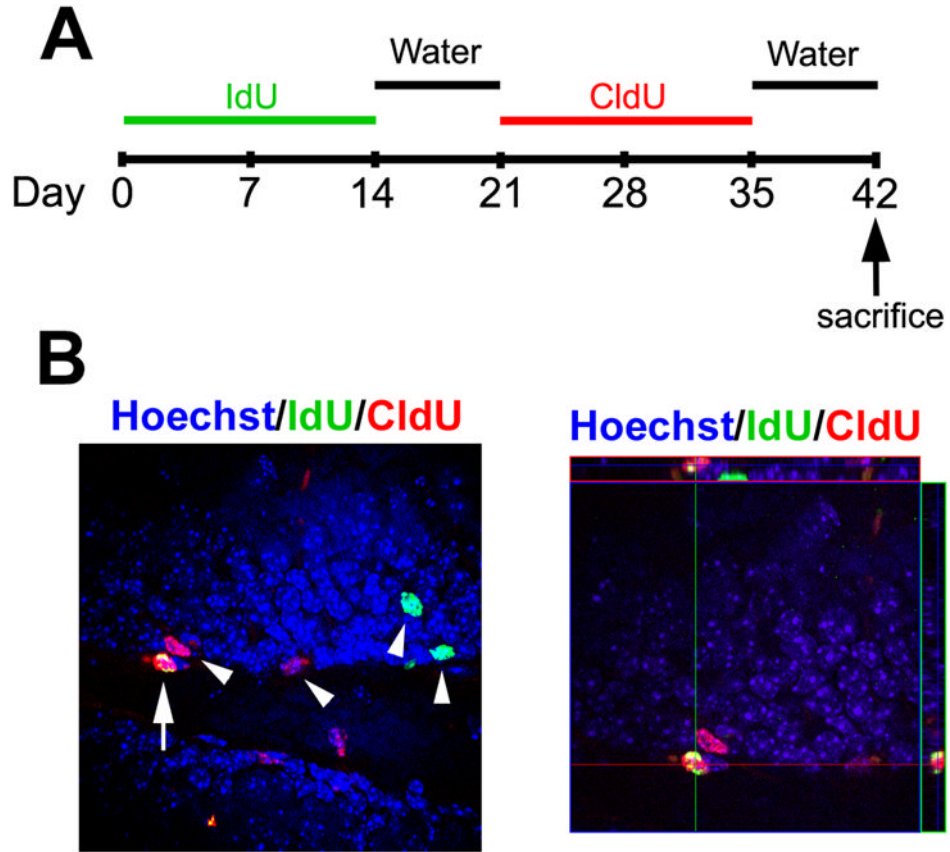
Figure 5.
BMP signaling is present, but largely inhibited in SGZ precursor cells *in vivo*. (A–C) Noggin protein is expressed in the SGZ, GCL, and hilus of the adult dentate gyrus. BMP signaling was assessed by immunofluorescence for the signal transducer phospho-SMAD 1/5/8 (D–H) and target gene ID3 (I–K) in precursor cells labeled with BrdU for 3 days. (D–H) Np-SMAD is decreased in proliferating cells in the SGZ and specifically within GFAP⁺BrdU⁺ cells. Arrows point to BrdU⁺GFAP⁺ cells, arrowheads to BrdU⁺GFAP⁻ cells. (I–K) ID3 reveals the presence of BMP signaling in some SGZ precursor cells (% of BrdU⁺ cells that co-express ID3). (J) Confocal orthogonal views demonstrate the co-localization of ID3 and BrdU. (K) The percentage of SGZ precursors that express ID3 increases in BMP-overexpression (NSE-BMP4) animals and decreases in Noggin overexpression (NSE-Noggin) animals. Arrows point to ID3⁺BrdU⁺ cells. GCL: granule cell layer; SGZ: subgranular zone; h: hilus; np-SMAD: nuclear phosho-SMAD1/5/8; NSE: neuron specific enolase; NN: NSE-Noggin; WT: wild-type; NB: NSE-BMP4. Error bars represent mean ± SEM of at least 20 cells from 3 separate wild-type mice (H) or 3 mice of each genotype (K).

*Differs from the BrdU⁻ group at P<0.01 (H) or wild-type group at P<0.005 (K) by ANOVA with the Bonferroni post hoc test.

Figure 6.
Noggin increases stem/progenitor cell proliferation. Noggin was overexpressed under the control of the NSE promoter. (A–E) BrdU labeling and immunofluorescence reveals an increase in multiple precursor populations: GFAP[+] putative stem cells, PSA-NCAM[+] migratory precursors, and GFAP[−] PSA-NCAM[−] putative transient amplifying progenitors. (F) Although all three precursor populations are expanded, the proportion of GFAP[+] putative stem cells is increased at the expense of other precursors. Arrows point to GFAP[+] BrdU^{+} cells, arrowheads to PSA-NCAM[+] BrdU^{+} cells. NCAM: PSA-NCAM. Error bars represent mean $\pm$ SEM of three experiments *$P<0.05$; **P 0.01 by unpaired Student's t-test.

Figure 7.
Noggin increases relatively quiescent stem cell numbers in the SGZ. (A) Adult wild-type and NSE-Noggin mice were administered two thymidine analogs orally for 14 days each and regular drinking water for 7 days after each analog before euthanasia. (B) Putative quiescent stem cells (IdU⁺CldU⁺, arrows) are distinguishable from more committed progeny (IdU⁺CldU⁻ or IdU⁻CldU⁺, arrowheads) in the SGZ. Noggin overexpression significantly increased the number of label retaining cells in the SGZ ($29.7 \pm 2.1\%$ CldU⁺ IdU⁺ cells/mm²; $P=0.040$) compared to wild-type controls (17.7 ± 3.4 CldU⁺ IdU⁺ cells/mm²).