LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells

Michael A. Bonaguidi*, Tammy McGuire, Min Hu, Lixin Kan, Jayshree Samanta and John A. Kessler

Davee Department of Neurology, Northwestern University's Feinberg School of Medicine, Chicago, IL 60611, USA
Author for correspondence (e-mail: m-bonaguidi@northwestern.edu)

Accepted 13 October 2005

Development 132, 5503-5514
Published by The Company of Biologists 2005
doi:10.1242/dev.02166

Summary

Bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) signaling both promote the differentiation of neural stem/progenitor cells into glial fibrillary acidic protein (GFAP) immunoreactive cells. This study compares the cellular and molecular characteristics, and the potentiality, of GFAP$^+$ cells generated by these different signaling pathways. Treatment of cultured embryonic subventricular zone (SVZ) progenitor cells with LIF generates GFAP$^+$ cells that have a bipolar/tripolar morphology, remain in cell cycle, contain progenitor cell markers and demonstrate self-renewal with enhanced neurogenesis – characteristics that are typical of adult SVZ and subgranular zone (SGZ) stem cells/astrocytes. By contrast, BMP-induced GFAP$^+$ cells are stellate, exit the cell cycle, and lack progenitor traits and self-renewal – characteristics that are typical of astrocytes in the non-neurogenic adult cortex. In vivo, transgenic overexpression of BMP4 increases the number of GFAP$^+$ astrocytes but depletes the GFAP$^+$ progenitor cell pool, whereas transgenic inhibition of BMP signaling increases the size of the GFAP$^+$ progenitor cell pool but reduces the overall numbers of astrocytes. We conclude that LIF and BMP signaling generate different astrocytic cell types, and propose that these cells are, respectively, adult progenitor cells and mature astrocytes.

Key words: GFAP, Glia, Astrocyte, Neural stem cell, Leukemia inhibitory factor, Bone morphogenetic protein, Noggin

Introduction

Neural stem/progenitor cells (NSCs) in the early embryonic ventricular zone (VZ) do not express GFAP, the classical astrocytic marker, but, by late embryonic development, NSCs do begin to express it (Imura et al., 2003). In the adult brain, GFAP$^+$ neurogenic progenitors exist in the subventricular zone (SVZ) of the lateral ventricle (Altman, 1969; Doetsch et al., 1999) and the subgranular zone (SGZ) (Altman and Das, 1965; Seri et al., 2001) of the hippocampal dentate gyrus. Thus, some GFAP$^+$ cells in the postnatal brain have stem cell potential (Imura et al., 2003; Laywell et al., 2000; Morshead et al., 2003), and a morphologically distinct subpopulation of GFAP$^+$ cells is the predominant source of constitutive adult neurogenesis (Garcia et al., 2004). However, most GFAP-expressing cells in the generative zones, as well as GFAP-expressing astrocytes outside of these zones, do not act as progenitors in the normal adult brain (Laywell et al., 2000). It is currently unclear how these mature astrocytes are derived, and the lineage relationships between GFAP$^+$ progenitors and mature astrocytes are not well defined.

NSCs express GFAP in response to several signaling molecules, including the leukemia inhibitory factor (LIF)/ciliary neurotrophic factor (CNTF) and BMP families (Gross et al., 1996; Johe et al., 1996). Canonically, LIF/CNTF activates the JAK/STAT pathways, whereas BMPs signal primarily through SMAD pathways. Nevertheless, their signaling pathways have points of convergence in the regulation of GFAP, leading to suggestions that these cytokine families activate astrogliogenesis through the same mechanisms (Nakashima et al., 1999a; Sun et al., 2001). However, whereas BMP signaling promotes the generation of astrocytes from SVZ forebrain stem cells both in vitro and in vivo (Gomes et al., 2003; Gross et al., 1996), LIF signaling inhibits the restriction of early embryonic forebrain stem cells to a glial lineage and helps to maintain a stem cell phenotype (Shimazaki et al., 2001). Furthermore, BMP2 treatment of progenitor cells cultured from LIFR$^{-/-}$ animals induces astrogliogenesis (Koblar et al., 1998), indicating that signaling from this receptor is not necessary for the generation of astrocytes. It is currently unclear whether LIF and BMP signaling generate GFAP-expressing cells with similar characteristics and developmental potential. We therefore used a combined in vitro and in vivo approach to compare the properties of GFAP-expressing cells that are generated in response to LIF versus BMP signaling. Our findings suggest that LIF signaling induces GFAP$^+$ progenitor cells, whereas BMP signaling promotes a mature astrocyte phenotype that lacks stem/progenitor cell potential.

Materials and methods

Animals

The generation of NSE-BMP4 and NSE-Noggin transgenic mice is described elsewhere (Gomes et al., 2003; Guha et al., 2004). Timed-pregnant CD1 mice were obtained from Charles River (Wilmington, MA).

Immunochemistry

Postnatal day 15 (P15) brains were fresh-frozen on dry ice, cut into 10 μm coronal sections, fixed in 4% paraformadehyde (PFA), and 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 were as follows: Ki67 (rabbit polyclonal, 1:1000; Novocastra), GFAP (mouse IgG1 or rabbit polyclonal, 1:400; Sigma), vimentin (mouse IgM, 1:4; Developmental Studies Hybridoma Bank). Primary antibodies were visualized with mouse or rabbit Cy2- or Cy3-conjugated secondary antibodies (Jackson Laboratories). Nuclei were counterstained with Hoechst 33342 (Sigma). Cells were counted in the dentate SGZ (a two-nucleus-wide band below the apparent border between the GCL and the hilus and inner third of the GCL) and ML (superior/dorsal to the GCL), and normalized to the area analyzed in mm^2 (Kempermann et al., 2003).

Bromodeoxyuridine (BrdU) labeling

BrdU (10 mM) was added to differentiating neural cells on day 6, and processed on day 7 after 16 hours. Cells were fixed with 4% PFA and processed with 2N HCl for 45 minutes, then 0.1 M Borax (pH 8.5) for 15 minutes before immunochemistry.

Immunochemistry of cultures

Prior to PFA fixation, 15 μl/ml O4 (mouse IgM, Chemicon) and 5 μg/ml LeX/CD15 (mouse IgM, clone MMA; BD Biosciences) were added to cells for 30 minutes at 4°C. Fixed coverslips were blocked with serum for 45 minutes and incubated with primary antibodies at room temperature for 2-3 hours. Antibodies were as follows: BrdU (mouse IgG2a, 1:1000; clone BU-1, Chemicon), βIII-tubulin (mouse IgG2b, 1:400; Sigma), SOX1 (rabbit polyclonal, 1:1000; a kind gift from Dr Hisato Kondoh, Osaka University). Primary antibodies were visualized with Alexa 647- (infrared) Alexa 555/594- (red), Alexa 488- (green) and Alexa 350- (blue) conjugated secondary antibodies (Molecular Probes). Cells were counted in seven alternate fields of each coverslip and verified in a minimum of three independent experiments.

Generation of progenitor cell neurospheres and differentiation cultures

The ganglionic eminences of E18.5 mice were dissociated and grown in serum-free medium (SFM) with EGF (20 ng/ml, human recombinant, Biosource) for 7 days, as previously described, to generate neurospheres (Mehler et al., 2000; Zhu et al., 1999). Primary spheres were grown for 3-4 days in vitro (DIV), and then passaged by dissociating with 0.25% trypsin (Invitrogen) for 2 minutes followed by incubation with a soybean trypsin inhibitor (Sigma), a 5-minute spin, and repeated trituration. Secondary spheres were grown for an additional 3-4 DIV and used for subsequent studies. For differentiation studies, neurospheres were dissociated and plated at a density of 1×10^4 cells/cm^2 onto poly-D-lysine-coated (PDL, Sigma, 20 μg/ml for >1 hour) coverslips within 24-well culture plates, and then grown for 7 DIV in SFM plus 2 ng/ml EGF and 250 ng/ml Noggin (R&D Systems), 20 ng/ml LIF (Chemicon) or LIF+Noggin, or 20 ng/ml BMP4 (R&D Systems). Cells were re-fed on day 3.

Retrovirus production and neurosphere infection

The EGFP-N1 cassette (Clontech) was cloned into the *Bgl*II and *Bst*BI sites on the pLXRN retrovirus shuttle vector (Clontech) and replaced the G418r cassette. The rat 1.9 kb GFAP promoter (Sun et al, 2001) was partially digested and inserted into the *Hpa*I and *Bgl*II shuttle sites, which excised the P_{RSV}. Virus was packaged by co-transfecting the shuttle (rGFAPp-EGFP or control P_{RSV}-EGFP) with VSVG into GP2-293 cells (Clontech), using Lipofectamine 2000 (Invitrogen). Supernatant collected on days 2, 3 and 6 was concentrated 1000× and stored at –80°C until use. Secondary neurospheres were passaged, infected with 15 μl virus in 10 ml medium the day following dissociation, cultured and passaged once more before plating for differentiation.

Fluorescent-activated cell sorting (FACS) and neurosphere-forming assay

Plated cells were harvested, resuspended at a density of 1×10^6 cells/ml in SFM, and sorted on the basis of forward-side scatter and GFP expression at 1000 events/second. Sorted cells were plated at a density of 1000 cells/well into non-adherent 96-well plates containing SFM plus 20 ng/ml EGF, or 2 ng/ml EGF plus 20 ng/ml LIF where denoted. Cell survival was >85% by Trypan Blue exclusion analysis. The numbers of free-floating spheres were counted at day 7 in a minimum of three independent experiments.

RT-QPCR (reverse transcriptase-quantitative polymerase chain reaction)

Plated cells were treated with cytokines for 20 hours before harvesting RNA using RNeasy, according to the manufacturer's protocol (Qiagen). Reverse transcriptase (RT) was performed using Thermoscript (Invitrogen), and QPCR using Platinum SYBR Green (Stratagene). Specificity of the PCR reaction was confirmed by running PCR products on a 2% agarose gel. Two replicates were run for each cDNA sample with the test and control primers. An amplification plot showing cycle number versus the change in fluorescent intensity was generated by the Sequence Detector program (Applied Biosystems).

Results

LIF and BMP both promote GFAP expression by cultured NSCs, but induce different morphologies

Embryonic NSCs proliferate in vitro in the presence of FGF2 and/or EGF to form floating cell clusters termed neurospheres (Reynolds and Weiss, 1996). We prepared E18.5 EGF-responsive neurosphere cultures to investigate the mechanisms generating different populations of $GFAP^+$ cells. BMP4 is expressed endogenously (Fig. 1A) by neurosphere cells; BMP7 and BMP2 are also produced but at lower levels (Gross et al., 1996; Nakashima et al., 1999b). Endogenous BMPs regulate GFAP expression by these cells, and treatment with the BMP inhibitor noggin decreases GFAP transcripts fourfold (Fig. 1B,C). Thus, study of the effects of LIF signaling independent of BMP signaling requires the inhibition of endogenous BMPs. In fact, treatment with noggin decreases GFAP transcript levels twofold in the presence of LIF relative to LIF alone (Fig. 1B). Treatment with LIF increases levels of GFAP transcripts 16-fold, whereas treatment with BMP4 increases them more than 32-fold (Fig. 1B). Changes in the number of cells that express GFAP parallel the changes in levels of GFAP transcripts (Fig. 1G). Treatment with noggin significantly reduces the number of $GFAP^+$ cells ($P<0.05$). Conversely, LIF treatment increases the number of $GFAP^+$ cells ($P<0.05$), and BMP4 treatment results in an even larger increase, such that more than 90% of BMP4-treated cells express GFAP ($P<0.001$).

Although treatment with LIF or BMP4 each increases the number of cells expressing GFAP, the morphologies of those cells differ drastically (Fig. 1D-F,H-J). Treatment with LIF alone promotes a mixture of elongated bipolar/tripolar and stellate morphologies (Fig. 1E). However, when noggin is included to inhibit endogenous BMP signaling, LIF treatment promotes the bipolar/tripolar morphology at the expense of a stellate one (Fig. 1F,I). By contrast, treatment with BMP4 leads to cells with a stellate morphology characteristic of some

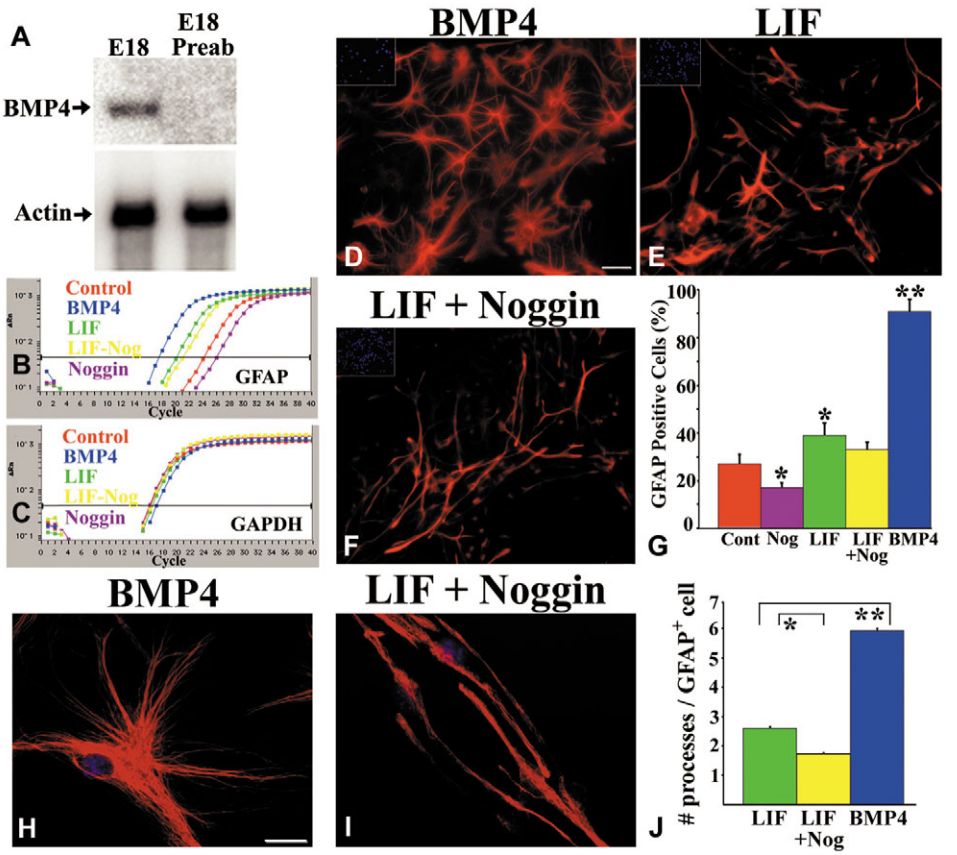


Fig. 1. LIF and BMP both increase GFAP expression but promote different morphologies. (A) Western analysis demonstrates that endogenous BMP4 is present in E18.5 EGF-responsive neurosphere cultures. Preabsorption of the antibody with BMP4 (preabsorb) eliminates the band. (B,C) Quantitative PCR analysis illustrates that noggin inhibition of endogenous BMP decreases GFAP levels, whereas exogenous LIF and BMP4 increase GFAP levels in dissociated neurospheres plated for a 20-hour differentiation. Note that BMP treatment produces the highest level of GFAP. (D-F,H,I) Immunofluorescence reveals that 7-day LIF and BMP4 treatments promote different GFAP-expressing cell morphologies. Red, GFAP; blue insets, Hoechst nuclear counter stains. (D,H,J) BMP4 treatment produces a stellate morphology with an increased process number. (E,J) LIF treatment alone induces a mixture of elongated bipolar/tripolar and stellate morphologies. (F,I,J) In the presence of noggin, LIF promotes the bipolar/tripolar morphology and reduces process number. (G) Quantitation of GFAP immunofluorescent cells. Noggin treatment significantly reduces the number of GFAP$^+$ cells. Conversely, LIF, and LIF plus noggin, treatment increases the number of GFAP$^+$ cells and BMP4 treatment results in an even larger increase. (G) *P<0.05, **P<0.001; (J) *P<0.01, **P<0.001 ANOVA. Error bars are ±s.e.m. Scale bars: 20 μm in D-F;10 μm in H,I.

mature astrocytes (Fig. 1D,H). GFAP$^+$ cells generated after BMP4 treatment had an average of six major processes per cell, whereas GFAP$^+$ cells generated in the presence of LIF and noggin have an average of slightly more than two major processes per cell (Fig. 1J).

BMP4 but not LIF prompts exit of GFAP$^+$ cells from cell cycle

To determine whether GFAP$^+$ cells generated by BMP4 and LIF signaling differ with respect to their ability to enter the S-phase of the cell cycle, the cells were labeled with a long (16 hour) pulse of BrdU on day 6 of the 7-day differentiation protocol. In control cultures, a small number of GFAP$^+$ cells were labeled with BrdU (18.9±2.6%, Fig. 2A,F). Treatment with BMP4 decreased BrdU incorporation to an almost quiescent state [2.6±0.4%, P<0.01; Fig. 2E,F (Gross et al., 1996)]. Conversely, noggin inhibition of endogenous BMPs increased BrdU incorporation by 130% (44.0±4.0%, P<0.01; Fig. 2B,F). LIF alone did not significantly alter proliferation (29.1±1.8%; Fig. 2C,F). However, LIF treatment in the presence of noggin increased BrdU incorporation by 63% compared with noggin alone, and approximately 70% of the GFAP$^+$ cells incorporated BrdU under these conditions (70.2±4.9%, P<0.01; Fig. 2D,F). BMP4-treated cells did not incorporate BrdU even after a prolonged 2-day pulse on days 9-10 of differentiation, indicating that the cells had not simply paused in G0 (M.A.B., unpublished). BMP4 treatment did not alter cell survival either (see Table S1 in the supplementary material for clonal analysis).

LIF increases, whereas BMP4 decreases, neural precursor markers in GFAP$^+$ cells

The foregoing observations suggested that the GFAP$^+$ cells generated by LIF signaling might represent stem/progenitor cells, whereas the quiescent GFAP$^+$ cells generated by BMP signaling might represent more differentiated astrocytes. We therefore compared these populations of cells with respect to the expression of neural stem/progenitor cell markers. We first examined the SRY transcription factor SOX1, which is expressed by both early and adult progenitors cells, but not by astrocytes (Bylund et al., 2003). SOX1 was expressed by just 7.0±1.1% of the control GFAP$^+$ cells (Fig. 3A,F). However noggin, LIF and LIF plus noggin all increased the number of SOX1$^+$GFAP$^+$ precursors by approximately 200% (Fig. 3B-D,F; P<0.005). Conversely, BMP4 decreased the number of SOX1$^+$GFAP$^+$ precursors by 43% (Fig. 3E,F; P<0.05). The glycoprotein LeX (CD15/SSEA1) is also expressed by neural stem/progenitor cells (Capela and Temple, 2002; Kim and Morshead, 2003). In control cultures, 11.7±1.5% of GFAP$^+$ cells expressed LeX (Fig. 3G,L). Inhibition of endogenous BMP by noggin did not alter LeX expression by GFAP$^+$ cells (Fig. 3H,L). However, LIF treatment increased the number of LeX$^+$GFAP$^+$ cells by 81% (20.2±2.2%, P<0.005; Fig. 3I,L), and LIF plus noggin increased LeX$^+$GFAP$^+$ cells by 127%

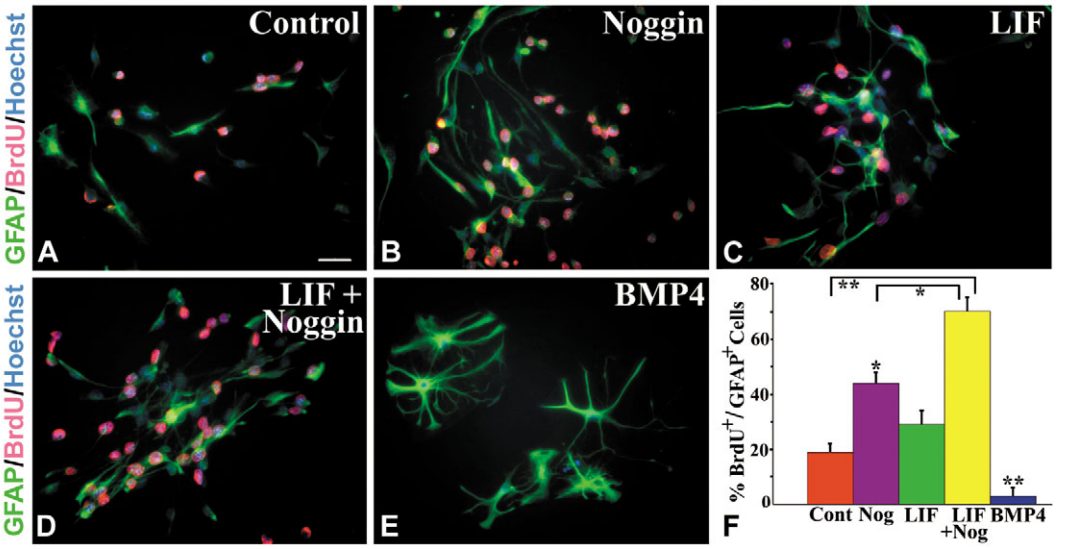


Fig. 2. BMP4 prompts, whereas LIF reduces, exit of GFAP$^+$ cells from the cell cycle. Neurosphere cells were plated and allowed to differentiate with or without cytokines for 6 days, followed by 16 hours of labeling with BrdU. (A,F) Only a small percentage of GFAP$^+$ cells (green) incorporate BrdU (red). (B,F) Treatment with noggin to inhibit endogenous BMP signaling enhances BrdU incorporation. (C,D,F) Treatment with LIF alone does not significantly alter BrdU incorporation. However, LIF treatment in the presence of noggin greatly increases BrdU incorporation compared with treatment with noggin alone. (E,F) BMP4 treatment virtually abolishes BrdU incorporation. (F) Quantitation of the percentage of GFAP$^+$ cells that incorporate BrdU. *P<0.01, **P<0.001 ANOVA. Scale bars: 20 μm in A-E.

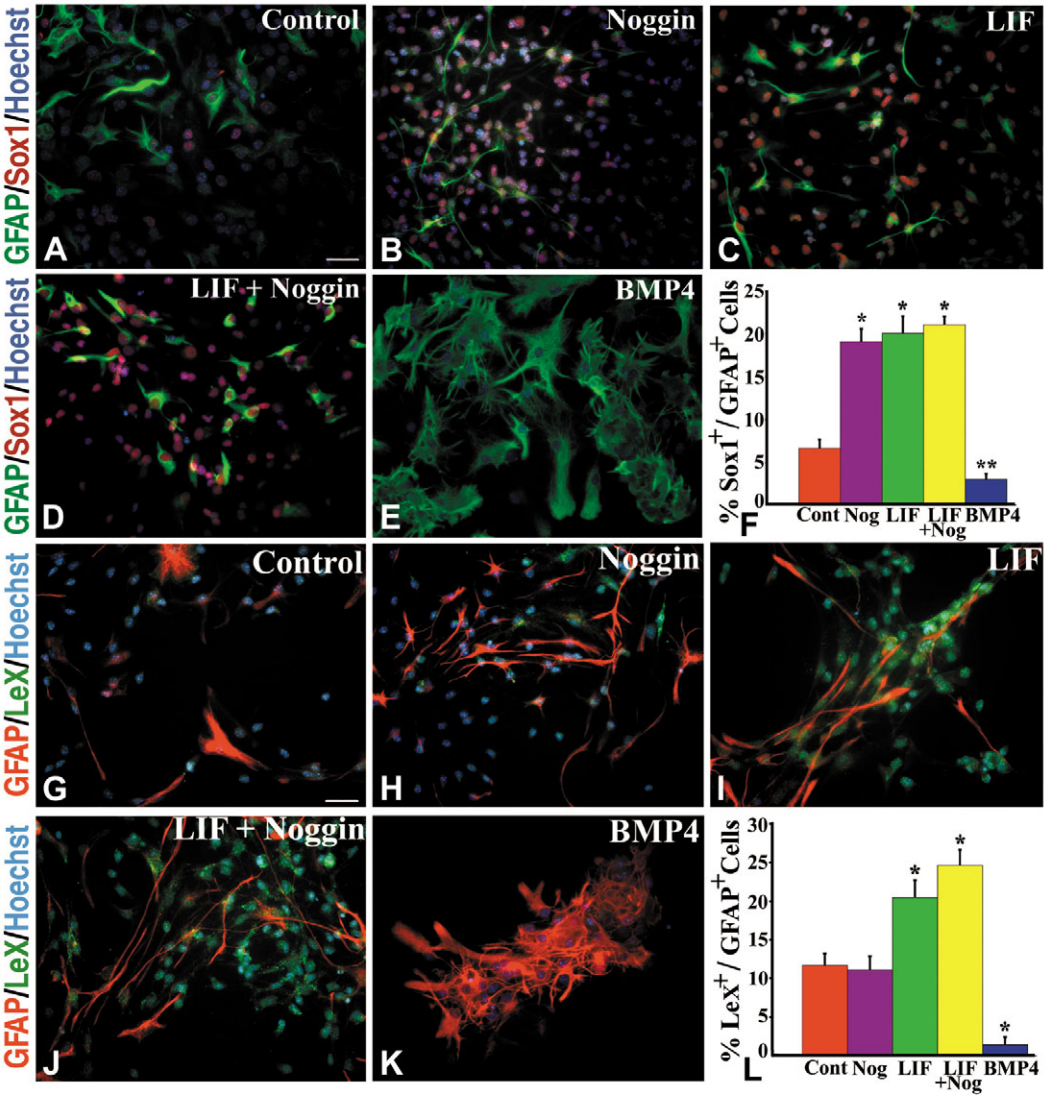


Fig. 3. LIF increases, whereas BMP4 decreases, neural precursor markers in GFAP$^+$ cells. Neurosphere cells were plated and allowed to differentiate with or without cytokines for 7 days before immunocytochemical examination. (A,F) Few differentiated GFAP$^+$ cells express the progenitor marker SOX1. (B-D,F) Treatment with LIF, noggin, and LIF plus noggin all increase the percentage of SOX1$^+$GFAP$^+$ precursors. (E,F) Conversely, BMP4 treatment decreases the percentage of SOX1$^+$GFAP$^+$ precursors. (G,L) Similarly, few differentiated GFAP$^+$ cells express the neural stem cell marker LeX. (H,L) Treatment with noggin does not alter LeX expression on GFAP$^+$ cells. (I,J,L) LIF treatment with or without noggin increases the number of LeX$^+$GFAP$^+$ cells. (K,L) By contrast, BMP4 treatment decreases the number of LeX$^+$GFAP$^+$ cells. Thus LIF and BMP have opposite effects on stem/progenitor cell markers in GFAP$^+$ cells. *P<0.05, **P<0.005 ANOVA. Scale bars: 20 μm in A-F,H-K.

(25.1±2.0%, P<0.005; Fig. 3J,L). By contrast, BMP4 treatment decreased the number of LeX$^+$GFAP$^+$ cells by 92% (1.0±0.8%, P<0.005; Fig. 3K,L), and virtually no LeX$^+$ cells were present in the BMP4-treated cultures. Additionally, the same pattern of findings was observed with a third neural progenitor marker, the intermediate filament vimentin (M.A.B., unpublished).

LIF promotes, whereas BMP4 inhibits, a GFAP$^+$ multipotential stem/progenitor cell fate

As LIF and BMP appear to induce discrete GFAP$^+$ cell populations, we hypothesized that those cells would display different potentialities. To directly test whether the GFAP$^+$ cells induced by cytokines could behave as stem cells, we used the well-characterized rat GFAP promoter (rGFAPp) (Sun et al., 2001; Takizawa et al., 2001), FACS, and neurosphere-generating assays to select, purify and assess stem cell activity in the GFAP$^+$ cell populations. Neural stem cell activity is defined as the ability to form self-renewing neurospheres and to generate multiple neural lineages: neurons, astrocytes and oligodendrocytes. A retroviral vector was used to introduce the rGFAPp driving eGFP into expanding neurospheres. After passage, the neural stem cells were plated for differentiation, with or without cytokines, for 5 days. To determine whether eGFP expression faithfully mirrored GFAP expression, the cells were examined by immunocytochemistry for GFAP, the neuronal marker βIII-tubulin (TuJ1), and the oligodendrocyte marker O4, to identify which cell types displayed GFP fluorescence. EGFP fluorescence was exhibited by 29.4±3.3% of the GFAP$^+$ cells, indicating infection efficiency. Importantly, 99.7±0.3% of the GFP cells were also GFAP$^+$ positive, demonstrating that the virus faithfully selects GFAP$^+$ cells. Also, eGFP did not localize to any neurons or oligodendrocytes (see Fig. S1 in the supplementary material). Furthermore, the cells expressing eGFP displayed morphologies similar to those observed after GFAP staining of control and cytokine-treated cultures (see Fig. S1 in the supplementary material and Fig. 1). After 5 days of cytokine treatment, GFAP$^+$ cells were sorted on the basis of forward-side (FS) scatter and eGFP levels. Dead cells were omitted by FS scatter and eGFP$^+$ cells were clearly discernable (Fig. 4A, see

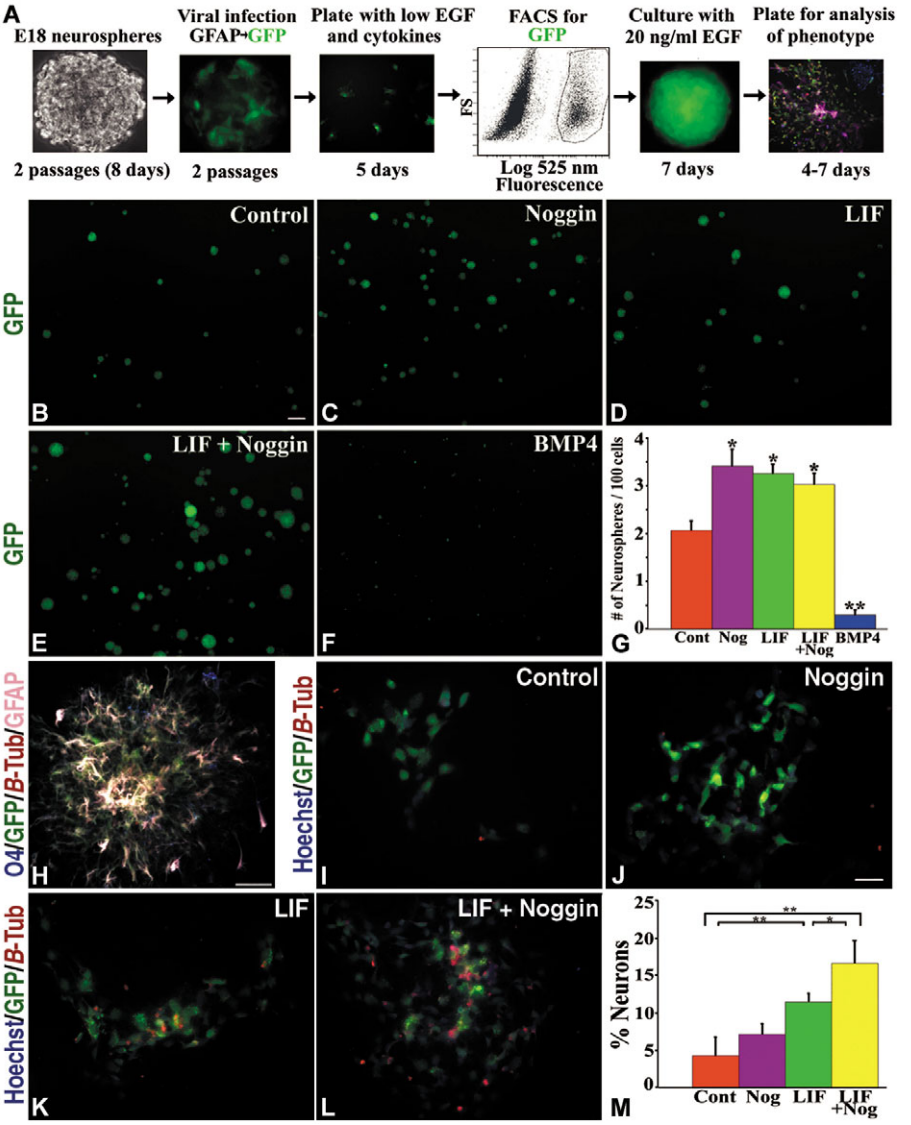


Fig. 4. LIF promotes, whereas BMP4 inhibits, a GFAP-expressing multipotential stem cell fate and neuron production. (A) Experimental paradigm for the cytokine induction, rGFAP promoter and FACS selection, and stem cell analysis of GFAP$^+$ cells. Neurospheres were expanded, infected with the rGFAPp-EGFP retrovirus, further expanded, plated for 5 days to differentiate with or without cytokines and then sorted on the basis of eGFP expression (green). Sorted cells were plated in a neurosphere-forming assay to assess self-renewal and subsequent spheres were plated for differentiation to assess the ability to form neurons (red), astrocytes (purple) and oligodendrocytes (blue). (B,G) In the neurosphere-forming assay, 2% of control GFAP$^+$ cells self-renew (green indicates cells that are GFP positive). (C-E,G) LIF and noggin treatments each increase the percentage of GFAP$^+$ cells that form neurospheres (green). LIF treatment also increases neurosphere size, which is further increased by additional BMP inhibition. (F,G) BMP4 treatment prevents the self-renewal of GFAP$^+$ cells. (H) A single GFP-positive neurosphere plated for differentiation gives rise to oligodendrocytes (O4, blue), neurons (β-tubulin, red) and astrocytes (GFAP, pink), demonstrating multipotentiality. (I-M) GFP-expressing neurosphere populations were dissociated to assess neuronal progeny. (I,M) GFAP-expressing (GFP positive, green) cells produce few neurons (red). (J,M) Noggin treatment alone does not significantly change neuron production. (K,M) LIF treatment significantly increases the number of neurons generated. (L,M) LIF treatment with BMP inhibition further increases neuron production. (G) *P<0.05, **P<0.01; (M) *P<0.01, **P<0.005 ANOVA. Scale bars: 100 μm in B-F; 20 μm in H-L.

also Fig. S1 in the supplementary material). Sort purity was 99.2±0.7%, and cell survival was 85.0±1.2% as measured by Trypan Blue exclusion.

After 7 days in the neurosphere-forming assay, 2.0±0.1% of the GFAP$^+$ cells that were not treated with cytokines formed neurospheres (Fig. 4B,G). BMP4 treatment drastically reduced the ability of the cells to form neurospheres (0.0±0.1%, P<0.01; Fig. 4F,G), while inhibition of BMP (noggin) increased the number of GFAP$^+$-generated neurospheres by approximately 66% (3.4±0.1%, P<0.05; Fig. 4C,G). LIF treatment not only increased the number of GFAP$^+$-generated neurospheres by approximately 58% (3.3±0.1%, P<0.05; Fig. 4D,G), but also increased neurosphere size. LIF treatment plus noggin increased neurosphere numbers by approximately 47% compared with controls and further increased neurosphere size, but did not enhance neurosphere numbers relative to LIF alone (3.0±0.1%, P<0.05; Fig. 4E,G). These data demonstrate that LIF-generated cells have an enhanced ability to form neurospheres, whereas BMP-generated GFAP$^+$ cells rarely form neurospheres under these conditions. To determine whether the neurosphere cells actually had stem cell potential, single neurospheres were plated for differentiation in the absence of cytokines. Neurons, astrocytes and oligodendrocytes emerged from spheres generated under all conditions (Fig. 4H) except for treatment with BMP4 (where neurospheres rarely formed), demonstrating that the neurosphere cells are multipotent. Hence, LIF promotes, whereas BMP4 inhibits, a GFAP$^+$ multipotential stem cell fate.

LIF increases neuron production from GFAP-expressing cells

To determine whether exposure to LIF alters the potential of GFAP$^+$ cells, control or cytokine-treated GFAP$^+$-derived neurospheres (derived from the FACS-sorted cells) were dissociated and plated for 4-5 days of differentiation. The resultant cells were processed for βIII-tubulin to determine neuron numbers. Control spheres produced a small number of neurons (3.8±2.5%; Fig. 4I,M). BMP inhibition (noggin) did not significantly change the number of neurons generated, although there was a trend towards an increase (6.8±1.5%; Fig. 4J,M). However, LIF increased neuron numbers by 90% (11.0±1.1%, P<0.005; Fig. 4K,M). Moreover, LIF plus noggin further increased neurogenesis by 55% relative to LIF alone (17.1±2.8%, P<0.005; Fig. 4L,M). These observations demonstrate that LIF-induced GFAP$^+$ cells have an increased ability to produce neurons that is further enhanced by suppressing BMP signaling.

LIF maintains GFAP-expressing multipotent progenitors for prolonged periods of time

We then investigated whether LIF could maintain the GFAP$^+$ progenitor cell state for prolonged periods of time. Because high mitogen levels may reprogram more committed cells to exhibit progenitor characteristics that they might otherwise not display (Kondo and Raff, 2000; Anderson, 2001), neural progenitors were plated with only low (1-2 ng/ml) levels of EGF with LIF, and were passaged repeatedly. Neural cells could be propagated this way as a monolayer for at least 10 passages and for several months (Fig. 5A). By contrast, in the absence of LIF, the cells became progressively sparser with attempted passaging and could only be passaged a few times. We then examined whether GFAP-expressing cells generated in the presence of LIF could still proliferate (incorporate BrdU) and express the immunocytochemical characteristics of progenitor cells at the higher passages. In the LIF-treated cultures, GFAP-expressing cells at passage 7 (p7) still incorporated BrdU (2-hour pulse) and expressed the progenitor marker LeX (Fig. 5B). LIF-treated cultures were also able to generate neurons at high passages (>p7), whereas nearby GFAP-expressing cells remained in cell cycle, as assayed by Ki67 expression (Fig. 5C). To directly assess the self-renewal and multipotentiality of GFAP$^+$ cells maintained by LIF, higher passage (>p7) cells were infected with the rGFAPp-EGFP retrovirus, selected by FACS, plated in a neurosphere-forming assay (2 ng/ml EGF plus 20 ng/ml LIF) and subsequent spheres plated for differentiation. GFAP-expressing cells maintained by LIF were able to self-renew at high passages, as evidenced by neurosphere formation, and they retained multipotentiality and, specifically, the ability to produce neurons (Fig. 5E). Thus, LIF maintains GFAP-expressing cells as multipotential stem/progenitor cells for prolonged periods of time, independent of exposure to high levels of other mitogens.

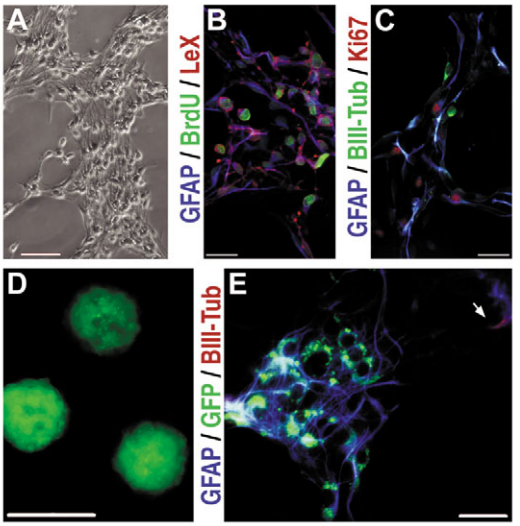
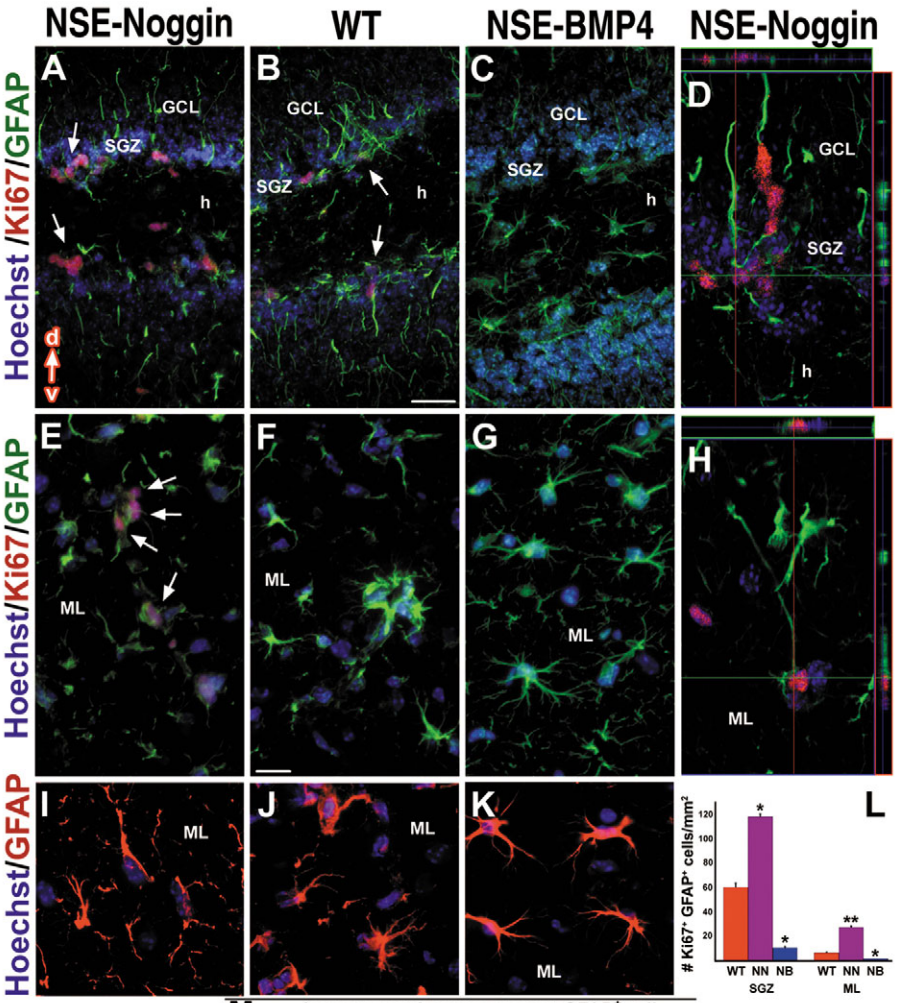


Fig. 5. LIF maintains GFAP-expressing multipotent progenitors. (A) LIF and low (1-2 ng/ml) EGF propagate neural progenitor cells as a monolayer for at least 10 passages. (B) At higher passages (p7 is shown), GFAP-expressing cells proliferate, as shown by BrdU incorporation (green, 2-hour pulse), and exhibit the progenitor marker LeX (red). (C) Higher passage cultures are still able to generate neurons (green), whereas nearby GFAP-expressing cells remain in cell cycle, as demonstrated by Ki67 expression (red). (D,E) To directly test the ability of GFAP-expressing cells for self-renewal and multipotentiality, higher passage cells were infected with the rGFAPp-EGFP retrovirus, selected by FACS, plated in a neurosphere-forming assay to assess self-renewal (in the absence of high mitogen), and subsequent spheres plated for differentiation to assess the ability to form neurons (red) and astrocytes (blue). (D) GFAP-expressing cells are able to self-renew at high passages, as shown by neurosphere formation (green, GFP). (E) Spheres formed from high passage GFAP-expressing cells retain the ability to produce neurons (red; blue indicates astrocytes; green, GFP). Scale bars: 20 μm in A-C,E; 100 μm in D.



M	Average number process per GFAP$^+$ cell		
	Wild-type	Noggin	BMP4
	4.1 ± 0.1 (18)	2.4 ± 0.1 (19)**	5.5 ± 0.1 (18)*

Fig. 6. BMPs regulate morphology and are necessary and sufficient for the cell-cycle exit of GFAP-expressing cells in vivo. Transgenic animals overexpressing BMP4 or noggin were analyzed at P15. (A-K) Immunofluorescence for GFAP and Ki67 in the hippocampus SGZ (A-D) and ML (E-K). (L) Plot of Ki67$^+$GFAP$^+$ cells in the SGZ (*P<0.03) and the ML (*P<0.01, **P<0.04). (M) Average number of processes per GFAP$^+$ cell. Numbers in parentheses indicate the number of cells analyzed. *P<0.01, **P<0.001 ANOVA. (A,B,L) GFAP-expressing cells in the SGZ remain in cell cycle and are increased in number when noggin is overexpressed. Arrows indicate clusters of Ki67$^+$ cells. (C,L) Overexpression of BMP4 in the SGZ promotes cell-cycle exit of GFAP$^+$ cells. (D) High magnification confocal image demonstrating co-localization of Ki67 and GFAP in the SGZ of noggin mice. (F,G,L) Few GFAP-expressing cells in the ML remain in cell cycle in wild-type or BMP4-overexpressing mice. (E,L) BMP inhibition in the ML maintains GFAP$^+$ cells in cell cycle. Arrows (E) indicate Ki67$^+$GFAP$^+$ cells. (H) High magnification confocal image demonstrating co-localization of Ki67 and GFAP in the ML of noggin mice. (J,K,M) GFAP$^+$ cells in the ML exhibit branched morphologies that become further ramified with BMP4 overexpression. (I,M) Noggin overexpression in the ML prevents the formation of stellate morphology, and these cells resemble the thin elongated GFAP$^+$ cells in the SGZ (H,D). GCL, granule cell layer; SGZ, subgranular zone; h, hilus; ML, molecular layer; WT, wild type; NN, NSE-Noggin; NB, NSE-BMP4. Scale bar in B: 20 μm for A-C,E-G; 10 μm for D,H-K.

BMPs regulate the morphology of GFAP-expressing cells in vivo, and are necessary and sufficient for cell-cycle exit

In vivo, GFAP-expressing astrocytes are derived from radial glia during the postnatal period (Schmechel and Rakic, 1979; Voigt, 1989). Most of these cells lose progenitor function, but radial glia also give rise to GFAP$^+$ adult stem cells in the SVZ and hippocampus (Eckenhoff and Rakic, 1984; Merkle et al., 2004; Rickmann et al., 1987; Voigt, 1989). To test the hypothesis that BMPs developmentally regulate the maturation of GFAP$^+$ cells in vivo, we generated transgenic animals that overexpress either BMP4 or its antagonist noggin under the control of the neuron-specific enolase (NSE) promoter (Gomes et al., 2003; Guha et al., 2004). Transgene expression begins before gliogenesis at embryonic day 16 (E16), peaks postnatally, and persists into adult life (Gomes et al., 2003). Therefore, these animals serve as an excellent model to study the development of GFAP-expressing cells. BMP4 overexpressing animals have an increased number of GFAP$^+$ cells in the brain, whereas the noggin overexpressing animals conversely have significantly reduced numbers of GFAP$^+$ cells (Gomes et al., 2003) (see also Fig. S2 in the supplementary material). Similarly, BMP4 overexpressing animals have increased numbers of S100β-expressing cells, whereas numbers of these cells are significantly reduced in the noggin transgenic animals (Gomes et al., 2003) (Fig. S2 in the supplementary material). In the adult brain, GFAP$^+$ progenitor cells in neurogenic regions including the hippocampal SGZ remain in cell cycle (Seri et al., 2001; Garcia et al., 2004). As the NSE transgene is expressed at the highest levels in the hippocampus (Gomes et al, 2003), we examined the effects of BMP signaling on the proliferation of GFAP$^+$ cells in the SGZ using Ki67 and GFAP double labeling. Overexpression of BMP4 depleted the SGZ of Ki67$^+$GFAP$^+$ cells, whereas inhibition of BMP signaling by noggin significantly increased the number of these cells (Fig. 6). Specifically, at P15, 60.6±3.8 GFAP-expressing cells/mm^2 (in 10 μm sections) remained in cell cycle in the SGZ of wild-type animals (Fig. 6B,L). Inhibition of BMP signaling significantly increased the number of Ki67$^+$GFAP$^+$ cells by nearly 100% (118.4±2.4 cells/mm^2, P<0.03; Fig. 6A,L). Conversely, overexpression of BMP4 reduced the number of these cells by 92% (10.4±1.3 cells/mm^2, P<0.03; Fig. 6C,L). High magnification confocal images demonstrated the co-localization of Ki67 and GFAP in the SGZ

(Fig. 6D, and Fig. S3 in the supplementary material). To determine whether BMP signaling is necessary for promoting the cell-cycle exit of GFAP$^+$ cells, we investigated co-labeling with Ki67 in the hippocampal molecular layer (ML), an area that does not normally contain proliferative GFAP$^+$ cells in the adult (Garcia et al., 2004). We found only rare Ki67$^+$GFAP$^+$ cells in the ML in P15 wild-type mice (6.2±0.3 cells/mm^2; Fig. 6F,L), and BMP4 overexpression almost completely depleted this small population of cells (1.1±0.2 cells/mm^2, P<0.04; Fig. 6G,L). However, noggin overexpression markedly increased the number of cycling GFAP$^+$ cells remaining in the ML (27.4±1.5 cells/mm^2, P<0.01; Fig. 6F,H,L), demonstrating that BMP is necessary for the normal exit of GFAP$^+$ cells from the cell cycle in this region.

Because GFAP-expressing progenitors in the adult neurogenic SVZ and SGZ contain fewer processes than do multipolar astrocytes in non-neurogenic regions (Garcia et al., 2004), we compared the morphology of GFAP$^+$ cells in the hippocampal SGZ and ML regions in control and transgenic animals (Fig. 6I-M). GFAP$^+$ cells in the ML of wild-type animals exhibited highly branched stellate morphologies (4.1±0.1 branches/cell, Fig. 6J,M), and overexpression of BMP4 resulted in further ramification of astrocytic processes (5.5±0.1 branches/cell, P<0.05; Fig. 6K,M). Conversely, noggin overexpression in the ML prevented the formation of the stellate morphology, and GFAP$^+$ cells in the ML of these animals maintained the elongated morphology typical of GFAP$^+$ cells in the SGZ (2.4±0.1 branches/cell, P<0.01; Fig. 6D,H-I,M). Therefore, BMPs regulate the mature morphology of GFAP$^+$ cells in the non-neurogenic hippocampus ML. Notably, the morphology and proliferative state of GFAP$^+$ cells in the SGZ of wild-type and noggin animals, and in the ML in noggin animals, are similar to that of the LIF-generated GFAP$^+$ cells in vitro (Fig. 6A,B,D,E,J,L; Fig. 1F; Fig. 2C,F).

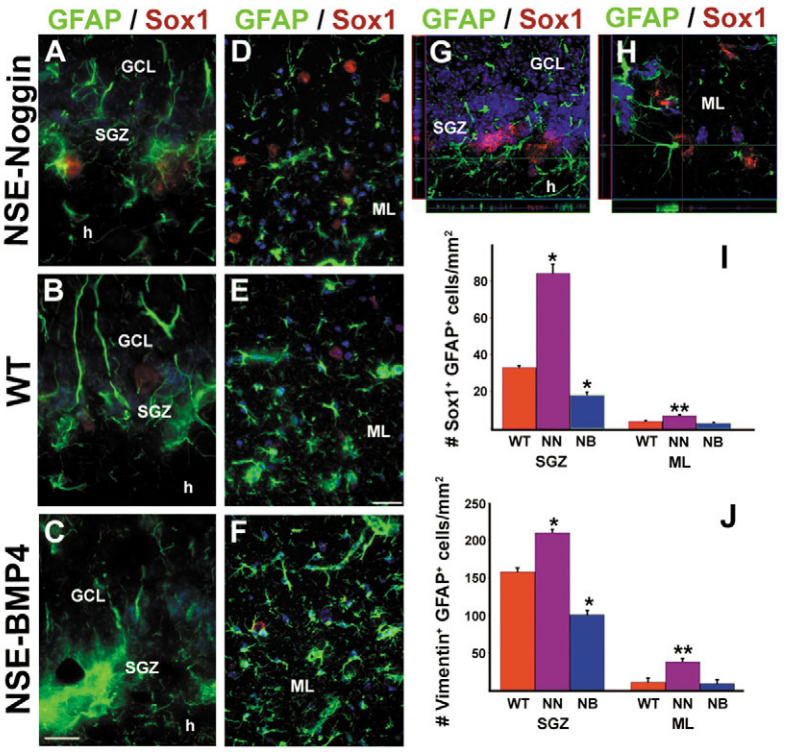


Fig. 7. BMPs regulate the maturation of GFAP-expressing cells in vivo. (A-H) GFAP (green), and SOX1 or vimentin (red) in the hippocampus SGZ (A-C,G) and ML (D-F,H) in P15 wild-type (B,E), NSE-noggin (A,D,G,H) and NSE-BMP4 (C,F) animals. Cells were counterstained with Hoechst (blue). (I,J) Number of SOX1- (I) and vimentin- (J) expressing GFAP$^+$ cells. (A,B,D,I,J) GFAP-expressing cells in the SGZ remain as progenitors and increase in number when noggin is overexpressed, as assessed by SOX1 and vimentin co-labeling. (C,I,J) Overexpression of BMP4 in the SGZ promotes the loss of progenitor markers in GFAP$^+$ cells. (D) Confocal image demonstrating co-localization of SOX1 and GFAP in the SGZ of noggin mice. (F,G,I,J) GFAP-expressing cells in the ML rarely co-express SOX1 or vimentin in wild-type or overexpressed BMP4 mice. (E,I,J) BMP inhibition in the ML increases progenitor markers in GFAP$^+$ cells. (H) Confocal image demonstrating co-localization of SOX1 and GFAP in the ML of noggin mice. (I) *P<0.02, **P<0.05; (J) *P<0.03, **P<0.01 ANOVA. Scale bar: in C, 10 μm for A-C,G,H; in E, 20 μm for D-F.

BMPs regulate the maturation of GFAP-expressing cells in vivo

The foregoing observations suggested that BMPs regulate the maturation of GFAP-expressing cells in vivo by promoting cell-cycle exit and increasing process ramification. Consequently, we further investigated whether BMP signaling regulates the maturation of GFAP$^+$ progenitor cells into astrocytes in vivo by examining the expression of progenitor cell markers in the hippocampal SGZ and ML. Because LeX staining was difficult to quantitate in vivo, we instead used the progenitor marker vimentin, in addition to SOX1 (Garcia et al., 2004; Seri et al., 2004). The wild-type SGZ contained 32.5±0.7 cells/mm^2 that were GFAP$^+$SOX1$^+$ and 157.6±0.2 cells/mm^2 that were GFAP$^+$vimentin$^+$ (Fig. 7B,I-J). In noggin overexpressing animals, the number of GFAP$^+$ progenitors was significantly increased, as assayed both by SOX1 (83.9±4.6 cells/mm^2, P<0.02, Fig. 7A,D,I) and by vimentin (209.8±4.4 cells/mm^2, P<0.03; Fig. 7J, see also Fig. S4 in the supplementary material). By contrast, BMP4 overexpression in the developing SGZ reduced the number of GFAP$^+$ progenitors, as assessed by SOX1 (18.2±1.5 cells/mm^2, P<0.02; Fig. 7C,I) and vimentin (101.1±1.2 cells/mm^2, P<0.03, Fig. 7J, Fig. S4). Thus, BMP signaling promotes the loss of progenitor markers, as well as a quiescent state in GFAP$^+$ cells in neurogenic areas. To determine whether BMPs are necessary for the maturation of GFAP$^+$ cells in non-neurogenic regions, we analyzed SOX1 and vimentin expression in the ML of the noggin-overexpressing animals. GFAP$^+$ cells in the ML rarely co-express SOX1 or vimentin in wild-type mice (SOX1, 3.9±0.2 cells/mm^2; vimentin, 10.5±3.1 cells/mm^2; Fig. 7F,I,J), or in BMP4 overexpressing mice (SOX1, 3.0±0.2 cells/mm^2; vimentin, 8.7±5.0 cells/mm^2; Fig. 7G,I,J; Fig. S5 in the supplementary material). However, BMP inhibition (noggin overexpression) in the ML increased the number of GFAP$^+$ cells maintaining progenitor cell markers, suggesting that BMP signaling is necessary for astrocyte

Table 1. Comparison of GFAP-expressing cells generated in response to LIF versus BMP4

Phenotype	BMP4	LIF
GFAP expression	+++	+
Stellate morphology	+	–
Cell-cycle exit	+	–
LeX expression	–	+
Sox1 expression	–	+
Vimentin expression	–	+
Neurosphere formation (self-renewal)	–	+
Neuron production by progeny	–	+

LIF and BMP4 induce discrete GFAP$^+$ cell populations from embryonic stem cells, when endogenous BMPs are taken into account. LIF-induced GFAP$^+$ cells have a bipolar/tripolar, morphology, re-enter the cell-cycle, express proteins characteristic of progenitors, and function as multipotential stem/progenitor cells capable of self-renewal and the generation of neuronal progeny. By contrast, BMP-induced GFAP$^+$ cells have a mature astrocytic phenotype as demonstrated by stellate GFAP$^+$ morphology, exit from the cell cycle, loss of stem/progenitor markers and the inability to self-renew.

maturation in this region (SOX1, 7.3±0.2 cells/mm^2, P<0.05; vimentin, 37.9.±5.1 cells/mm^2, P<0.01; Fig. 7E,H-J and Fig. S4). Thus, BMP signaling in vivo causes GFAP$^+$ cells to exit the cell cycle and lose progenitor markers in normally neurogenic regions, similar to the observations in vitro. Conversely, inhibiting BMP signaling in non-neurogenic regions in vivo prevents GFAP$^+$ cells from maturing, as assessed by exit from the cell cycle and the loss of progenitor markers that typically occur in this region (Hutchins and Casagrande, 1989; Bylund et al., 2003).

Discussion

We show here that LIF and BMP signaling result in the commitment of NSCs to distinct GFAP$^+$ cell populations that differ with respect to morphology, proliferation, gene expression and developmental potential (Table 1). LIF signaling generates proliferative, bipolar/tripolar GFAP$^+$ cells with stem/progenitor cell properties, whereas BMP signaling generates stellate, GFAP$^+$ astrocytes that lack stem/progenitor cell potential. Using transgenic animals that overexpress either BMP4 or noggin, we confirm that BMP signaling in vivo is both necessary and sufficient to induce mature GFAP$^+$ astrocytes, whereas the inhibition of BMP signaling promotes a proliferative, GFAP$^+$ progenitor phenotype characteristic of astrocytes in the adult SGZ.

LIF signaling generates GFAP-expressing progenitors

Numerous previous studies have demonstrated that LIF/CNTF signaling induces GFAP expression by cultured embryonic NSCs (Bonni et al., 1997; Johe et al., 1996; Morrow et al., 2001; Rajan and McKay, 1998; Song and Ghosh, 2004; Sun et al., 2001). The GFAP$^+$ cells induced in these studies had a partially ramified morphology, and no effect on proliferation was noted (Bonni et al., 1997). Our findings regarding the effects of LIF differ from these numerous previous studies, because we performed our experiments in the absence of BMP signaling. Cultured neural stem/progenitor cells produce BMPs endogenously (Fig. 1) (see also Gross et al., 1996; Nakashima et al., 1999b), and this alters the phenotype adopted by the cells. By including the BMP inhibitor noggin along with LIF, we were able to more precisely define the role of LIF signaling. We found that LIF signaling not only induces the expression GFAP, but also promotes re-entry into the cell cycle and maintenance of the progenitor cell traits (Figs 1-5).

Substantial evidence has been generated previously indicating that LIF signaling exerts effects on re-entry into cell cycle and on the maintenance of stem/progenitor cell traits. Null mutation of the LIF receptor (LIFR) decreases the number of progenitors derived from E14 brain in vitro, and a reduction in the levels of gp130 decreases progenitor cell re-entry into cell cycle in vivo (Hatta et al., 2002; Shimazaki et al., 2001). Conversely, LIF injection in vivo or treatment of E14 progenitors in vitro increases re-entry into cell cycle (Hatta et al., 2002; Pitman et al., 2004). In the adult, LIFR$^{+/-}$ mice show a loss of EGF-responsive progenitors derived from the SVZ, whereas CNTF injection increases the number of multipotential adult progenitors in vivo and in vitro at the expense of glial-restricted cells (Shimazaki et al., 2001). These observations are fully concordant with our finding that LIF signaling promotes a stem/progenitor cell state. In most neurosphere cultures, only about 2% of the cells are typically ‘stem’ cells that have the capacity to self-renew; the remainders are multipotent progenitor cells. This same principle probably applies to the GFAP$^+$ cells in the LIF plus noggin conditions. These cells are definitely not terminally differentiated astrocytes, as they proliferate, express progenitor cell markers, and display multipotentiality. In fact, when LIF-generated GFAP$^+$ cells were subsequently treated with BMP4, they adopted the morphology and characteristics of BMP4-generated astrocytes (M.A.B., unpublished). These findings are consistent with a hypothesis that LIF signaling generates a GFAP$^+$ progenitor cell that differentiates into an astrocyte under the influence of BMP signaling (Fig. 8).

Mechanisms for astrocyte generation

Astrocyte generation has been postulated to occur through the convergence of LIF/CNTF and BMP signaling. The transcriptional co-activator p300 bridges the LIF and BMP

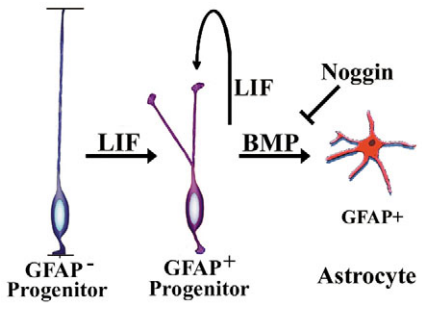


Fig. 8. Model for the affects of LIF and BMP on astrocyte differentiation. Stem cells in the developing rodent brain are initially GFAP$^-$, but later express GFAP during the postnatal and adult periods. LIF fosters maintenance of stem/progenitor trait throughout the continuum, but converts GFAP$^-$ cells into GFAP$^+$ ones. By contrast, BMPs induce exit from cell cycle and the loss of stem/progenitor cell traits, resulting in what we have termed a mature astrocyte. Inhibition of endogenous BMPs is required to prevent maturation of adult stem cells into the mature astrocytic phenotype.

signaling targets STAT3 and SMAD1, and mediates their cooperative effects on GFAP expression (Nakashima et al., 1999a). Our studies do not address the question of whether BMP signaling can promote astrogliogenesis without some prior activation of STAT signaling. BMP2 treatment of progenitor cells cultured from LIFR$^{-/-}$ animals induces astrogliogenesis (Koblar et al., 1998), indicating that the LIFR is not required, but it is possible that STAT signaling is activated by cytokines that do not utilize the LIFR such as EGF (Shuai et al., 1993; Zhong et al., 1994). In this regard, it is noteworthy that BMP signaling does not promote astroglial differentiation of early embryonic neural stem cells, but rather promotes neuronal differentiation (Mabie et al., 1999). This may reflect both the presence of high levels of neurogenin1/2 (NGN1/2) that sequester the CBP-SMAD1 transcription complex away from astrocyte differentiation genes (Sun et al., 2001), and the absence of signaling from the EGFR (Eagleson et al., 1996; Gross et al., 1996; Zhu et al., 1999). EGFR is associated with a switch in bias of the cells from neurogenesis to gliogenesis (Burrows et al., 1997), and EGFR regulates the ability of stem cells to interpret LIF, but not BMP, as a GFAP$^+$-inducing agent (Viti et al., 2003). EGFR expression is upregulated in neural stem cells between E13 and E16 in mice (Burrows et al., 1997), and GFAP expression in response to LIF and BMP can first be observed at approximately E14.5 (Eagleson et al., 1996; Gross et al., 1996). FGF2 signaling also primes neural stem cells for gliogenesis, at least in part, by the removal of histone methylation at the STAT-binding site on the rat GFAP promoter (Song and Ghosh, 2004). This may reflect an FGF2-mediated increase of EGFR expression (Lillien and Raphael, 2000). Concurrent with increased EGFR expression levels, there is a decrease in the expression of genes that inhibit gliogenesis (*Ngn1/2*) (Sun et al., 2001), and an increase in the expression of other putative proglial genes, such as hairy-enhancer of split 1/5 (*Hes1/5*) and Hes-related genes 1 and 2 (Takizawa et al., 2003). Thus, the ability of neural stem cells to interpret BMPs as pro-astrocytic differentiation factors depends upon the status of other signaling pathways and the intrinsic regulation in the cell, but may not depend upon the LIF/CNTF-mediated conversion of GFAP$^-$ NSCs into GFAP$^+$ progenitor cells (Fig. 8).

BMP4 signaling generates mature astrocytes

The ability of noggin to prevent the maturation of GFAP$^+$ progenitor cells into astrocytes in vivo indicates that BMP signaling normally regulates astrocytic lineage commitment. However, BMP4 and other BMPs are abundantly expressed throughout the nervous system (Furuta et al., 1997; Mehler et al., 1997). How then is the progenitor cell phenotype maintained in the adult brain? Noggin is normally expressed in the SVZ and SGZ of adult animals, and helps to maintain a niche for adult neurogenesis (Chmielnicki et al., 2004; Lim et al., 2000). Furthermore, antisense noggin reduces proliferation in the adult dentate gyrus (Fan et al., 2004). These observations are consistent with our findings that noggin preserves the GFAP$^+$ progenitor cell phenotype and prevents the astrocytic differentiation of these cells. Thus, noggin not only maintains the proliferation of cells within the niche, but more generally maintains a multipotent progenitor cell phenotype by inhibiting BMP-directed differentiation. Our data further suggest that the number of GFAP$^+$ progenitor cells is inversely proportional to the amount of BMP signaling in the developing hippocampus. Because noggin is expressed in the anterior subiculum in neonates, and in the dentate gyrus from one week of age into adulthood (Fan et al., 2003), it was not clear whether noggin overexpression in this area would have much effect. However, we found that noggin overexpression markedly increased the number of GFAP$^+$ progenitor cells in the SGZ, indicating that the levels of endogenous noggin expression are insufficient to fully inhibit BMP signaling in this area. The almost complete depletion of GFAP$^+$ progenitor cells from the SGZ of BMP-overproducing animals highlights the essential role played by BMP inhibitors such as noggin in maintaining the progenitor cell phenotype.

Mature astrocytes and adult progenitors are separate cell populations

The molecular characterization of GFAP and the relatively limited number of cell types that express the protein led to its use as a surrogate marker for the astrocyte phenotype. The lack of an unambiguous biochemical marker has complicated the precise definition of astrocyte identity and of the astrocytic lineage (Gotz and Steindler, 2003; Kimelberg, 2004). Although it is clear that some GFAP$^+$ cells in the adult brain have stem cell potential (Doetsch et al., 1999; Garcia et al., 2004; Imura et al., 2003; Morshead et al., 2003; Seri et al., 2001), only a morphologically distinct subpopulation of GFAP$^+$ cells produce new neurons (Garcia et al., 2004). This has led to terms such as radial astrocyte and horizontal astrocyte, which are based on morphological criteria in vivo, to help to distinguish the unique subsets of GFAP$^+$ cells in the brain that display progenitor cell traits (Seri et al., 2004). The lineage relationship between adult progenitor cells and other astrocytes has been unclear. Our findings suggest a lineage relationship in the rodent brain in which GFAP$^+$ progenitors generate mature astrocytes in response to BMP signaling (Fig. 8), and, further, that these represent distinct and separable cell types. It may therefore be inappropriate to continue to use the same term – astrocyte – for these disparate cell types, particularly as GFAP$^+$ progenitors cells also generate other lineages in the normal adult brain (Garcia et al., 2004). It might be more accurate to use terms such as radial progenitor or horizontal progenitor, and to reserve the use of the term ‘astrocyte’ for more terminally differentiated phenotypes, as these cells differ in their morphology, molecular characteristics and potentiality (Morest and Silver, 2003).

We thank M. Paniagua and J. Nelson for technical assistance with FACS, V. Sahni for tissue processing, W. Fu for molecular cloning, C. Czeisler for graphic illustrations, and Marika Bonaguidi for the cover art. We are grateful to M. Greenberg for providing the rGFAP promoter and H. Kondoh for the SOX1 antibody. The vimentin antibody was developed by A. Alvarez-Buylla and obtained from DSHB. This work is funded by NIH training grant NS41234 to M.A.B., and NIH grants NS20013 and NS20778 to J.A.K.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/24/5503/DC1

References

Altman, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J. Comp. Neurol.* **137**, 433-457.

Altman, J. and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124**, 319-335.

Anderson, D. J. (2001). Stem cells and pattern formation in the nervous system: the possible versus the actual. *Neuron* **30**, 19-35.

Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D. A., Rozovsky, I., Stahl, N., Yancopoulos, G. D. and Greenberg, M. E. (1997). Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science* **278**, 477-483.

Burrows, R. C., Wancio, D., Levitt, P. and Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron* **19**, 251-267.

Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.

Capela, A. and Temple, S. (2002). LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* **35**, 865-875.

Chmielnicki, E., Benraiss, A., Economides, A. N. and Goldman, S. A. (2004). Adenovirally expressed noggin and brain-derived neurotrophic factor cooperate to induce new medium spiny neurons from resident progenitor cells in the adult striatal ventricular zone. *J. Neurosci.* **24**, 2133-2142.

Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-716.

Eagleson, K. L., Ferri, R. T. and Levitt, P. (1996). Complementary distribution of collagen type IV and the epidermal growth factor receptor in the rat embryonic telencephalon. *Cereb. Cortex* **6**, 540-549.

Eckenhoff, M. F. and Rakic, P. (1984). Radial organization of the hippocampal dentate gyrus: a Golgi, ultrastructural, and immunocytochemical analysis in the developing rhesus monkey. *J. Comp. Neurol.* **223**, 1-21.

Fan, X., Xu, H., Cai, W., Yang, Z. and Zhang, J. (2003). Spatial and temporal patterns of expression of Noggin and BMP4 in embryonic and postnatal rat hippocampus. *Brain Res. Dev. Brain Res.* **146**, 51-58.

Fan, X. T., Xu, H. W., Cai, W. Q., Yang, H. and Liu, S. (2004). Antisense Noggin oligodeoxynucleotide administration decreases cell proliferation in the dentate gyrus of adult rats. *Neurosci. Lett.* **366**, 107-111.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Garcia, A. D., Doan, N. B., Imura, T., Bush, T. G. and Sofroniew, M. V. (2004). GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat. Neurosci.* **7**, 1233-1241.

Gomes, W. A., Mehler, M. F. and Kessler, J. A. (2003). Transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment. *Dev. Biol.* **255**, 164-177.

Gotz, M. and Steindler, D. (2003). To be glial or not-how glial are the precursors of neurons in development and adulthood? *Glia* **43**, 1-3.

Gross, R. E., Mehler, M. F., Mabie, P. C., Zang, Z., Santschi, L. and Kessler, J. A. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron* **17**, 595-606.

Guha, U., Mecklenburg, L., Cowin, P., Kan, L., O'Guin, W. M., D'Vizio, D., Pestell, R. G., Paus, R. and Kessler, J. A. (2004). Bone morphogenetic protein signaling regulates postnatal hair follicle differentiation and cycling. *Am. J. Pathol.* **165**, 729-740.

Hatta, T., Moriyama, K., Nakashima, K., Taga, T. and Otani, H. (2002). The Role of gp130 in cerebral cortical development: in vivo functional analysis in a mouse exo utero system. *J. Neurosci.* **22**, 5516-5524.

Hutchins, J. B. and Casagrande, V. A. (1989). Vimentin: changes in distribution during brain development. *Glia* **2**, 55-66.

Imura, T., Kornblum, H. I. and Sofroniew, M. V. (2003). The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. *J. Neurosci.* **23**, 2824-2832.

Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic, M. M. and McKay, R. D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* **10**, 3129-3140.

Kempermann, G., Gast, D., Kronenberg, G., Yamaguchi, M. and Gage, F. H. (2003). Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* **130**, 391-399.

Kim, M. and Morshead, C. M. (2003). Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J. Neurosci.* **23**, 10703-10709.

Kimelberg, H. K. (2004). The problem of astrocyte identity. *Neurochem. Int.* **45**, 191-202.

Koblar, S. A., Turnley, A. M., Classon, B. J., Reid, K. L., Ware, C. B., Cheema, S. S., Murphy, M. and Bartlett, P. F. (1998). Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor. *Proc. Natl. Acad. Sci. USA* **95**, 3178-3181.

Kondo, T. and Raff, M. (2000). Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. *Science* **289**, 1754-1757.

Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C. and Steindler, D. A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. USA* **97**, 13883-13888.

Lillien, L. and Raphael, H. (2000). BMP and FGF regulate the development of EGF-responsive neural progenitor cells. *Development* **127**, 4993-5005.

Lim, D. A., Tramontin, A. D., Trevejo, J. M., Herrera, D. G., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2000). Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* **28**, 713-726.

Mabie, P. C., Mehler, M. F. and Kessler, J. A. (1999). Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype. *J. Neurosci.* **19**, 7077-7088.

Mehler, M. F., Mabie, P. C., Zhang, D. and Kessler, J. A. (1997). Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* **20**, 309-317.

Mehler, M. F., Mabie, P. C., Zhu, G., Gokhan, S. and Kessler, J. A. (2000). Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. *Dev. Neurosci.* **22**, 74-85.

Merkle, F. T., Tramontin, A. D., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl. Acad. Sci. USA* **101**, 17528-17532.

Morest, D. K. and Silver, J. (2003). Precursors of neurons, neuroglia, and ependymal cells in the CNS: what are they? Where are they from? How do they get where they are going? *Glia* **43**, 6-18.

Morrow, T., Song, M. R. and Ghosh, A. (2001). Sequential specification of neurons and glia by developmentally regulated extracellular factors. *Development* **128**, 3585-3594.

Morshead, C. M., Garcia, A. D., Sofroniew, M. V. and van Der Kooy, D. (2003). The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. *Eur. J. Neurosci.* **18**, 76-84.

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K. and Taga, T. (1999a). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science* **284**, 479-482.

Nakashima, K., Yanagisawa, M., Arakawa, H. and Taga, T. (1999b). Astrocyte differentiation mediated by LIF in cooperation with BMP2. *FEBS Lett.* **457**, 43-46.

Pitman, M., Emery, B., Binder, M., Wang, S., Butzkueven, H. and Kilpatrick, T. J. (2004). LIF receptor signaling modulates neural stem cell renewal. *Mol. Cell. Neurosci.* **27**, 255-266.

Rajan, P. and McKay, R. D. (1998). Multiple routes to astrocytic differentiation in the CNS. *J. Neurosci.* **18**, 3620-3629.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.

Rickmann, M., Amaral, D. G. and Cowan, W. M. (1987). Organization of radial glial cells during the development of the rat dentate gyrus. *J. Comp. Neurol.* **264**, 449-479.

Schmechel, D. E. and Rakic, P. (1979). A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anat. Embryol.* **156**, 115-152.

Seri, B., Garcia-Verdugo, J. M., McEwen, B. S. and Alvarez-Buylla, A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci.* **21**, 7153-7160.

Seri, B., Garcia-Verdugo, J. M., Collado-Morente, L., McEwen, B. S. and Alvarez-Buylla, A. (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *J. Comp. Neurol.* **478**, 359-378.

Shimazaki, T., Shingo, T. and Weiss, S. (2001). The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. *J. Neurosci.* **21**, 7642-7653.

Shuai, K., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z. and Darnell, J. E. (1993). Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. *Nature* **366**, 580-583.

Song, M. R. and Ghosh, A. (2004). FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. *Nat. Neurosci.* **7**, 229-235.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., Fan, G. and Greenberg, M. E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* **104**, 365-376.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M. and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Dev. Cell* **1**, 749-758.

Takizawa, T., Ochiai, W., Nakashima, K. and Taga, T. (2003). Enhanced gene activation by Notch and BMP signaling cross-talk. *Nucl. Acids Res.* **31**, 5723-5731.

Viti, J., Feathers, A., Phillips, J. and Lillien, L. (2003). Epidermal growth factor receptors control competence to interpret leukemia inhibitory factor as an astrocyte inducer in developing cortex. *J. Neurosci.* **23**, 3385-3393.

Voigt, T. (1989). Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. *J. Comp. Neurol.* **289**, 74-88.

Zhong, Z., Wen, Z. and Darnell, J. E., Jr (1994). Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* **264**, 95-98.

Zhu, G., Mehler, M. F., Mabie, P. C. and Kessler, J. A. (1999). Developmental changes in progenitor cell responsiveness to cytokines. *J. Neurosci. Res* **56**, 131-145.