

Fibroblast Growth Factor-2 Activates a Latent Neurogenic Program in Neural Stem Cells from Diverse Regions of the Adult CNS

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During development of the mammalian brain, both neurons and glia are generated from multipotent neural stem cells. Although neurogenesis ceases in most areas at birth, stem cells continue to generate neurons within the subventricular zone and hippocampal dentate gyrus throughout adult life. In this work, we provide the first demonstration that precursors native to regions of the adult brain that generate only glia can also generate neurons after exposure to FGF-2 *in vitro*. When progenitors isolated from hippocampal tissue were directly compared with cells isolated from the neocortex, both populations were able to initiate a program of proliferative neurogenesis. Genetic mark-

ing and lineage analysis showed that a majority of the cells able to generate neurons were multipotent precursors; however, progeny from these precursors acquired the competence to differentiate into neurons only after exposure to FGF-2. The recruitment of similar FGF-2-responsive cells from the adult optic nerve, a structure well isolated from the neurogenic zones within the brain, confirmed that neuron-competent precursors naturally exist in widely divergent tissues of the adult brain.

Key words: neural stem cells; adult; hippocampus; cortex; optic nerve; density gradient

Cell proliferation in the adult mammalian brain is ubiquitous but is primarily confined to the measured production of glia. Except for discrete regions in the hippocampus and the subventricular zone (SVZ), neurogenesis is conspicuously absent (Altman and Das, 1965, 1966; Bayer, 1982; Kaplan and Bell, 1984; Levison et al., 1993; Lois and Alvarez-Buylla, 1993; Luskin, 1993). The reasons why these areas continue to generate neurons are unknown, but primary cell cultures from the adult rodent brain are beginning to provide some insights. Cultures initiated from adult SVZ or hippocampal (HC) tissues contain proliferative neuronal and glial-restricted progenitors, as well as multipotent precursors with the characteristics of neural stem cells, i.e., the ability to self-renew and the ability to generate both neurons and glia (Gage et al., 1995b; Temple and Qian, 1996; Weiss et al., 1996; McKay, 1997). More recently, Johansson et al. (1999) have shown evidence that some of these stem-like cells may actually be ependyma. If ependymal cells are actually stem cells, it seems increasingly unlikely that neurogenesis is absent in other regions because of the lack of multipotent stem cells. In past work, we have suggested that stem cells may be more widely distributed because cells from non-neurogenic areas repeatedly passaged in the presence of high concentrations of basic fibroblast growth factor (FGF-2) do begin to generate neurons *in vitro* (Palmer et al., 1995; Shihabuddin et al., 1997). This observation is consistent with the isolation of neuronal progenitors from these areas, but the protracted times in culture suggest another explanation. It is known that stem cell cultures initiated from hippocampal tissues

will spontaneously transform because of accumulated genetic abnormalities. Abnormalities in chromosome number can occur in as little as 30 population doublings (Palmer et al., 1997) and, as cells become increasingly aneuploid, it is possible that glial-restricted progenitors acquire capabilities beyond those available *in vivo*.

With current methodologies, it has been difficult to distinguish between the activation of a latent potential versus *in vitro* mutation. Unlike fetal tissues, which are easily dissociated and yield relatively abundant precursor populations, adult tissues yield few progenitors, and the progenitor preparations are contaminated with differentiated cells and tissue debris. The myelin-rich debris inhibits cell attachment and growth, whereas differentiated cells complicate the evaluation of lineage potential in acutely isolated cultures. Past studies have evaluated “progenitors” only after repeated passaging had eliminated the debris and differentiated cells (Gage et al., 1995a; Palmer et al., 1995, 1997). Even if these cells had remained diploid, they may have been altered dramatically in prolonged culture. A newly developed progenitor enrichment protocol has resolved many of these issues and has allowed us to evaluate the lineage potential of proliferative cells from cortex and hippocampus immediately after isolation from adult tissues. Surprisingly, both tissues yielded populations of multipotent precursors.

MATERIALS AND METHODS

Tissue dissection. Three areas were dissected from adult rat brains as follows (Fig. 1). Rats (170–190 gm, Fisher 344 males or females; Harlan Sprague Dawley, Indianapolis, IN) were deeply anesthetized with a mixture of ketamine, xylazine, and acepromazine. Animals were decapitated, and whole brains were removed. First, ~1.5 mm of each optic nerve were harvested rostral to, but not including, the optic chiasma. The brain was then bisected longitudinally, and each hippocampal lobe was separated from the overlaying cortical white matter using the natural separation line along the alveus hippocampus. The white matter of the fimbria and subiculum was removed as much as possible. Some white matter remained. Finally, a 1.5-mm-wide cortical ribbon containing parietal and frontal segments was dissected longitudinally, proximal to the

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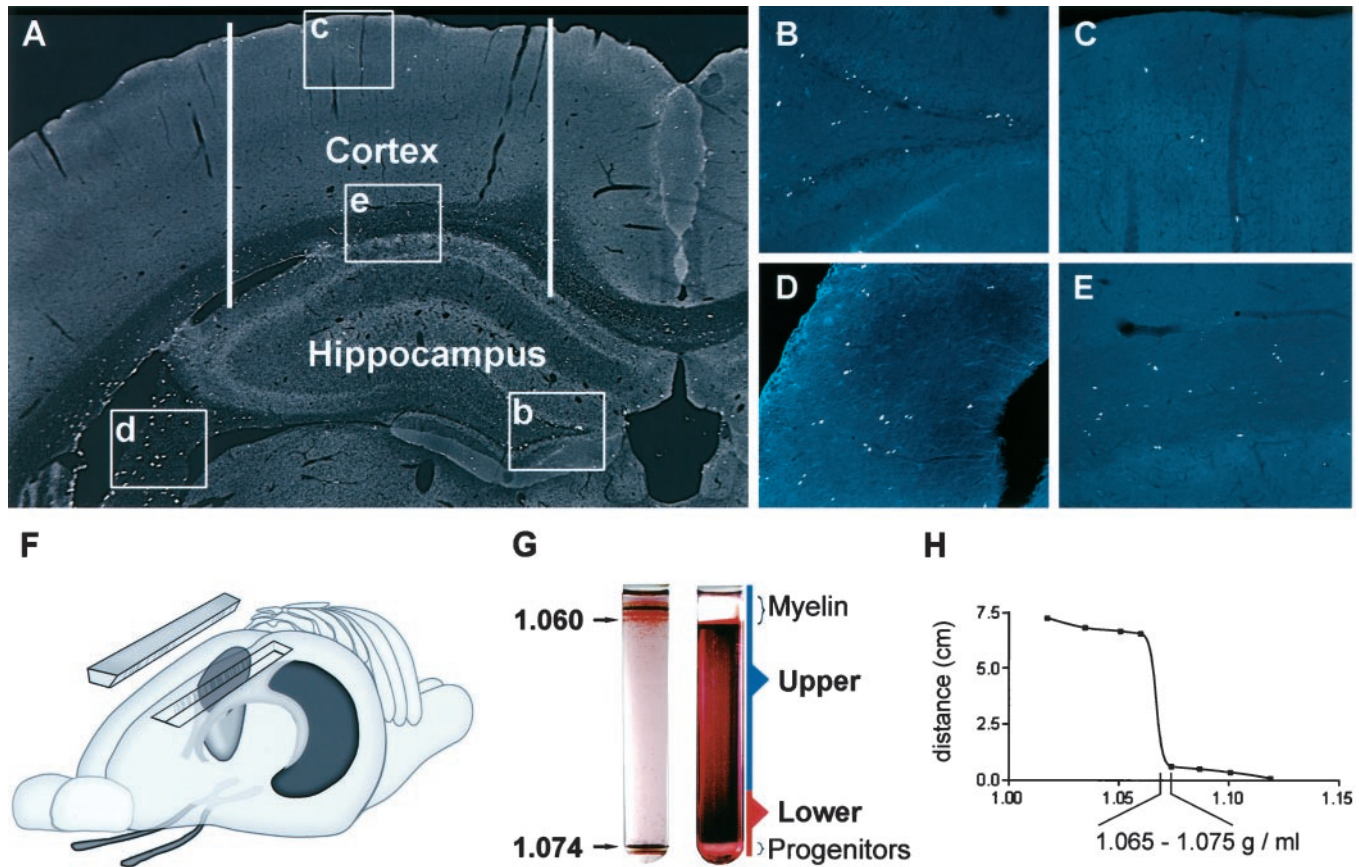


Figure 1. Isolation of endogenously proliferating cells from the adult hippocampus and cortex. *A*, Coronal section of the cortex and hippocampus of an adult rat immunostained for BrdU-labeled nuclei (white spots). Animals were injected with BrdU once each day for 6 d and then evaluated on the seventh day. Proliferative cells were abundant within the subgranule zone of the hippocampal dentate gyrus (*b*, *B*) and fimbrial ridge of the hippocampus (*d*, *D*). Closely juxtaposed pairs of daughter cells are clearly seen in white matter tracts, suggesting division *in situ* (*D*, *E*). Acid-stable vimentin immunoreactivity (blue) is clearly shown in the specialized ependymal cells lining the fimbrial surface. Although BrdU-positive proliferative cells were present in the cortical gray matter (*c*, *C*), they were much more abundant in subcortical white matter and adjacent ventricular zones (*e*, *E*). *F*, Tissues used for cultures included whole hippocampal lobes, cortical ribbons lateral to the midline (approximate lateral boundaries show by white bars in *A*), and sections of optic nerve rostral to the optic chiasma. *G*, Tissue was enzymatically dissociated and fractionated over a 50% Percoll gradient. Colored beads of known buoyancy were used to calibrate the gradient (left). The specific gravity of beads flanking the separation zone is shown (in grams per milliliter). Fractionated tissues generated two major bands of nucleated cells (right). The top band contained differentiated cells, myelin, and tissue debris. The bottom band contained undifferentiated progenitors and ependymal cells. A band of RBCs also formed immediately below the progenitor population (not visible here). *H*, The migration of progenitors in the gradient relative to that of calibrated beads demonstrates that adult-derived neural progenitors have a relatively high-buoyancy density of 1.065–1.075 gm/ml.

central fissure. The pial and callosal surfaces were trimmed from each cortical ribbon to remove a majority of the meninges and white matter.

Tissue dissociation and fractionation. As described previously (Gage et al., 1995a; Palmer et al., 1995), tissues were finely minced and digested in a solution of papain (2.5 U/ml; Worthington, Freehold, NJ), DNase (250 U/ml, Worthington), and neutral protease (1 U/ml Dispase; Boehringer Mannheim, Indianapolis, IN) dissolved in HBSS. Cells and tissue fragments were washed three times with DMEM containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). Whole digested tissue was then suspended in DMEM–10% FBS, filtered through a sterile 107 μ m nylon mesh and thoroughly mixed with an equal volume of Percoll solution. The Percoll solution was made by mixing nine parts of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) with one part 10 \times PBS (Irvine Scientific, Santa Ana, CA). The cell suspension was then fractionated by centrifugation for 30 min, 18°C, at 20,000 \times *g*. Cell fractions were harvested and washed free of Percoll by three or more rinses in DMEM–10% FBS.

Cell culture. Percoll gradients were optimized using perpetuated hippocampal stem cell clones AP31 and PZ5a (Palmer et al., 1997). Cells were maintained on polyornithine/laminin (PORN/LAM)-coated dishes in growth medium consisting of DMEM/F-12 (1:1) supplemented with N2 supplement (Life Technologies, Gaithersburg, MD) and 20 ng/ml recombinant human FGF-2 prepared in *Escherichia coli* (kindly provided by A.

Baird, San Diego, CA). Cells fractionated on Percoll gradients were washed free of Percoll and plated onto PORN/LAM-coated multiwell slides (Fisher Scientific, Houston, TX) or PORN/LAM-coated tissue culture dishes (Fisher Scientific). For 36 hr acute cultures, cells were allowed to attach to glass slides for 36 hr in DMEM/F-12 (1:1) containing 10% FBS and subsequently fixed for 10 min using 4% paraformaldehyde in PBS. For long-term primary cultures, isolated cells were maintained for 24 hr in 10% FBS. The medium was then replaced with serum-free growth medium. Seventy-five percent of the medium was replaced with new growth medium every 48 hr. Cultures were passaged at confluence by exposing cells to trypsin–EDTA solution (Irvine Scientific). Detached cells were then rinsed once with DMEM/F-12 and replated at one-half of their original density in growth medium supplemented with 30% conditioned medium (medium exposed to cells for 24 hr before passaging). To promote differentiation, growth medium was replaced with DMEM/F-12 containing 1% FBS, 100 ng/ml all-*trans* retinoic acid, and 1 ng/ml FGF-2 (differentiation medium).

Retroviral vectors and virus preparation. Steven Suhr (Salk Institute, La Jolla, CA) kindly provided the retrovirus-containing plasmid pNIT-GFP. pNIT-GFP contains replication-defective MoMLV-based retroviral elements designed to carry and express sequences encoding neomycin phosphotransferase (neo), tetracycline (tet) transactivator protein (Gossen and Bujard, 1992), and enhanced GFP. pNIT-GFP provides consti-

tutive neo and tet-transactivator expression under the control of the MoMLV LTR. GFP is expressed under the control of the tetracycline-suppressible *tet* operator system. A stable NIT-GFP packaging cell line was generated by cotransfecting 293 cells with pLNIT-GFP and the packaging constructs pMD.G and pCMV-gp as described previously (Emi et al., 1991; Burns et al., 1993). The transiently produced virus was used to infect 293 cells carrying a stable integrant of pCMV-gp (293gp). Clones of G418-resistant 293gp/NIT-GFP cells were screened for single, unrearranged NIT-GFP integrants. Clone 293gp/NIT-GFPc4 was chosen for use in these studies. Virus containing supernatants was harvested from 293gp/NIT-GFPc4 after transfection with pMD.G. Viral stocks were then concentrated 100-fold by centrifugation at $50,000 \times g$ for 90 min. Viral pellets were suspended in normal saline and again pelleted by centrifugation. Viral pellets were then resuspended in normal saline ($\sim 0.001 \times$ the volume of medium initially harvested). Final virus titers were $\sim 4 \times 10^8$ neo^r colonies/ml of virus as measured by G418-resistant colony formation on National Institutes of Health 3T3 cells. No helper virus was detected (>1 colony-forming units per milliliter of unconcentrated supernatant) using a marker rescue assay. Concentrated virus was stored in small aliquots at -70°C before use.

Progenitor marking and clonal analysis. Acutely isolated progenitors were cultured for 7 d in growth medium, detached with trypsin–EDTA solution, washed one time with DMEM/F-12, and suspended to a final concentration of 10^6 cells/ml in growth medium supplemented with $1 \mu\text{g/ml}$ polybrene. Volumes of NIT-GFP virus sufficient to infect 5–50 cells were added to 0.5 ml of cells (500,000 cells) and then incubated for 30 min at 37°C . The cells were pelleted, resuspended in growth medium containing 30% conditioned medium, and plated into 10 cm Porn/Lam-coated tissue culture dishes. Twenty-four to 36 hr later, the locations of individual green cells were marked on each dish. The cells were then allowed to grow to confluence with monitoring of individual infected cells on each day (7 d). Adjacent clones closer than 1 cm apart were excluded from the study. The growth medium was replaced every other day and then switched on the seventh day to differentiation medium. Differentiation medium was replaced every day for 7 d, and then the confluent monolayers were fixed for 10 min with 4% paraformaldehyde and evaluated using immunofluorescence.

Immunofluorescent staining. Paraformaldehyde-fixed cells in suspension, cell monolayers, or $30 \mu\text{m}$ floating tissue sections were rinsed with PBS and then blocked for 30 min at room temperature in PBS containing 0.3% Triton X-100 and 5% preimmune donkey serum (PBS²⁺). Samples were then incubated in PBS²⁺ containing dilutions of up to four primary antibodies for 24–48 hr at 4°C . Samples were then washed twice with PBS for 10 min at room temperature and then a third time for 30 min at room temperature. Samples were then incubated at 4°C for an additional 24–48 hr with secondary antibodies conjugated to aminomethyl coumarin, fluorescein isothiocyanate, Texas Red, or cyanin 5. Secondary antibodies (donkey; Jackson ImmunoResearch, West Grove, PA) were used at a final dilution of 1:500 in PBS²⁺. The samples were then washed as above, treated with 10 mg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) for 10 min and coverslipped in 20% polyvinylalcohol (20,000–30,000 MW; Air Products and Chemicals, Allentown, PA) in 50% glycerol (w/v) containing 2.5% w/v 1,4-diazobicyclo-[2.2.2]-octane (Sigma).

Primary antibodies generated in mouse (mo), rat (rt), rabbit (rb), and guinea pig (gp) were used at the following concentrations: mo anti-type III β -tubulin (1:2000; Babco, Richmond, CA), mo anti-microtubule-associated protein 2abc (1:5000; Sigma), mo anti-neuronal nuclear antigen (1:20; hybridoma supernatant kindly provided by R. Mullen, University of Utah, Salt Lake City, UT), mo anti-O4 (1:4; hybridoma supernatant kindly provided by O. Boegler, University of California, San Diego, CA), mo anti-receptor-interacting protein (1:20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), gp anti-glial fibrillary acidic protein (GFAP) (1:500; Advanced Immunochemical, Inc., Long Beach, CA), mo anti-A2B5 (1:100; Boehringer Mannheim), rt anti-bromodeoxyuridine (BrdU) (1:500; Accurate Chemicals, Westbury, NY), mo anti-Ox42 (1:1000; Chemicon, Temecula, CA), mo anti-Nestin (1:2000; Rat401; PharMingen, San Diego, CA), mo anti-vimentin (1:500; Amersham Pharmacia Biotech), rb anti-galactocerebroside (1:250; Advanced Immunochemicals), and rb anti-fibronectin (1:100; Telios Pharmaceuticals, San Diego, CA). Fluorescent samples were evaluated using a Bio-Rad (Hercules, CA) MRC1024UV confocal imaging system, which allows simultaneous evaluation of up to four separate fluorophores. When it was necessary to show nuclei in addition to four immunological

markers, cells were first evaluated for immunological staining in the absence of DAPI and then counterstained with DAPI and reimaged.

RESULTS

A progenitor enrichment protocol based on buoyant density

Density gradient media have been used frequently to fractionate cells on the basis of buoyant density (Poduslo and Norton, 1975; Lisak et al., 1981; Pertoft and Laurent, 1982; Shank and Campbell, 1984). Recently, mitotic cells have been separated rapidly from postmitotic cells in the late embryo using density gradients generated with colloidal silica (Percoll; Pertoft and Laurent, 1982; Maric et al., 1997). In these studies, fetal postmitotic cells were found to have buoyant densities lower than 1.043 gm/ml, whereas mitotic cells and progenitors have densities higher than ~ 1.056 gm/ml. To evaluate Percoll gradients for their use in fractionating multipotent precursors from the adult rat brain, we first determined the buoyant density of cells from perpetuated hippocampus-derived stem cell cultures (Palmer et al., 1997). These cultures are stem cell-derived and contain a mixture of lineages at various stages of differentiation. The most immature cells had remarkably high densities ranging from 1.065 to 1.075 gm/ml. On the assumption that the most immature cells within the adult brain would have similarly high densities, we optimized gradients using beads of known densities to generate steep density profiles spanning 1.060–1.075 gm/ml (Fig. 1). All cells with densities lower than 1.060 gm/ml were expected to form a band at the top of the gradient, whereas those with a buoyancy similar to the immature cultured progenitors would migrate into the gradient. Stem-like cells in perpetuated cultures typically formed a discrete band at the bottom of the gradient.

BrdU labeling was first used to mark endogenously proliferating cells for identification *in situ*. Adult rats were injected with BrdU once each day for 6 consecutive days, and then brains were collected for evaluation on day 7 (Fig. 1A). Two percent of all labeled nuclei within the hippocampus were found within the putative subventricular residuum, an area arbitrarily defined as a thin lamina extending inward $50 \mu\text{m}$ from the ependymal surface, including the hippocampus alveus but excluding ependymal cells. Ependymal cells proper accounted for 4% of the total labeled population, and a similarly small proportion was found within the neurogenic zone of the subgranular zone (SGZ) (8%) (Fig. 1B). In contrast, 52% were present in the white matter of the fimbrial ridge (Fig. 1D), and the remainder were scattered throughout the parenchyma. A similar comparison of neocortical gray and white matter showed that 15% of the BrdU-labeled cells were present in the parenchyma of the cortex (Fig. 1C). The remaining 85% were present in the subcortical white matter and associated SVZ (Fig. 1E). Although one might expect many of the BrdU-labeled cells to be derived from the local ventricular zone (Levison and Goldman, 1993; Luskin, 1993), ongoing studies indicate that most marked cells undergo DNA synthesis as resident populations within both gray and white matter (T. D. Palmer and F. H. Gage, unpublished observations).

To determine whether endogenously proliferating progenitors could be isolated from adult tissue on the basis of buoyant density, adult rats were injected with BrdU four times over a 48 hr period. Whole hippocampal lobes or cortical ribbons (Fig. 1F) were dissociated and fractionated over Percoll gradients. Three visible bands of cells were formed (Fig. 1G). A layer of red blood cells (RBCs) formed near the bottom of the gradient. A band of nucleated cells with buoyant densities similar to those of cultured

Table 1. Markers expressed by freshly isolated cells or after 36 hr in culture (%)

Lineage	Marker	Hippocampus			Cortex		Optic nerve
		Lower 0 hr	Lower 36 hr	Upper 36 hr	Lower 0 hr	Lower 36 hr	Lower 36 hr
Progenitor	Nestin	15	0.1	<0.1	7	<0.1	<0.1
	A2B5	1	0.5	nd	0.8	0.7	1.2
	O4	12	24	2	15	29**	31
Glia	Vimentin*	37 (34)	87 (36)	68 (15)	35 (30)	89 (15)	76 (18)
	GFAP	<0.1	0.1	26	<0.1	<0.1	<0.1
	RIP	<0.1	<0.1	14	<0.1	<0.1	<0.1
	GalC	nd	<0.1	12	<0.1	<0.1	<0.1
Neuron	β Tubulin	0.1	0.1	9	<0.1	<0.1	<0.1
	Map2	<0.1	<0.1	18	<0.1	<0.1	<0.1
	NeuN	nd	<0.1	15	<0.1	<0.1	<0.1
Other	Fibronectin	<0.1	<0.1	2	<0.1	<0.1	nd
	Ox42	<0.1	<0.1	1	<0.1	<0.1	nd
	ED-1	<0.1	<0.1	<0.1	<0.1	nd	nd
	BrdU	0.7	0.8	<0.01	0.1	0.2	nd

*Total immunoreactive cells versus acid-stable immunoreactive cells; nd, not determined; means are reported for three to seven separate cell isolates; **O4 immunoreactivity in 36 hr low-buoyancy cortical cultures showed the largest relative SEM and was 14% of the mean or 29 ± 4 . Map2, Microtubule-associated protein 2; RIP, receptor-interacting protein. NeuN, Neuronal nuclear antigen; ED-1, microglial marker; GalC, galactocerebroside.

progenitors formed just above the RBC layer, and a majority of the remaining differentiated cells, as well as tissue fragments and myelinated neuropil, formed a large band at the top of the gradient. A small number of cells were diffusely distributed throughout the gradient. Hippocampal tissues yielded 1015 ± 31 cells/mg of tissue in the lower band, and cortical tissues yielded 732 ± 24 cells/mg of tissue (mean \pm SEM; $n = 5$).

Cells from the upper and lower region of each gradient (Fig. 1G) were collected. A fraction of each population was immediately fixed and evaluated for BrdU and lineage-specific markers (Table 1). The upper fractions were so contaminated with cell debris and myelin that it was very difficult to determine phenotype, with the exception of BrdU immunoreactivity in cell nuclei. For both HC and cortical tissues, there were very few BrdU-labeled cells in the high-buoyancy fraction (less than one cell detected in 5000 nuclei scored for both HC and cortex), and the few cells not trapped within aggregates of debris nearly all expressed neuronal or glial markers. BrdU-labeled cells were found predominantly in the lower band, with 0.7 or 0.1% of all hippocampal or cortical cells labeled. In both hippocampal and cortical preparations, <0.1% of the low-buoyancy cells were GFAP-immunoreactive astrocytes. Although a few immature neurons (β -tubulin) were present in the hippocampal population (0.1%), none were detected in the low-buoyancy cortical fraction. A significant fraction of cells were immunoreactive for vimentin (37 and 35% in hippocampus and cortex, respectively), and vimentin expression was rapidly upregulated with 87 and 89% of all cells expressing vimentin after 36 hr in culture. Vimentin, a marker attributed to ependymal cells, immature astrocytes, and radial glia, is also known to be expressed by multipotent precursors in perpetuated neural precursor cultures (Levison and Goldman, 1993; Palmer et al., 1995; Luskin et al., 1997). Some cells in both fresh and 36 hr populations were weakly immunoreactive for O4, a marker first attributed to immature oligodendrocytes (Sommer and Schachner, 1981, 1982) and also expressed by FGF-2 stimulated multipotent precursors *in vitro* (Palmer et al., 1997). Nestin, a marker for immature precursors (Lendahl et

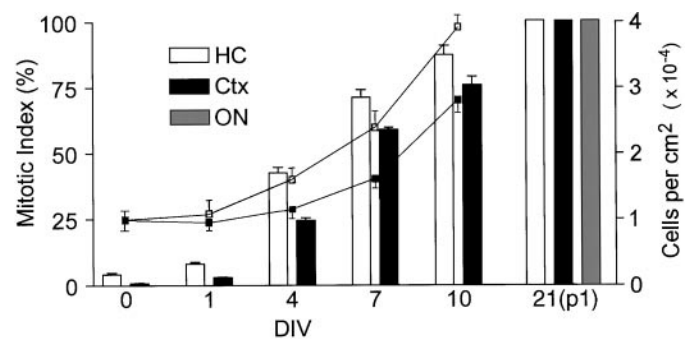


Figure 2. Proliferation of progenitors after exposure to FGF-2. Cells from the low-buoyancy fraction were plated at a density of $10^4/\text{cm}^2$ into defined medium containing 20 ng/ml FGF-2. To monitor proliferation, cells were pulsed with BrdU for 24 hr before being fixed after the indicated number of DIV, with the exception of day 21 in which each culture was passaged once and the cells labeled with BrdU for 72 hr before being fixed. At day 21, cultures initiated from optic nerve were also included, and >50,000 cells were scored in each culture. All cells were labeled, demonstrating that all cells were proliferative. The total number of cells per square centimeter was determined by counting nuclei (lines), whereas the percent of cells labeled with BrdU (mitotic index) was determined using immunofluorescent staining (bars). Values are mean \pm SEM; $n = 3$ independent isolates. Ctx, Cortex; ON, optic nerve.

al., 1990), was detected in 15 or 7% of the freshly isolated cells from HC or cortex, respectively, but was then transiently downregulated. At 36 hr in culture, very few cells expressed detectable nestin, yet 1 week later, virtually all cells in both hippocampal and cortical cultures were nestin-positive. Our paradigm involves an initial plating in 10% FBS. We suspect that exposure to serum followed by treatment with FGF-2 may be responsible for this modulation.

The quality of the vimentin staining in the low-buoyancy fraction also proved to be informative. After 36 hr in culture, vimentin staining was intense in cells with a flattened, neurepithelial-like morphology and weak in phase-bright cells reminiscent of

progenitors in long-term cultures. Upon acid pretreatment (required for the immunological detection of BrdU), only the intensely staining cells remained immunoreactive. This staining pattern was also seen *in vivo* in which the acid-stable vimentin immunoreactivity was restricted to the ependyma proper (Fig. 1D), whereas glia in the parenchyma exhibited a weaker, acid-labile staining. In addition, some of the cells in the low-buoyancy fraction were motile during the first few hours of culture. When stained with Coomassie blue, many of the vimentin-positive cells had easily detectable cilia typical of ependymal cells, and a small portion of cells were motile within the freshly isolated lower fraction (data not shown). On the basis of acid-stable vimentin staining, as many as 30–35% of the cells in the low-buoyancy fraction from both HC and cortex may have been ependymal cells.

Although BrdU-labeled cells fractionated to the low-buoyancy population, most of the isolated cells were unlabeled, consistent with the isolation of immature, yet relatively quiescent, precursors. To determine whether the unlabeled cells were competent to proliferate or simply lineage marker-negative terminally differentiated cells, hippocampus or cortex was fractionated, and cells from the low-buoyancy fraction were cultured in defined medium containing 20 ng/ml FGF-2 (DMEM/F-12 containing N2 supplement and 20 ng/ml FGF-2, growth medium). Cell division was monitored by counting cells and by treating replicate cultures with BrdU at different times after plating (Fig. 2). After a delay of several days, cells began an exponential growth pattern that reached a steady state in 7–10 d. After 10 d, growth rates were similar to those of the perpetuated cultures, with ~85% of the cells dividing in a given 24 hr period and >99% of the cells labeled after a 48 hr exposure to BrdU.

The freshly isolated cells also displayed a density-dependent growth that was similar to that seen in perpetuated stem cell cultures. Plating densities of ~10,000 cells/cm² or higher were required for optimum proliferation, whereas cells plated at clonal densities (<1 cell/cm²) grew very slowly or not at all. By fractionating cells, not only was it possible to eliminate debris and differentiated cells, but those cells remaining could be plated immediately into culture at densities that promoted the recruitment of cells into cycle.

Analysis of lineage potential

The lineage potential of progenitors from cortex or hippocampus was determined by culturing low-buoyancy cells in growth medium for 14 d and then allowing cells to differentiate under conditions shown previously to stimulate both neuronal and glial differentiation (differentiation medium: 1 ng/ml FGF-2, 1% fetal bovine serum, and 100 nM all-*trans* retinoic acid) (Palmer et al., 1997; Takahashi et al., 1999). At 14 d, few of the cells expressed markers for neurons or glia. Of the total population in HC or cortical cultures, 0.8 or 0.2%, respectively, were immunoreactive for β -tubulin (neurons), 1.1 or 1.7% were immunoreactive for GFAP (astrocytes), and 24 or 25% were immunoreactive for O4, a marker often attributed to immature oligodendrocytes (Sommer and Schachner, 1981) but also expressed by multipotent progenitors in long-term FGF-2-stimulated cultures (Palmer et al., 1997). There were no galactocerebroside-positive oligodendrocytes (<0.01%) detected in either culture. After differentiation for 7 d, both cultures contained numerous cells from all three lineages. Cortical cultures tended to contain a higher proportion of astrocytes (36 vs 28% for the hippocampus). Both hippocampal and cortical cultures contained similar numbers of highly arborized oligodendrocytes (1 and 2%, respectively), and each

contained significant numbers of neurons (8 and 3%, respectively). Figure 3A shows a field of cells from cortical cultures in which all three lineages are present.

To determine whether the neurons in the cortical populations were derived from proliferative multipotent precursors, retroviral marking was used to evaluate the lineage potential of cells within the low-buoyancy fractions. Cells were first stimulated with FGF-2 for 7 d to induce proliferation (a prerequisite for retroviral infection), and then retroviruses carrying a GFP transgene were used to infect the proliferating population. Individual infected cells were marked and allowed to proliferate within the noninfected bulk population for an additional 7 d. The resulting colonies were then induced to differentiate in differentiation medium (marking scheme shown in Fig. 3B). Figure 3C shows a typical population of colonies generated using an excess of virus [multiplicity of infection (m.o.i.), ~0.01]. To avoid overlapping colonies, ~50 infectious units were used in each assay below (m.o.i. of ~10⁻⁴). The number of colonies generated per 10 cm assay dish ranged from 4 to 21. To determine whether individual colonies were indeed clonal, the positions of individual marked cells were documented 24–36 hr after infection, and then colony growth was monitored daily (Fig. 3D–F). The incidence of closely juxtaposed cells giving rise to overlapping colonies with such low virus concentrations was insignificant (two clones were excluded in 543 clones scored). The remaining quantitation was performed as a “colony assay” on the assumption that all colonies separated by a centimeter or more were derived from single cells.

Clones were grouped into five categories based on the expression of lineage-specific markers and morphology (Fig. 3G–J, K–L). Neuron-only clones were infrequent (5.3%) in the hippocampal preparations and rare (<0.1%) in cortex-derived cultures (Fig. 3G). Some clones were glial-restricted and contained only GFAP-positive astrocytes and/or O4-positive oligodendrocytes (Fig. 3H). A small but significant proportion of the marked cells (21 and 17% from hippocampus and cortex, respectively) produced a mixture of glia and neurons (Fig. 3I). The remaining clones were negative for all three lineage markers. The marker-negative cells were further divided into two clone types; one type was very large and consisted of flattened phase-dark cells strongly immunoreactive for the acid-stable vimentin epitope (vimentin staining not shown). One such colony can be seen in Figure 3C (*bottom left*). The remaining marker-negative clones were small and contained large bipolar cells with simple, large-caliber processes (Fig. 3J).

When scored by size (Fig. 3L), neuron-only clones contained few cells, whereas glial-restricted progenitors and multipotent progenitors generated colonies of intermediate size. The largest clones were the lineage marker-negative, vimentin-positive clones. Although the large size may suggest a faster growth rate in the 7 d after infection, we found this large size to be an artifact caused by continued growth in differentiation medium. When observed during the first 7 d after viral infection, the clones with the flattened phase-dark morphology typical of these large lineage-negative clones actually grew more slowly than the other clones being monitored. Continued growth in differentiation medium was confirmed by repeating these experiments in the presence of BrdU during differentiation. We found that the large marker-negative clones were uniformly labeled with BrdU, whereas cells from the smaller neuron-only, glia-only, or mixed clones were unlabeled (data not shown).

The clonal analysis demonstrated that approximately half of the lower fraction of cells from both cortex and hippocampus was

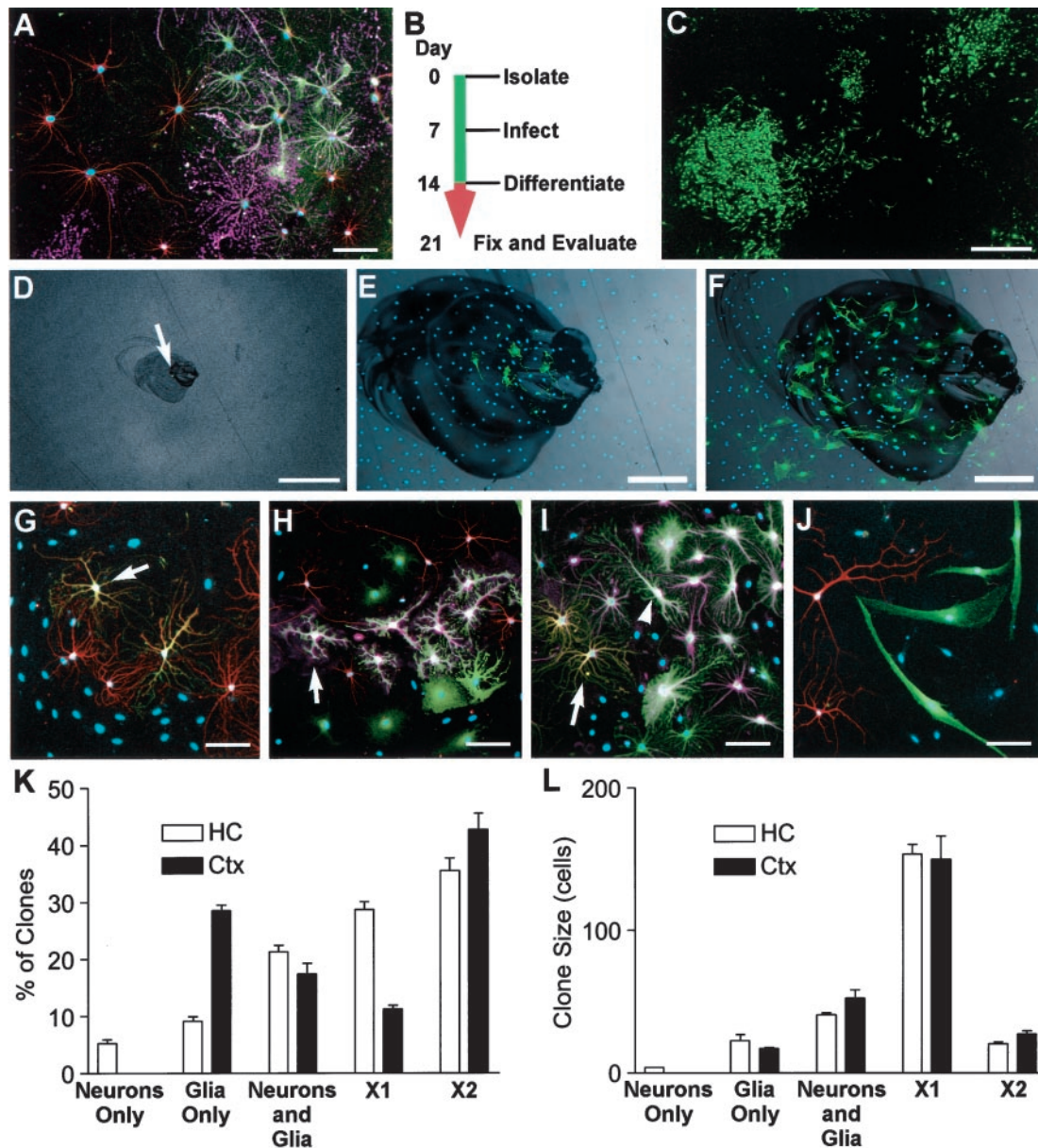


Figure 3. Lineage potential of freshly isolated progenitors. To determine the lineage potential of low-buoyancy cells from cortex or hippocampus, cells were cultured for 14 d and then allowed to differentiate (cortical cells shown in *A–J*). *A*, Bulk populations contained all three neural lineages. β -Tubulin-positive neurons are shown in *red*, GFAP-positive astrocytes in *green*, immature O4-positive oligodendrocytes in *magenta*, and nuclei in *blue*. *B*, To evaluate the lineage potential of single cells, cultures were treated with retroviral vectors on day 7, allowed to proliferate, and then induced to differentiate. Cell phenotypes were evaluated on day 21. *C*, A large variability in clone sizes and morphologies can be seen in the GFP-positive clones generated from an excess of virus. One hundred-fold less virus was used to generate well-separated clones scored in *D–L*. *D–F*, The locations of single green cells were marked 24–36 hr after infection (*arrow* in *D*). The same clone is shown at 5 d after infection and after fixation at day 21 (*E, F*). *G–J*, Lineage-specific markers were evaluated within each clone. Tubulin is shown in *red*, GFAP in *magenta*, and nuclei in *blue*. *G*, A two-cell clone containing only neurons. The neuronal cluster contains seven or more cells, but only two are marked with GFP (*orange* where *red* and *green* overlap), suggesting that the virally marked cell was resident within a larger clone of neuroblasts. *H*, A clone containing only glia. Although neurons are present in this field (*red*), the GFP-marked clone (*green*) only contains GFAP-positive astrocytes (*magenta* within the *green* staining cell bodies, *arrowhead*). *I*, Both β -tubulin-positive (*orange*, *arrow*) and GFAP-positive cells (*magenta* and *green* overlay, *arrowhead*) are present in this clone, demonstrating that the infected precursor was multipotent. *J*, Many small clones of large bipolar cells were negative for neuronal or glial markers. These cells grew slowly and were eventually outgrown by the neural progenitors in long-term cultures. *K, L*, Clones from at least three independent tissue preparations were scored for lineage and size (number of GFP-labeled cells per clone). *Neurons Only*, Only β -tubulin-positive cells; *Glia Only*, only GFAP- and/or O4-positive cells; *Neurons and Glia*, colonies with β -tubulin- and GFAP-positive cells; *X1*, large vimentin-positive clones negative for neuronal or glial markers similar to the large clone in the *bottom left corner* of *C*; *X2*, small clones containing large bipolar cells as in *J*. Values are mean \pm SEM. Scale bars: *A, E–J*, 50 μ m; *C, D*, 400 μ m.

made up of cells that formed large colonies but did not differentiate into neurons or glia. Although the acid-stable vimentin staining in these cells is consistent with an ependymal origin, the lineage of these cells has not been determined. The remaining

cells consisted of neural progenitors of mixed lineage potential. Approximately 20% of all cells isolated were able to generate both neuronal and glial progeny, suggesting that both cortex and hippocampus contained multipotent precursors.

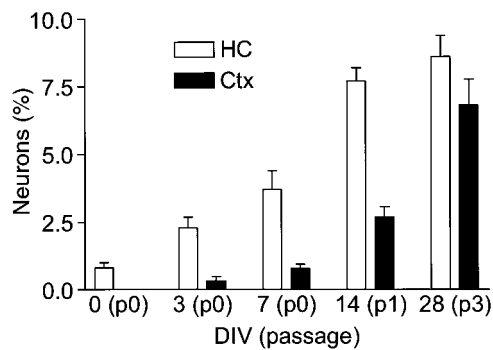


Figure 4. Exposure to FGF-2 activates a neurogenic potential in progenitors from cortex. Cells from the hippocampus or cortex were cultured for various times in FGF-2 and then allowed to differentiate for 7 d. Without previous exposure to FGF-2, cortex-derived cells were unable to generate β -tubulin-positive neurons ($<0.001\%$ at day 0). With as little as 3 d of FGF-2 exposure, some cortical progenitors began to generate neurons and, with increasing passage, cortical and hippocampal cultures normalized to a steady-state population, $\sim 10\%$ of which were capable of differentiating into neurons.

A novel role for FGF-2 in neurogenesis

Neurogenesis is not detected in the adult cortex, yet a significant number of the progenitors from cortical tissues were competent to generate neurons once removed from their *in vivo* environment. Activation of this neurogenic program could be triggered by several mechanisms. One possibility is that multipotent precursors may yield progeny that are competent to differentiate into neurons but are suppressed by cell-extrinsic signals *in vivo*. If so, the simple act of removing them from the *in vivo* environment may disinhibit or activate a latent neuronal differentiation program. To test this possibility, low-buoyancy cells were isolated from adult tissue and immediately plated into differentiation medium. The presence of β -tubulin-positive neurons was scored after 10 d [0 days *in vitro* (DIV) in FGF-2] (Fig. 4). Both cortical and hippocampal populations generated abundant populations of glia, but only the hippocampal preparations generated β -tubulin-positive neurons. The absence of neurons in cortical preparations suggested that precursors from non-neurogenic tissues require signals provided in culture to acquire the competence to differentiate into neurons.

To determine how rapidly cortical cells acquired the competence to differentiate into neurons in culture, cells were isolated and cultured in growth medium for various times, allowed to differentiate in differentiation medium, and then evaluated for the presence of neurons (Fig. 4). In control cultures, cells were cultured in 10% fetal bovine serum or 5 ng/ml recombinant platelet-derived growth factor (PDGF) instead of FGF-2. After 3 d in FGF-2, the occasional neuron was observed in cortical cultures, and this number gradually increased with lengthening exposure to FGF-2. By 4 weeks, both cultures had been passaged three times, and $\sim 10\%$ of the cells formed neurons when induced to differentiate. Although cells were rapidly recruited into cell cycle in serum or PDGF, no neurons were formed (data not shown). Cultures were also treated with BrdU for the first 10 d, and the newly generated neurons were evaluated for evidence of cell division. All neurons scored were labeled ($>99\%$). This demonstrated that exposure to FGF-2 was necessary for the recruitment of neuron-competent precursors and that proliferation invariably accompanied the recruitment process.

Neuron-competent progenitors are found in areas distant from the proliferative zones of the anterior SVZ

Cortical gray matter contains a small population of endogenously dividing glial progenitors (Mares et al., 1975; Kaplan and Hinds, 1980), but the underlying subcortical white matter and ventricular zone have relatively abundant populations of dividing cells (Fig. 1A,E). Although we expect that many of the “cortical” progenitors were derived from this underlying proliferative zone, it was also possible that contaminating cells from the more rostral neurogenic areas of the lateral ventricle may have been present in the cortical preparations. Noble, Raff, and others have shown that the adult optic nerve retains an active population of glial progenitors (Raff et al., 1987; Miller et al., 1989; Wolswijk and Noble, 1989), and the optic nerve rostral to the optic chiasma can be easily harvested without risk of contamination from the SVZ of the lateral ventricle.

To determine whether there were progenitors in the optic nerve with a latent ability to generate neurons, optic nerve was harvested, dissociated, and fractionated. Low-buoyancy cells were cultured for 14 d in the presence of high FGF-2 and then allowed to differentiate for an additional 14 d. BrdU was added during the last 72 hr of FGF-2 treatment (day 14). On day 28, cultures were evaluated for the presence of neurons (β -tubulin), and those neurons present were scored for BrdU immunoreactivity. Figure 5A–C shows a typical culture from the optic nerve after 1, 3, or 7 d *in vitro*. Although many glia rapidly differentiated in the primary culture (Fig. 5A, arrows), clusters of proliferative precursor-like cells were readily detected (Fig. 5B,C). After several weeks in culture, a small but significant minority of these responding cells were able to generate β -tubulin-positive neurons when induced to differentiate ($0.8 \pm 0.3\%$ of the total population; mean \pm SEM; $n = 5$) (Fig. 5). Neuronal markers, such as β -tubulin, 200 kDa neurofilament, and tau, were never detected in GFAP-positive or O4-positive glia, suggesting that the neuron-like cells were authentic neurons rather than glia that inappropriately expressed neuronal markers. Neurons were often found in small clusters, suggesting a clonal derivation, and all cells, including neurons, were labeled with BrdU during the last 72 hr of FGF-2 treatment (less than one unlabeled cell per 50,000 total nuclei) (Figs. 2, 5D–K). This data indicates that all neurons were derived from proliferative precursors. The fact that these neurons are generated from cells isolated from the optic nerve dispels any concerns of contamination from known neurogenic zones and demonstrates that a latent neurogenic potential is retained by precursors from divergent regions of the adult brain.

DISCUSSION

The use of acutely isolated cells in this study suggests several possibilities regarding the presence of multipotent progenitors in the adult brain. First, the potential of cells from non-neurogenic areas to generate neurons does not appear to be an artifact of perpetuated cultures because new neurons can be recruited within days of isolating cells from normal adult tissues. Second, this normal progenitor population appears to exist as an early multipotent progenitor or stem cell (Anderson, 1994; Temple and Qian, 1996; Morrison et al., 1997), because the competence to differentiate into neurons is not intrinsic to these cells but is only gained by progeny of the original cell after the instructional influence of exogenous cues (i.e., removal from *in vivo* cues and the application of high concentrations of FGF-2). Third, because white matter areas rich in proliferative glial progenitors also yield populations of multipotent cells, it seems likely that stem cells

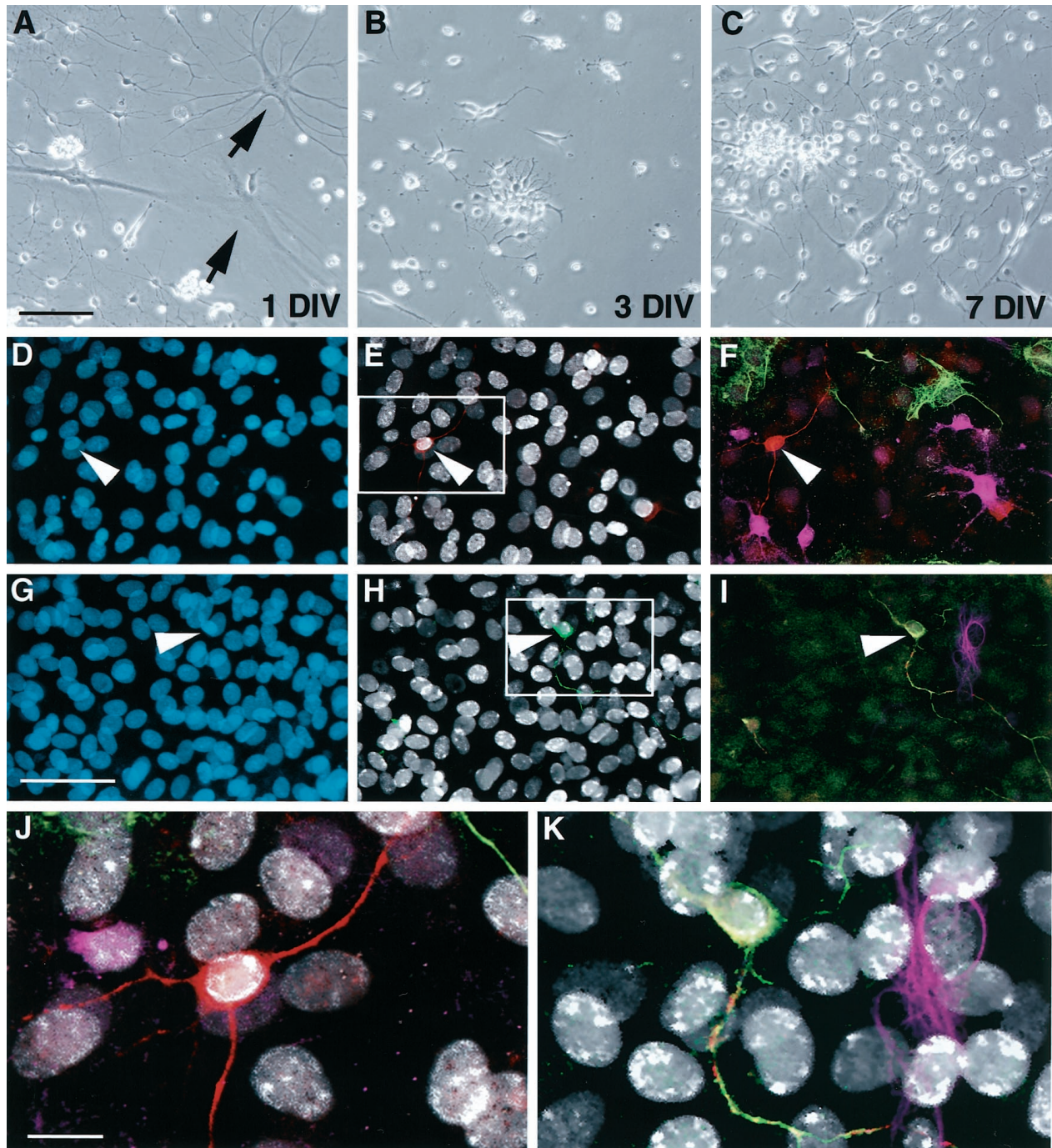


Figure 5. Neurogenesis in cultures initiated from adult optic nerve. Low-buoyancy cells were isolated from adult optic nerve and placed in culture. At 1 DIV, a mixture of immature and differentiated cell morphologies was present (*A*). Arrows in *A* show typical glial profiles present at 1 DIV. On days 3 (*B*) and 7 (*C*), small proliferating clusters became apparent. After 3 weeks in the presence of FGF-2, a small but significant proportion of cells (0.8%) were able to differentiate into β -tubulin-positive neurons. In *D–K*, cells were treated with BrdU for 72 hr (days 12–14) and then switched to differentiation medium on day 14. Cells were fixed on day 28 and evaluated for BrdU and lineage-specific markers. Total nuclei are shown in blue (*D*, *G*). BrdU-positive nuclei (white) in the same fields are shown along with β -tubulin (red, *E*) or 200 kDa neurofilament (green, *H*). *F* and *I*, respectively, show multiple labeling for β -tubulin (red), GFAP (green), and O4 (magenta) or 200 kDa neurofilament (green), tau (red), and GFAP (magenta). Enlargements of the areas boxed in *E* and *H* clearly show that the neuronal nuclei are BrdU-positive (white in *J* and *K*). Scale bars: *A–C*, 60 μ m; *D–I*, 35 μ m; *J*, *K*, 15 μ m.

continue to participate in ongoing gliogenesis in the adult. This could be an active process, i.e., multipotent cells actively proliferate to generate glial progeny, or an indolent process by which a quiescent population of multipotent cells slowly replenishes the proliferative pool of glial progenitors.

The exact number of multipotent stem cells in adult tissues is

still open for debate. The clonal analysis suggests that ~20% of all proliferative cells present after 1 week of culture were multipotent stem-like cells. However, because progenitors at different stages of commitment may cycle at different rates, it would be difficult to calculate the exact number of multipotent precursors isolated from each tissue. A crude estimate might be made as

follows. The number of low-buoyancy cells recovered from a known weight of adult tissue was ~1000 cells/mg for hippocampus and ~700 cells/mg for cortex. If 20% are multipotent upon isolation, then ~200 or 140 multipotent cells are present in each milligram of HC or cortical tissue, respectively. Although this number is exceedingly small relative to the total cell complement, it is probably quite significant given the considerable proliferative capacity of these stem-like cells and the ability to generate neurons. In combination with our past observations, the present findings suggest that stem-like cells can be isolated from very diverse regions of the adult CNS, including septum, striatum, cortex, spinal cord, and optic nerve (Gage et al., 1995a; Palmer et al., 1995; Shihabuddin et al., 1997).

Although multipotent cells may be broadly distributed, this distribution is likely to be nonuniform. Of the total low-buoyancy cell complement from hippocampus, cortex, and optic nerve, 7.7, 2.7, or 0.8%, respectively, differentiate into neurons after 2–3 weeks of proliferation in the presence of FGF-2. Consistent with these results, preliminary experiments on carefully subdivided tissues indicate that the ventricular zone of the lateral ventricle is particularly rich in low-buoyancy multipotent cells, an observation consistent with the numerous works of Weiss, van der Kooy, and others (Cepko, 1988; Reynolds et al., 1992; Morshead et al., 1994) and an observation consistent with the recent report of stem cells within or immediately adjacent to the ependymal layer proper (Doetsch et al., 1999; Johansson et al., 1999). White matter of the corpus callosum and spinal cord also yield abundant but lesser populations of FGF-2-responsive neurogenic cells, and samples of gray matter conservatively trimmed of white matter or ependymal surfaces yield lower, yet still significant, numbers of cells. This finding implies that multipotent precursors are most abundant in ventricular areas, i.e., the residuum of the developmental neurogenic zone, but also suggests that cells with multilineage potential may be present within the parenchyma as well. When comparing results from Johansson et al. (1999) to the recent findings of Doetsch et al. (1999), it is unclear what form the multipotent stem cell takes under normal conditions (i.e., ependymal vs astrocyte-like or some undetermined phenotype); however, the implication of both works is that a common and widespread cell type can generate neurons under appropriate conditions, an observation first suggested for optic nerve by Omlin and colleagues (Omlin and Waldmeyer, 1989; Omlin and Riederer, 1992).

The fact that the ependymal zone retains a population of stem cells may provide an explanation for our ability to isolate such cells from so many different areas of the adult brain, especially if these cells indeed turn out to be astrocyte-like cells. However, a remaining question is why multipotent progenitors are not used to make neurons in all regions. The naturally elicited neurogenic potential in the SGZ and SVZ may involve either escape from several layers of glial-directed cues present in the other regions or simple activation of a dormant program. In our studies, simple removal of cells from *in vivo* cues does not allow cells from cortical tissues to differentiate into neurons, suggesting that active local inhibition alone does not account for the lack of neurogenesis. Neuronal competence is only acquired after one or more divisions in the presence of high concentrations of FGF-2. The replication of the DNA of a cell may be essential for chromatin restructuring associated with alterations in gene expression patterns, particularly when overcoming the relatively stable chromatin structure associated with silenced regions of the genome (Aparicio and Gottschling, 1994). Replication alone, however,

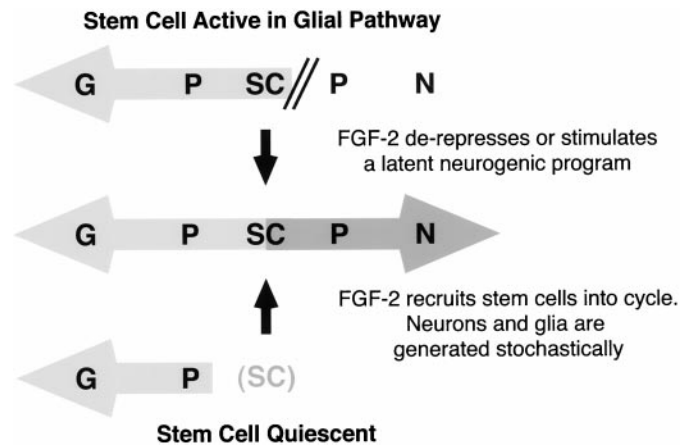


Figure 6. Potential influence of FGF-2 on the adult neural stem cell. If multipotent precursors or stem cells (SC) exist in non-neurogenic areas of the brain, they may persist in one of two states. Multipotent cells may actively participate in the gliogenic program by replenishing a pool of committed progenitors (P). Removal of cells from this environment and treatment with FGF-2 may de-repress or stimulate a latent neurogenic program. Alternatively, stem cells may represent a vestigial population that is inactive in most areas of the brain. Native gliogenesis (and/or neurogenesis) would then be mediated by committed progenitors, and the primary effects of FGF-2 on stem cells isolated in culture would be mitogenic. Once actively dividing in culture, stem cells may generate glia (G) or neurons (N) in a stochastic manner.

does not appear to be sufficient to activate a neuronal potential. Most of the cells proliferate in the presence of serum or recombinant PDGF, but the responding cells were limited to glial precursors. A high concentration of FGF-2 appears to be an essential component of the environment that allows stem cell progeny to acquire the competence to form neurons.

FGF-2 has long been known for its pleiotropic effects on neural progenitors (Walicke, 1988; McKinnon et al., 1990; Anderson, 1993; Sensenbrenner et al., 1994), and many groups have noted that FGF-2 is a necessary mitogen for maintaining proliferative multipotent precursors *in vitro* (Richards et al., 1992; Vescovi et al., 1993; Kilpatrick and Bartlett, 1995; Gritti et al., 1999; Tropepe et al., 1999), but little is known regarding the potential of FGF-2 to alter the lineage potential of a cell. Work by Qian et al. (1997) suggests that a developmental change in the concentration of FGF-2 may play a role in regulating the fate of neural stem cells. FGF-2 expression *in vivo* is upregulated concurrently with the stem cells switch from a neuron-only program to one that also generates glia. *In vitro*, treatment of embryonic day 10 (E10) cortical progenitors with low levels of FGF-2 (0.1 ng/ml) retains cells in a neuron-only program. Treatment of these same cells with higher concentrations of FGF-2 (10 μ g/ml) stimulates stem cell proliferation (i.e., the average clone size increases) and encourages progeny to generate glia in addition to neurons. Although the authors argue that FGF-2 acts to activate a gliogenic program, the observation that FGF-2 stimulates the production of multiple lineages in a population ordinarily limited to generating one cell type may actually be very similar to our findings. The obvious difference is that the predominant differentiation program of "adult" precursors is to generate glia and not neurons. If stem cells are involved in adult gliogenic processes, perhaps both adult and embryonic precursors are "normalized" to a multi-lineage program by high concentrations of FGF-2 (Fig. 6), e.g., FGF-2 may activate both neuronal and glial programs in responding cells. Because the neuronal pathway is

fully active in E10 stem cells, only changes in glial production would be measured. In fact, treatment of E10 progenitors with FGF-2 had very little effect on the number of neurons generated by each clone, although the average number of total cells in each colony increased dramatically. In a similar sense, the glial pathway may be fully activated in adult stem cells, and the only measurable effect of FGF-2 would be to stimulate neuron production. Whether this is through a recruitment process in which a latent potential is activated (our favored hypothesis because of the rapidity of neuronal recruitment *in vitro*) or because of abrupt FGF-2-mediated reprogramming of progenitors normally committed to the glial lineage remains to be determined.

An alternative hypothesis not addressed by our paradigm is that multipotent precursors might not participate in adult gliogenesis at all, and FGF-2 may simply act as a mitogen to recruit a vestigial population of stem-like cells that cofractionate with committed glial progenitors. Intraventricular administration of FGF-2 seems to argue against a pure mitogenic effect because precursors in the ventricular zone only respond with moderate increases in proliferation (Kuhn et al., 1997). Instead, FGF-2 seems to have a stronger effect on the ratio of newborn neurons and glia that survive after treatment, suggesting that FGF-2 may equally influence proliferation and cell fate *in vitro*.

From birth to senescence, the brain may exhibit a continuum of “developmental” plasticity retained by stem-like cells that respond to different environments in different manners. This plasticity may be gradually attenuated but persists at some level throughout life. Once resident, it may remain unused throughout life. Alternatively, stem-like cells may play an active role in the ongoing gliogenesis found throughout the adult brain. If the latter is true, the latent potential to generate neurons may simply never be invoked outside of the neurogenic regions of the hippocampus and rostral ventricular zone. In the context of our present work, it seems likely that the multipotent precursor recruited by high concentrations of FGF-2 represents a common precursor to both neuronal and glial progenitor populations generated in the adult brain. Although these cells are probably quite rare relative to the total cellular complement in adult brain tissues, the ability to isolate and enrich for these cells on the basis of buoyant density provides an opportunity to elucidate the regulatory mechanisms governing their activity *in vitro*. In addition, the rapid isolation of progenitors from adult tissues provides a unique opportunity to evaluate native progenitor populations in grafting models for cell replacement and brain repair.

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