

Single factors direct the differentiation of stem cells from the fetal and adult central nervous system

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Identifying the signals that regulate stem cell differentiation is fundamental to understanding cellular diversity in the brain. In this paper we identify factors that act in an instructive fashion to direct the differentiation of multipotential stem cells derived from the embryonic central nervous system (CNS). CNS stem cell clones differentiate to multiple fates: neurons, astrocytes, and oligodendrocytes. The differentiation of cells in a clone is influenced by extracellular signals: Platelet-derived growth factor (PDGF-AA, -AB, and -BB) supports neuronal differentiation. In contrast, ciliary neurotrophic factor and thyroid hormone T3 act instructively on stem cells to generate clones of astrocytes and oligodendrocytes, respectively. Adult stem cells had remarkably similar responses to these growth factors. These results support a simple model in which transient exposure to extrinsic factors acting through known pathways initiates fate decisions by multipotential CNS stem cells.

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Most of the neuronal and glial cell types found in the mature central nervous system originate from precursor cells in the ventricular zone of the fetal brain and spinal cord (Jacobson 1991). Recently, several studies have examined cell lineage in the vertebrate central nervous system (CNS) to see whether neuronal and glial cell types originate from a common precursor or from distinct progenitors (Kornack and Rakic 1995; Reid et al. 1995; for review, see Luskin 1994). In cortex (Price and Thurlow 1988; Walsh and Cepko 1988), retina (Turner and Cepko 1987; Holt et al. 1988; Wetts and Fraser 1988), and optic tectum (Galileo et al. 1990), a single precursor cell can give rise to both neurons and glia as well as different types of neurons even at late stages of neurogenesis. These results imply that multipotential precursors persisting throughout CNS development differentiate in response to local cues.

At the same time, other lineage analyses using retroviral marking indicated that separate progenitors give rise to neurons and glia as well as to neuronal subtypes, suggesting that those cells are committed to a particular fate through multiple cell divisions (Parnavelas et al. 1991; Luskin et al. 1993; Mione et al. 1994). Thus, the results from *in vivo* lineage analysis are not uniform. It is generally acknowledged that extrinsic factors must be

important in generating the cellular diversity observed in the CNS (Ferri and Levitt 1995; Lillien 1995). However, heritable intrinsic determination of cell fates before mitosis is complete is also a key component of current hypotheses of mammalian CNS development (Chenn and McConnell 1995; Kornack and Rakic 1995; Soriano et al. 1995; Reid et al. 1995). The relative roles of extracellular signals and cell autonomous mechanisms regulating fate choice are not well understood.

Most lineage studies in the mammalian CNS have focused on mapping the actual fate of cells in specific environments *in vivo*. Fate maps do not contain information about the potential of the cells; thus, *in vivo* lineage analysis neither identifies the precursor cells nor defines the mechanism driving them to specific fates. To explore the developmental mechanisms that control the specification, determination, and potency of cells, they must be manipulated and not simply labeled. The developmental capacity of a given precursor cell can be tested directly by transplantation *in vivo* or by manipulating its environment in tissue culture. Results from transplantation experiments have demonstrated the plasticity of neuronal precursors by showing that cells from a single region can give rise to neurons appropriate to ectopic regions of the host brain (Brustle et al. 1995; Fishell 1995; Vicario-Abejon et al. 1995a).

Several *in vitro* approaches have been used to analyze the lineage of cells in the CNS. *In vitro* experiments have

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defined a bipotential precursor with the capacity to differentiate into either oligodendrocytes or type 2 astrocytes (Raff et al. 1983; for review, see Barres and Raff 1994). Mitotic cells from acutely dissociated embryonic cortex and hippocampus have been analyzed by retroviral labeling. Clones ranging from a single cell type to variable mixtures of neurons, astrocytes, and oligodendrocytes were observed (Williams et al. 1991; Gotz et al. 1995; Williams and Price 1995). Clones of proliferative cells from the embryonic brain have been analyzed for their differentiation capacity in a short-term microculture system and have provided additional evidence for multipotential stem cells in the CNS (Temple 1989; Davis and Temple 1994). Other types of precursor cells from developing and adult CNS have been expanded in longer term culture by the mitogenic actions of basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) (for review, see Gage et al. 1995; Kilpatrick et al. 1995; Temple and Qian 1996). However, although the differentiation potential of various neural precursors expanded in vitro have been explored previously, there are few examples of a systematic clonal analysis that is necessary to fully characterize the mechanisms that regulate the origin of neurons and glia.

To further explore the origin of cell types in the vertebrate CNS, we established an in vitro system that permits extensive proliferation and controlled differentiation of nestin-positive cells from different regions of the fetal brain. Quantitative clonal analysis reveals that cells expanded for up to 1 month maintain the capacity to differentiate into all three major cell types of the mature CNS: neurons, astrocytes, and oligodendrocytes. Defined extracellular factors act in an instructive mode to restrict the differentiation potential of these CNS stem cells to unipotent progenitors for either astrocytes or oligodendrocytes. Furthermore, we find that multipotential stem cells with similar responses to growth factors

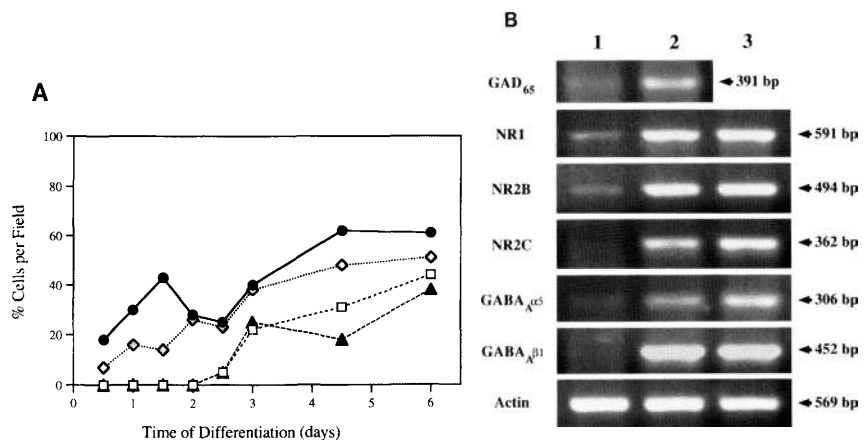
are present in the adult brain. These results suggest that the action of distinct extrinsic signals acting on a single class of multipotential stem cell is a central mechanism in the origin of cell types in the brain.

Results

Mitogen withdrawal initiates the differentiation of expanded CNS cells

Hippocampal cells isolated from embryonic rat brains were expanded by daily addition of bFGF in serum-free medium. A continuous supply of bFGF was important to repress differentiation and to maintain a homogeneous population of rapidly dividing cells expressing nestin, an intermediate filament protein characteristic for CNS precursor cells (Lendahl et al. 1990). Less than 1% of the cells expressed neuronal antigens, the astroglial marker GFAP or the oligodendroglial markers O4 and GalC. Withdrawal of bFGF initiated differentiation within 24 hr. Over a 6-day period, there was a progressive increase in the number of cells expressing several well-established neuron-specific antigens, including MAP2a, MAP2b and MAP2c, β -tubulin type III (TuJ1), tau, and neurofilaments (NFs) L, M, and H (Fig. 1A). Up to 50% of these cells expressed the neuronal antigens and exhibited a complex neuronal morphology. Varying proportions of the neurons were immunopositive for neuronal markers such as GABA, glutamate, calretinin, and calbindin (data not shown). Specific subtypes of GABA and glutamate receptors known to be present in the embryonic brain (Laurie et al. 1992; Bochet et al. 1994; Monyer et al. 1994) were also expressed in the differentiated cells (Fig. 1B). These results demonstrate that the long-term expanded cells differentiate efficiently to generate neurons. The remaining 50% of the differentiated cells expressed GFAP, GalC/O4, or nestin (data not shown). Al-

Figure 1. (A) Controlled differentiation of CNS stem cells at high density. Rapidly dividing nestin-positive precursor cells after 12 days in bFGF (10 ng/ml) were labeled with 10 μ M BrdU during the last 24 hr of proliferation. Differentiation was then initiated by withdrawal of bFGF (day 0) and continued for up to 6 days. At indicated times, cells were fixed and stained for BrdU and various neuronal antigens. Ratio of cells double-stained for BrdU and each neuronal antigen to total BrdU + cells per 40 \times field are shown. Up to 50% of BrdU + cells expressed neuronal antigens, and their expression was time dependent. (●) MAP2+; (shaded diamond) TuJ1+; (□) NF-L+; (▲) NF-M+. (B) RT-PCR analysis of gene expression in undifferentiated and differentiated cultured CNS stem cells. Total RNA isolated from undifferentiated stem cells (lane 1) or stem cells that had been differentiated for 6 days (lane 2) or 12 days (lane 3) was reverse-transcribed and analyzed by PCR as described in Materials and Methods. As indicated at left, oligonucleotides corresponding to the following gene products were used: glutamate decarboxylase (GAD_{65}); the NMDA receptor subunits NMDAR1 (NR1), NMDAR2B (NR2B), and NMDAR2C (NR2C); the GABA_A receptor subunits $\alpha 5$ (GABA_A $\alpha 5$) and $\beta 1$ (GABA_A $\beta 1$); and actin. The molecular mass of the PCR products is indicated at right.



though neurons and glia have been observed previously in expanded culture, these experiments establish that the differentiation of proliferating precursor cells to neurons and glia can be initiated at a precise time point and thus permit large-scale lineage analysis *in vitro*.

Multipotentiality of CNS precursor cells

To determine whether the precursor population contains separate committed progenitors that independently give rise to neurons and glia, rapidly dividing cells were plated at clonal density (200 cells per 10-cm plate) and well-isolated single cells were marked with 3-mm-diameter circles (Fig. 2A). Five to ten percent of the marked single cells survived and proliferated with a doubling time of 24 hr to generate clones of nestin-positive cells (Fig. 2B). After various periods of expansion (clone sizes ranging from 2^4 to 2^{10} cells), clones were differentiated by withdrawing bFGF and further cultured in serum-free medium without bFGF for 6 days. During the period after mitogen withdrawal, heterogeneous morphologies appeared rapidly and neurons differentiated in the clones (Fig. 2C,D). As in high-density culture, 50% of the cells in a clone expressed neuronal antigens including MAP2, tau, and β -tubulin type III (Table 1A). Neurofilament expression was delayed under these conditions, most likely owing to the low cell density. Other cell types within clones were analyzed by double-staining with combinations of cell type-specific antibodies. Eight percent of cells in the clones expressed GFAP and displayed a characteristic astrocytic morphology (Table 1A; Fig. 3A,C). In addition, 8% of the cells in the clones were GalC+ and had typical oligodendrocyte morphology (Table 1A; Fig. 3C). The remaining cells were unstained by any of the antibodies specific for differentiated cell types but reacted with A2B5 and/or antinestin antibodies that stain dividing precursors. A maximum of 20% of the cells died during differentiation. Almost all clones ana-

lyzed contained highly reproducible numbers of neurons, oligodendrocytes, and astrocytes.

Prior to differentiation, cells within clones displayed a uniform morphology and were immunonegative for antigens other than nestin. Yet, the separation of neuronal and non-neuronal morphologies occurred rapidly within 24 hr and only after mitogen withdrawal. The early neurons were evenly distributed throughout the clone without obvious polarity or localization, suggesting the absence of committed neuronal progenitors during clonal expansion. Moreover, the number of neurons increased linearly with increasing clone size and reproducibly constituted 50% of the clone (Fig. 4A,B). A very high proportion of clones were multipotential whether cells were obtained from acutely dissociated cells with no prior passage or from cells after four passages (26 days of expansion) (Table 1A). This finding shows that the multipotentiality of these cells is stable in culture. Furthermore, the absence of clones consisting of a single cell type shows that under these conditions committed progenitor cells are absent.

To further determine whether expanding clones consisted of distinct proliferating committed progenitors, individual clones were picked, dissociated, and replated again as single cells in the presence of bFGF. Ten to fifteen percent of the cells gave rise to second generation clones. After bFGF withdrawal, almost all of the subclones contained neurons, astrocytes, oligodendrocytes, and unstained cells (Table 1B). No subclone consisted of only one cell type. These data suggest strongly that, during the clonal expansion, the multipotential cells undergo symmetric divisions to generate daughter cells with multipotential capacity and are therefore stem cells.

Stem cells are abundant in the fetal brain

Cells with multipotential capacity were found through-

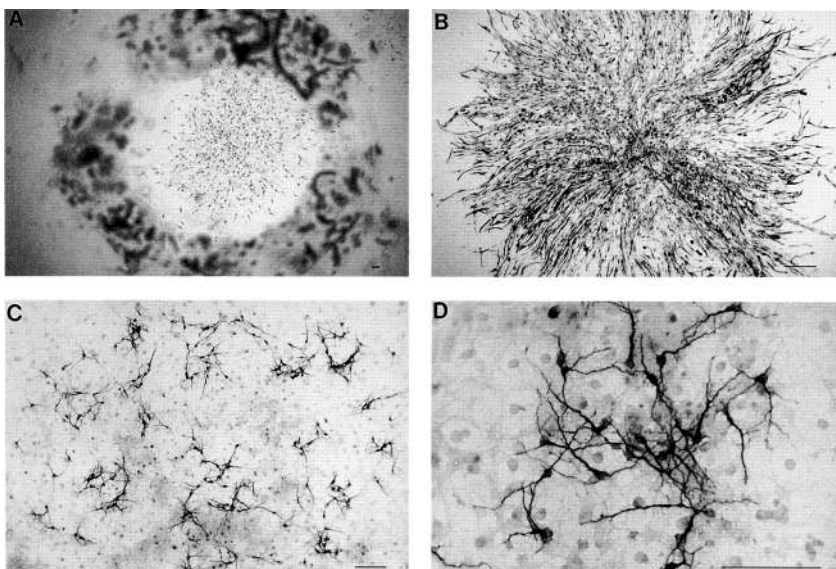


Figure 2. Clonal expansion and differentiation of CNS stem cells. (A) An example of a clone marked at a single-cell stage by a circle and subsequently expanded to large size and immunostained. (B) A typical clone expanded for 10 days, fixed without differentiation, and stained with antinestin antibody. Note the uniform radial morphology of the cells. (C) A typical clone after 6 days of differentiation, immunostained with neuron-specific antitubulin β III (TuJ1) antibody. (D) Higher magnification view of TuJ1+ neurons in the same clone shown in C.

Table 1. Cell type composition of differentiated clones

A. Clones of embryonic hippocampal stem cells ^a					B. Subclones from embryonic hippocampal stem cells ^c (continued)				
Passage	Clone size	MAP2+ (%)	Galc+ (%)	GFAP+ (%)	Subclone	Clone size	MAP2+ (%)	Galc+ (%)	GFAP+ (%)
1	319	145 (45)		41 (13)	H18.6	402	26 (6)	75 (19)	
1	451	245 (54)		0 (0)	H18.7	554		49 (9)	45 (8)
1	1237	634 (51)		9 (1)	H18.7	554		49 (9)	45 (8)
1	2197	956 (44)		42 (2)	H18.8	571		23 (4)	49 (9)
1	2779	1617 (58)		336 (12)	H18.9	662		41 (6)	118 (18)
4	71		10 (14)	5 (7)	H18.10	669	46 (7)	46 (7)	
4	139		14 (10)	4 (3)	H18.11	827	57 (7)	18 (2)	
4	296		21 (7)	139 (47)	H18.12	836	92 (11)	97 (12)	
4	341		54 (16)	38 (11)	H18.13	1084	104 (10)	53 (5)	
4	420		39 (9)	25 (6)	H18.14	1268	124 (10)	163 (13)	
4	600		35 (6)	60 (10)	H18.15	1284	75 (6)	193 (15)	
4	662		66 (10)	62 (9)					
4	141	42 (30)	4 (3)		Average ^d		20.1 ± 1.4%	8.9 ± 1.1%	10.0 ± 0.7%
4	427	220 (52)	15 (4)						
4	610	306 (50)	29 (5)						
Average ^b					C. Clones of adult striatal stem cells				
		48.6 ± 1.6%	8.4 ± 1.0%	7.8 ± 2.3%					
B. Subclones from embryonic hippocampal stem cells ^c					Passage	Clone size	MAP2+ (%)	Galc+ (%)	GFAP+ (%)
Subclone	Clone size	MAP2+ (%)	Galc+ (%)	GFAP+ (%)	1	73	6 (8)		37 (51)
H16.1	337	22 (7)	99 (129)		1	159	56 (35)		42 (26)
H16.2	338	13 (4)	157 (46)		1	173	57 (33)		26 (15)
H16.3	537	132 (25)	48 (9)		1	185	71 (38)		32 (17)
H16.4	565	98 (17)	28 (5)		1	230	97 (42)		39 (17)
H16.5	831	96 (12)	107 (13)		1	273	139 (51)		56 (21)
H16.6	886	158 (18)	134 (15)		1	387	117 (30)		45 (12)
H16.7	893	135 (15)	66 (7)		1	554	237 (43)		84 (15)
H16.8	950	154 (16)	53 (6)		1	675	280 (41)		74 (11)
H16.9	951	112 (12)	120 (13)		1	847	399 (47)		155 (18)
H16.10	970	105 (11)	95 (10)		1	496		23 (5)	92 (19)
H19.1	84	11 (13)		0 (0)	1	526		7 (1)	115 (22)
H19.2	211	45 (21)		0 (0)	1	644		19 (3)	26 (4)
H19.3	363	61 (17)		18 (5)	1	713		22 (3)	179 (25)
H19.4	697	172 (25)		5 (1)	1	1112		56 (5)	235 (21)
H19.5	861	135 (16)		57 (7)	0	278	153 (55)	6 (2)	
H19.6	1469	401 (27)		123 (8)	0	305	145 (48)	19 (6)	
H19.7	1841	486 (26)		179 (10)	1	411	156 (38)	68 (17)	
H18.1	88		4 (15)	0 (0)	0	513	242 (47)	3 (1)	
H18.2	104		3 (3)	0 (0)	0	532	246 (46)	26 (5)	
H18.3	193		16 (8)	28 (15)	0	538	283 (53)	10 (2)	
H18.4	237	14 (6)	39 (16)		0	584	277 (47)	32 (5)	
H18.5	384		65 (17)	119 (31)	0	1012	498 (49)	5 (0)	
					Average		41.7 ± 2.6%	4.2 ± 1.2%	19.6 ± 2.7%

^aA partial list of typical clones are presented. Clone size was determined by counting total number of cells in each clone. The numbers of cells stained positive for each of the three cell-type specific antigens are shown and their relative proportions are in parenthesis. A total of 48 clones was quantified from four different passages in six separate experiments.

^bAverage indicates the average composition of each cell type in the 48 clones.

^cA total of 84 subclones were quantified from 13 independent parental clones in two separate experiments. Representative subclones from three independent primary clones, HI 6, HI 8, HI 19, are shown.

^dAverage composition of each cell type from the 84 subclones.

out the developing neuroepithelium. Under identical culture conditions, similar cells could be prepared from other regions of the developing CNS including the cere-

bral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, and spinal cord (data not shown). From embryonic day 14 (E14) cortex and striatum and E16 hip-

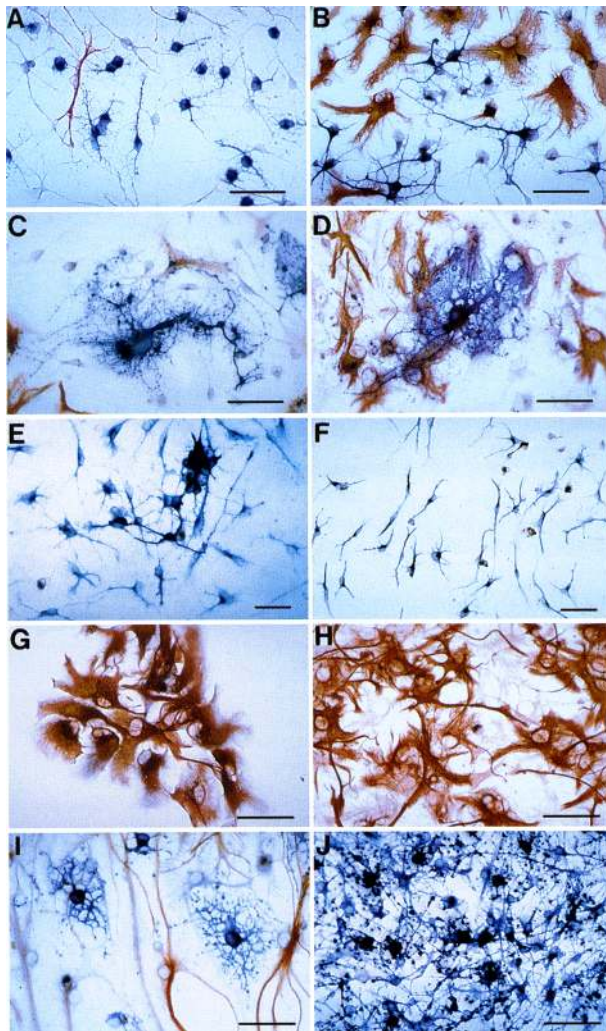


Figure 3. Demonstration of multipotentiality and influence of extracellular factors on cell-type specification. Representative clones of embryonic hippocampal cells (A,C,E,G,I) and adult subependymal cells (B,D,F,H,J) double-stained with combinations of antibodies to reveal different cell types within individual clones: anti-MAP2, neuronal; anti-GalC, oligodendrocytic; anti-GFAP, astrocytic. (A,B) Anti-MAP2 (blue) and anti-GFAP (red). (C,D) Anti-GalC (blue) and anti-GFAP (red). (E,F) Clones differentiated in the presence of PDGF; anti-MAP2 (blue) and anti-GFAP (red). Most cells are MAP2+ and few are GFAP+. (G,H) Clones differentiated in the presence of CNTF; anti-MAP2 (blue) and anti-GFAP (red). All cells are intensely GFAP+. (I,J) Clones differentiated in the presence of T3; anti-GalC (blue) and anti-GFAP (red). GFAP+ and, particularly, GalC+ cells increased. MAP2+ cells decreased (see Table 3).

pocampus, ~70% of acutely dissociated cells in high-density culture responded to bFGF by undergoing mitosis within 2 days of plating. Clonal analysis suggests that the stem cells constitute a large fraction of the cells in the starting culture. However, the low plating efficiency at clonal density did not permit a direct estimation of the proportion of CNS stem cells in the starting culture, im-

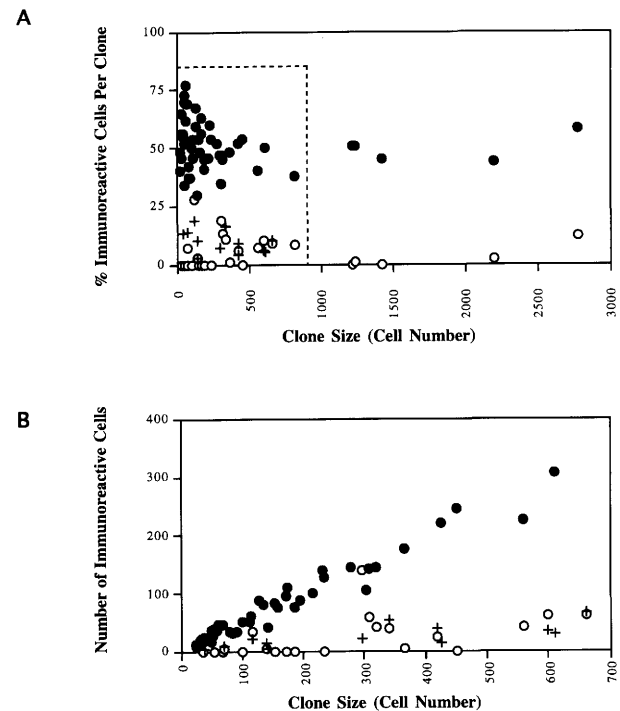


Figure 4. Proportion of multiple cell types in differentiated clones. (A) Clones of various sizes ranging from 39 cells to 2800 cells were differentiated for 6 days and analyzed for two cell types at a time by double immunohistochemistry. (●) MAP2+ neurons; (+) GalC+ oligodendrocytes; (○) GFAP+ astrocytes. A partial list of the clonal data is given in Table 1 and immunostaining shown in Fig. 3. Neuronal population constituted 50% of the clones, independent of the clone size. (B) Small clones within the area defined by a broken line in A replotted to emphasize that the number of neurons increased linearly with the increasing clone size.

mediately after dissociation of the tissues. As an alternative approach, acutely dissociated E14 cortical cells were plated at high density and clonal analysis was performed by infecting mitotic cells with a replication-defective retrovirus expressing β -galactosidase with a nu-

Table 2. Retroviral lineage analysis of E16 cortical cells

Frequency of clones (%) ^a	Clonal type ^b	Average clone size (cells) ^c
44	Multipotential	38.3 \pm 7.3
49	Astrocytic	19.5 \pm 5.3
4	Neuronal	7.7 \pm 2.0
3	Mixed glial	15.0 \pm 2.0

^aBased on total 68 X-gal positive clones triple-stained with anti-MAP2, GFAP, and O4 antibodies.

^b(Multipotential) mixed composition of MAP2+, GFAP+, and/or O4+ cells and unstained cells; (astrocytic) GFAP+ cells and unstained cells; (neuronal) MAP2+ cells and unstained cells; (mixed glial) mixture of GFAP+ cells, O4+ cells, and unstained cells.

^cAverage number of X-gal positive cells in a clone (\pm S.E.M.).

clear localization signal. Cells were differentiated by withdrawing bFGF, and the resulting cell types were identified by triple fluorescence staining with cell type-specific antibodies. Forty-four percent of the X gal+ clones were composed of multiple cell types, whereas 49% were astrocytic and 4% were neuronal (Table 2). These results show that a substantial proportion of the proliferative cells in the acutely dissociated neuroepithelium are multipotential and suggest that stem cells are abundant in the fetal brain.

Stem cells in the adult CNS

The subependymal layer of adult rat brain contains mitotic nestin-positive cells that could be expanded in aggregate culture in the presence of EGF but not bFGF (Reynolds and Weiss 1992). Cells in these aggregate cultures show neuronal and astrocytic properties. To further define the developmental capacity of proliferating nestin-positive cells in the adult brain, the mitotic population (1% of 1×10^5 cells/brain) lining the lateral ventricle of adult rat striatum was expanded in the presence of bFGF and compared with the embryonic precursors. The morphology and growth characteristics of these nestin-positive adult cells were similar to those of embryonic cells. Following bFGF withdrawal, marked clones differentiated into multiple cell types expressing MAP2, β -tubulin type III, GFAP, and GalC (Fig. 3B,D). Strikingly, the same high proportion of neurons were found in differentiated clones of adult cells as in the embryonic clones (Table 1C).

EGF was equally effective as bFGF as a mitogen for adult cells (Fig. 5), and, when these EGF-expanded clones

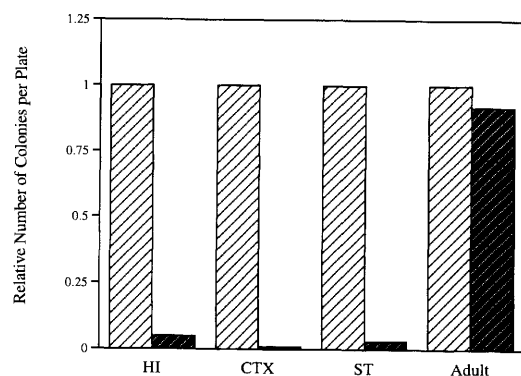


Figure 5. Distinct mitogenic efficacies of EGF and bFGF on acutely dissociated neuroepithelial cells. Cells acutely dissociated from E16 hippocampus (HI), E14 cortex (CTX) and striatum (ST), and adult subependymal layer (Adult) were plated initially at 1×10^4 cells per 10-cm plate and expanded with either EGF (20 ng/ml) or bFGF (10 ng/ml). Colonies arising after 10 days of expansion were stained for nestin and counted. Relative number of colonies averaged from at least two experiments for each region are shown (bFGF = 1). Twenty- to fiftyfold more nestin+ colonies per plate were present when the embryonic cells were grown in bFGF (light hatched bars) than in EGF (dark hatched bars).

Table 3. Effects of extracellular factors on cell-type specification

	Untreated (%)	+ PDGF (%)	+ CNTF (%)	+ T3 (%)
A. bFGF-expanded embryonic clones				
MAP2+	45.9 \pm 2.0	81.0 \pm 1.7	0.9 \pm 0.1	11.5 \pm 3.3
tau+	N.D.	84.4 \pm 7.0	N.D.	N.D.
TuJ1+	9.9 \pm 1.8	72.4 \pm 6.2	N.D.	N.D.
NF-M+	1.0 \pm 0.2	53.0 \pm 7.5	N.D.	N.D.
GalC+	7.4 \pm 2.8	2.8 \pm 0.7	4.5 \pm 1.2	21.2 \pm 2.7
GFAP+	6.3 \pm 2.5	2.0 \pm 0.7	97.3 \pm 1.8	20.7 \pm 7.5
Dead ^a	11.2 \pm 2.0	8.8 \pm 1.4	18.8 \pm 4.5	11.8 \pm 1.5
B. bFGF-expanded adult clones				
MAP2+	36.8 \pm 2.7	73.9 \pm 1.9	11.8 \pm 2.2	35.2 \pm 2.9
TuJ1+	47.9 \pm 2.7	72.4 \pm 7.8	N.D.	N.D.
GalC+	4.8 \pm 2.9	N.D.	N.D.	47.4 \pm 3.6
GFAP+	20.3 \pm 2.1	2.2 \pm 0.7	72.9 \pm 1.9	32.4 \pm 5.4
C. EGF-expanded embryonic clones				
MAP2+	42.0 \pm 6.5	54.0 \pm 4.0	9.1 \pm 2.6	9.2 \pm 2.4
GalC+	2.4 \pm 1.3	11.1 \pm 2.9	7.6 \pm 1.1	14.0 \pm 1.2
GFAP+	46.4 \pm 3.1	11.9 \pm 1.8	83.7 \pm 3.1	59.8 \pm 3.7

Clones were differentiated for 6 days either in the absence (untreated) or the presence of indicated factors: 10 ng/ml PDGF, 10 ng/ml CNTF, and 10 mM T3. Relative proportions of immunoreactive cells per clone are shown. $n = 6$ –23 clones. (N.D.) Not determined. \pm S.E.M.

^aDead cells were estimated by counting the cell ghosts left on the plate (see Materials and Methods).

were differentiated, they gave rise to all three cell types. For the embryonic cells from several different regions, EGF was at least 10-fold less effective than bFGF as a mitogen for acutely dissociated cells, independent of the initial cell density. However, EGF-expanded embryonic clones also differentiated into all three cell types upon withdrawal of the mitogen (Table 3C). Thus, with the exception of the proliferative effects of EGF, these data reveal that the multipotential cells from embryonic and adult CNS are remarkably similar.

Influence of extracellular factors on CNS stem cells

The clonal analysis suggests that the multipotential stem cells are not committed prior to mitogen withdrawal and that extracellular signals may regulate cell-type determination. We tested whether the proportion of the cell types generated within a clone could be influenced by diffusible molecules, growth factors, and cytokines during either proliferation or differentiation. Factors were added starting from 2 days prior to bFGF withdrawal and then for 6 days in the absence of bFGF. Thus, we tested for a possible window of influence during the last 2 days of proliferation, the last mitotic cycle, and the subsequent differentiation period.

Three soluble factors, platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), and triiodothyronine (T3), showed the most dramatic and contrasting effects on the differentiating clones. In embryonic clones, the proportion of cells expressing neuronal

antigens increased significantly in the presence of PDGF-AA, PDGF-AB, or PDGF-BB during differentiation. Up to 80% of the cells were neuronal with MAP2, tau, tubulin type III, or NF-M expression. Only 2% of the cells expressed O4, GalC, or GFAP (Fig. 3E; Table 3A). The cells expressing the neuronal antigens showed a less mature morphology under these conditions and continued to proliferate.

When treated with CNTF, clones gave rise almost exclusively to astrocytes (Fig. 3G; Table 3A). Remarkably, <1% of the cells were MAP2+ in CNTF. The CNTF-treated cells were intensely GFAP+, and all showed a flat, astrocytic morphology. Leukemia inhibitory factor (LIF) showed identical effects to CNTF. The thyroid hormone T3, influenced the differentiation of the multipotential precursors toward a mixed glial fate (Fig. 3I; Table 3A). Astrocytes and oligodendrocytes were both increased threefold, and there was a marked decrease in the proportion of neurons. As in the untreated clones, the GalC+ and O4+ cells showed characteristic oligodendrocytic morphologies. The clones were of similar size in all of the experiments, and numerical analysis of dead cells showed that there was no significant change in cell death (this issue is addressed further in data shown in Table 4). Thus, selective cell death could not account for the changes in the proportion of cell types shown. Similar results were obtained with multipotential stem cells from embryonic cortex and striatum.

The multipotential cells derived from the subependymal layer of the adult brain showed quantitatively similar differentiation responses to PDGF, CNTF, and T3 (Fig. 3F,H,J, respectively; Table 3B). Moreover, embryonic CNS stem cells expanded in EGF without exposure to bFGF also responded similarly to the three factors (Table 3C). CNS stem cells from adult and several embryonic brain areas responded equivalently to the three factors, suggesting that these factors are affecting a general mechanism of cell-type specification for mammalian CNS. Thus, the effects of the differentiation factors are independent of the source of the stem cells or the mitogen used to maintain them.

Immunohistochemistry with antibodies against GABA and glutamate shows that subsets of neurons in clones express these transmitters (not shown). A recent study shows that glutamate influences the proliferation of cells derived from the fetal cortex (LaMantia 1995; LoTurco et al. 1995), raising the interesting possibility that this neurotransmitter regulates the neuronal differentiation of CNS stem cells. bFGF-expanded stem cells exposed to GABA or glutamate showed no change in their proliferation (data not shown). Although it is likely that these neurotransmitters may influence later stages of differentiation, our data suggest that they do not initiate cell cycle withdrawal nor do they influence early steps in stem-cell differentiation.

Instructive action of extracellular factors

Because the extracellular factors were tested over the entire period of cell differentiation, it was important to demonstrate unambiguously whether the factors were acting directly on proliferating stem cells or only during the period of stem-cell differentiation. Thus, actively dividing stem-cell clones were exposed to PDGF, CNTF, or T3 at the four-cell stage (2 days after plating) in the presence of bFGF and allowed to expand for an additional 6 days in the presence of bFGF and one of the factors. Under these conditions, PDGF has no effect on antigen expression in stem cells showing that the induction of neuronal antigens requires the removal of bFGF.

CNTF induced the expression of GFAP in 98% of the cells in the presence of bFGF (Table 4). Very few cells expressed neuronal or oligodendrocytic antigens or morphologies, and there was no significant cell death. After 24 hr of CNTF treatment, mitotic stem-cell clones at only the four-cell stage began to undergo overt morphological transformation and express GFAP. When cultured for 6 days in bFGF and CNTF, almost all cells adopted astrocytic morphology and expressed GFAP (Table 4). The number of dead cells (Table 4) excludes the possibility that selective mechanisms account for the switch in antigen expression. These results indicate that

Table 4. Direct derivation of committed astroblasts from CNS stem cells by CNTF

Culture experience	Average cell number per clone	MAP2+	O4+/GC+	GFAP+	Unstained	Dead ^a
+F	213 ± 56	0%	0%	0%	99.9 ± 0.1%	0%
+F+C	194 ± 36	0%	0%	97.8 ± 2.1%	2.1 ± 2.0%	0%
+F+C → -F+C	235 ± 44	0%	2.6 ± 0.5%	80.8 ± 3.8%	16.6 ± 3.5%	11.9 ± 4.9%
+F+C → +F-C	634 ± 140	0%	0%	99.9 ± 0.1%	0%	0%
+F+C → +F-C → -F-C	1648 ± 673	3.6 ± 1.1%	8.9 ± 1.8%	79.9 ± 2.9%	11.1 ± 1.8%	3.7 ± 0.9%
+F+C → -F-C+E	775 ± 209	0%	0%	99.9 ± 0.1%	0%	14.8 ± 5.5%
+F+C → -F-C+E → -F-C-E	1677 ± 459	0%	7.1 ± 1.9%	80.5 ± 1.8%	12.3 ± 2.0%	14.2 ± 2.1%

Embryonic hippocampal clones were expanded in bFGF alone (+F, Control) or together with 10 ng/ml CNTF for 6 days (+F+C). Arrows indicate subsequent changes in culture condition. (-F+C) Without bFGF and with CNTF alone; (+F-C) with bFGF alone; (-F-C) without either bFGF or CNTF; (-F-C+E) switched to 10 ng/ml EGF alone; (-F-C-E) subsequent removal of EGF. At least six clones were counted under each condition. ±S.E.M.

^aDead cells were estimated by counting the cell ghosts left on the plate (see Materials and Methods).

CNTF acts directly to induce GFAP in the proliferating stem cell.

To further determine whether the astrocytic differentiation is transient or a stable change in the stem cell, clones initially grown in bFGF and CNTF were further expanded for 4 additional days in bFGF alone without CNTF. The cells continued to divide, evident from the increasing clone size, and maintained the astrocytic morphology and intense GFAP expression (Table 4). Thus, the continued presence of exogenous CNTF was not required to maintain GFAP expression. But, do the GFAP+ cells differentiate into astrocytes and only astrocytes? When bFGF was withdrawn from the culture, 80% of the cells continued to express GFAP intensely with the typical astrocytic morphology (Table 4). Interestingly, a fraction of the cells differentiated into MAP2+ or O4+/GalC+ cells. In the continuous presence of CNTF, expression of these antigens could be suppressed (Table 4). When the cells exposed to CNTF were treated with EGF for 4 days, the cell number per clone increased as in bFGF and almost all cells were astrocytic (Table 4). The mitogenic effects of bFGF and EGF for CNTF-treated stem cells were confirmed by BrdU double-labeling experiments (data not shown). These data demonstrate that a transient exposure to CNTF induces in the stem cell a stable fate restriction from a multipotent state to a unipotent and proliferative astroblast state.

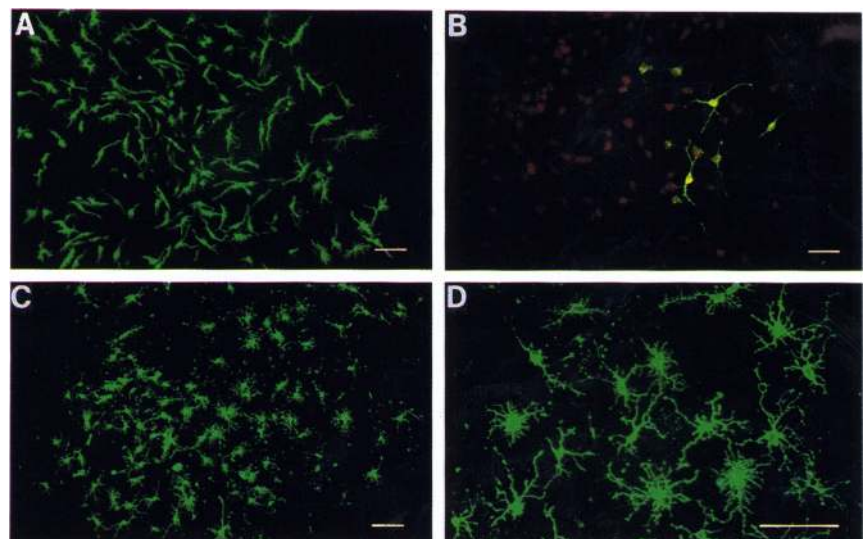
Under the same paradigm as with CNTF, when stem-cell clones were expanded in the presence of bFGF and T3 starting from the four-cell stage, some of the clones (~10%) consisted entirely of O4+ mitotic cells (Fig. 6A). Remaining clones contained variable proportions of O4+ cells as subsets within a clone. The O4+ cells were not distributed randomly but grouped together, suggesting that they were part of a subclone (Fig. 6B). Is the

expression of O4 antigen a stable property for cells exposed to T3? When clones exposed to T3 were grown subsequently for 4 days with bFGF alone without T3, clones entirely composed of O4+ cells with oligodendrocytic morphologies were present (Fig. 6C). In other clones, large subsets of O4+ cells with oligodendrocytic morphology were found clustered within a clone. The O4+ cells assumed more mature oligodendrocytic morphologies when bFGF was finally withdrawn (Fig. 6D). These data show that T3 exposure leads multipotent stem cells to glial lineage, where subsets of cells make a stable transition to proliferating oligodendrocytic precursors.

Discussion

A key feature of the studies presented here is that both proliferation and differentiation of the multipotential neuroepithelial stem cells can be controlled efficiently. This in vitro system permitted for the first time a quantitative analysis of the developmental capacity of CNS stem cells. Several lines of evidence indicate that most of the cells in the clones are multipotential and are not predetermined for any of the major cell types prior to mitogen withdrawal. First, the proportion of neurons generated is independent of clone size. The significance of this unchanged probability of neuronal generation is that it supports a model in which cellular properties are constant as clones expand. This stability is supported by the unchanged differentiation capacity in clones of acutely dissociated cells and in clones generated from cells that have been passaged. Second, subcloning experiments demonstrate that many multipotential secondary clones can be derived from a single primary clone, again showing that the multipotential cells are expanding in number. Asymmetric cell division may still be an im-

Figure 6. Direct derivation of committed oligodendroblast from CNS stem cells by T3. (A) A stem-cell clone expanding in the presence of both bFGF and T3, immunostained with anti-O4 antibody. Revealed by triple staining, all of the cells in this clone are O4+, MAP2-, and GFAP-, while they are mitotic. (B) Another stem-cell clone in the presence of bFGF and T3, double-stained with anti-O4 (green) and anti-GFAP (red) antibodies. O4+ mitotic cells appeared in a confined sector within the clone. The remaining cells expressed a low level of GFAP. (C) A clone of mitotic O4+ cells after the T3 treatment continuing to expand in bFGF alone. The oligodendrocytic fate was retained throughout successive cell division in the absence of T3. (D) A differentiating O4+ clone, 6 days after bFGF has been finally withdrawn. Oligodendrocytes stably express O4+ in the absence of T3 and bFGF and differentiate into more mature morphologies.



portant mechanism for cell-type specification *in vivo* (Chenn and McConnell 1995). However, a strict asymmetric model, in which only one of the daughter cells maintains multipotentiality, cannot account for the exponential increase in stem cells seen in culture.

Clonal analysis showed that multipotential stem cells could be isolated readily from many regions of the developing neuroepithelium. Initial plating efficiency at clonal density is inefficient. However, at high density and at short times after dissociation, a large proportion of cells survive and proliferate in response to bFGF. Almost half of the retrovirus-labeled clones in these short-term cultures are also multipotential, showing that CNS stem cells are abundant throughout the developing neuroepithelium. A model of cell-type specification that emerges from these data is that stem cells proliferate symmetrically to produce uncommitted daughter cells that form a major component of the neuroepithelium.

The distinct effects of extracellular factors acting at early stages of cell-type differentiation in the CNS are summarized in Figure 7. PDGF-AA, -AB, and -BB increased the proportion of neuronal cells but only in the absence of bFGF. All three forms of PDGF (AA, AB, and BB) act in the same manner, indicating that the α receptor mediates this effect of the factor. The expression of the α receptor and lower levels of the β receptor in cultured CNS stem cells has been established by Northern blotting (K. Forsberg-Nilsson and R. McKay, unpubl.). Stem cells treated with PDGF express several neuronal

antigens (MAP2a, MAP2b, MAP2c; tau; TuJ1; and neurofilament). PDGF does not induce the expression of these antigens in the presence of bFGF. These data suggest that PDGF is not responsible for a fate choice but is a survival and proliferation factor for immature neurons.

In contrast, CNTF and T3 induce glial fates and morphologies in the presence of bFGF. Transient exposure of stem cells to CNTF or T3 is sufficient to initiate a stable switch to a glial fate. The actions of CNTF and T3 are mediated clearly by a direct, instructive mechanism. These factors and/or their receptors are known to be present and functional during CNS development (Yeh et al. 1991; Orr-Urtreger et al. 1992; Davis et al. 1993; Ip et al. 1993; Barres et al. 1994; Ware et al. 1995). There are few examples of instructive action of soluble ligands on multipotential stem cells. Recently, GGF, BMP2, and TGF- β 1 have been shown to act instructively on PNS stem cells leading to neuronal, glial, or muscle fates (Shah et al. 1994, 1996). In principle, the difference between neuronal and glial fates might require a complex extracellular code. Our data show that single growth factors can rapidly and instructively direct lineage restrictions that are then maintained in the absence of the initiating signal.

Transplantation of immortalized and primary neuroepithelial cells also shows that local signals rather than cell autonomous mechanisms direct neuronal differentiation and that these signals may be retained in the adult cerebral cortex (Renfranz et al. 1991; Brustle et al. 1995; Shihabuddin et al. 1995; Vicario-Abejon et al. 1995). Thus, data from both transplantation and tissue culture stress the importance of extracellular signals and cellular plasticity in regulating the organization of the developing and adult brain.

There is evidence from lineage analysis *in vivo* and *in vitro* for the presence of lineage restrictions, including bipotential neuronal and oligodendrocyte precursors and committed neuronal progenitors (Luskin 1993; Davis and Temple 1994; Luskin and McDermott 1994; Williams and Price 1995). The identification of the bipotential oligodendrocyte precursor cell, O-2A, from the postnatal optic nerve most directly demonstrates that restricted progenitors are produced during development (Raff et al. 1983). Stem cells may differentiate into oligodendrocytes through an intermediate stage resembling an O-2A cell. However, stem cells are distinct from the O-2A cells as their origins, growth characteristics, morphology, antigenic profile, and developmental capacities differ. Most notably, stem cells efficiently give rise to neurons. We have shown previously that bFGF expanded cells differentiate in the presence of neurotrophins to express complex morphologies and antigens characteristic of specific neuronal subtypes (Vicario-Abejon et al. 1995b). Here, we show that the stem cells rapidly express receptors for excitatory and inhibitory neurotransmitters when they differentiate. In addition, electrophysiological experiments show that expanded cells form functional excitatory and inhibitory synapses (C. Vicario-Abejon, C. Collin, M. Segal, K. Johe, and R. McKay, unpubl.).

Neurons are not generated in large numbers in the

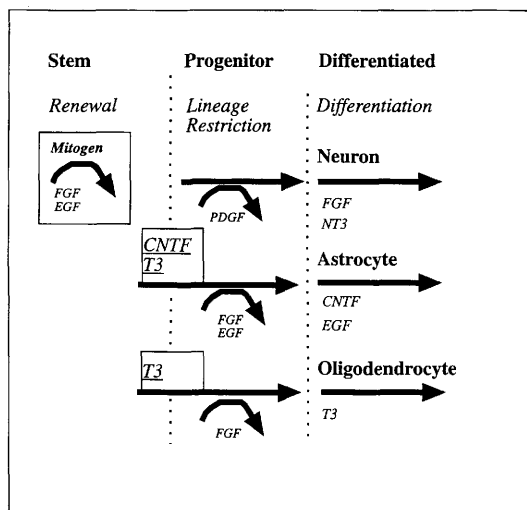


Figure 7. Cell-type transitions of CNS stem cells. This model illustrates the distinct effects of extracellular factors on CNS stem cells and the different cell types derived from stem cells. Three classes of cells are shown: multipotential stem cells, lineage restricted progenitors for neurons, astrocytes, and oligodendrocytes, and the three mature cell types. Mitogenic factors for stem and progenitor cells are shown with a bent arrow. Differentiation factors are shown next to a straight arrow. Differentiation factors of two kinds are shown: factors that promote differentiation and factors that cause lineage restriction (underlined).

adult mammalian CNS with the exception of the olfactory bulb (Altman 1969; Lois and Alvarez-Buylla 1992; Luskin 1993) and the hippocampal formation (Altman 1965; Cameron et al. 1993). Cultured adult precursors have been shown to differentiate into neurons and glia, but little is known about the mechanisms that regulate the differentiation of these cells (Gritti et al. 1996). Both the clonal analysis and the response to growth factors reported here show that cells derived from the adult CNS have properties that are very similar to stem cells in the fetal neuroepithelium. Further characterization of these general mechanisms that control the multipotentiality, self-renewal, and fate restriction of stem cells is clearly important to develop new therapies for cell regeneration and replacement in the adult nervous system.

Materials and methods

Isolation, mass expansion, and differentiation of embryonic CNS stem cells

Rat embryonic hippocampus (gestation day 16; day of conception is day 1; Taconic Farm) was dissected in Hank's buffered saline solution (HBSS) and dissociated by brief mechanical trituration in HBSS. The cells were collected by centrifugation and resuspended in a serum-free medium containing Dulbecco's modified Eagle medium (DMEM)/F12, glucose, glutamine, sodium bicarbonate, 25 μ g/ml of insulin, 100 μ g/ml of human apotransferrin, 20 nM progesterone, 100 μ M putrescine, 30 nM sodium selenite (pH 7.2) (Bottenstein and Sato 1979), plus 10 ng/ml of recombinant human bFGF (R&D, Inc.) (Vicario-Abejon et al. 1995b). Cells (1×10^6) were plated per 10-cm plastic tissue culture plate precoated with 15 μ g/ml of poly-L-ornithine and 1 μ g/ml of bovine plasma fibronectin (GIBCO). bFGF was added daily, and media change was every 2 days. Cells were passaged at 50% confluence (4 days after initial plating) by briefly incubating them in HBSS and scraping with a cell scraper.

After various periods of expansion, differentiation of CNS stem cells was initiated by culturing the cells in serum-free medium without bFGF. To analyze the time course of differentiation into neurons, rapidly dividing cells were incubated for the last 24 hr with 10 μ M BrdU (bromodeoxyuridine) prior to passaging. Cells were harvested by using trypsin and soybean trypsin inhibitor and plated in duplicate at 40,000 cells/cm² into multiwell chamber slides (LabTek) precoated with poly-L-ornithine (15 mg/ml) and fibronectin (1 mg/ml) and further cultured in serum-free medium without bFGF. At indicated times, the cells were fixed in 4% paraformaldehyde and stained first with various neuron-specific antibodies according to standard procedure, followed by postfixation and staining with monoclonal anti-BrdU antibody (Becton-Dickinson). Immunoreactive cells were counted under 400 \times magnification. At least five fields with total cell number >1000 per sample were counted. Antibody reagents used were anti-nestin antiserum (Nestin130; M. Marvin and R.D.G. McKay, National Institutes of Health, Bethesda, MD), monoclonal anti-MAP2 (clone HM-2; Sigma), anti-tau antiserum (Sigma), monoclonal anti-NFs L and M (clones NR4 and NN18; Boehringer Mannheim), anti- β -tubulin type III (TuJ1; gift of A. Frankfurter, University of Virginia, Charlottesville), monoclonal anti-GFAP (ICN), anti-GFAP antiserum (Chemicon), A2B5 (Boehringer Mannheim), O4 (gift of M. Schachner, ETH, Zurich, Switzerland), and monoclonal anti-galactoceroside (GalC; gift of M. Dubois-Dalcq, Pasteur Institute, Paris, France).

Isolation and expansion of adult CNS stem cells

Slices from forebrains of 250-gram adult rats (10–20 per experiment) were prepared, and the subependymal region of striatum lining the lateral ventricles was cut out under microscope in oxygenated HBSS. The cells were dissociated by incubating minced tissues in oxygenated HBSS containing trypsin (1 mg/ml), hyaluronidase (0.7 mg/ml), and kynurenic acid (0.2 mg/ml) for 10 min at room temperature. Cells were washed once in HBSS with 0.7 mg/ml of ovomucoid and 0.2 mg/ml of kynurenic acid, resuspended, and triturated in the same solution. Dissociated cells were recovered by centrifugation and cultured in the serum-free medium plus bFGF (10 ng/ml) as described for the embryonic cells.

Clonal analysis

At various passages, ~200 cells were plated per 10-cm plate and cultured under conditions as described above for mass culture. Both embryonic and adult clones were generated under identical culture conditions. Within 24 hr of plating when most cells had not yet divided, well-isolated single cells were marked with a 3-mm circle (Nikon) on the bottom of the plate. Initial viability of the marked single cells was 5–10%, and each plate typically yielded two to eight marked clones. The subsequent population of cells within each circle is progeny of the initial marked cell. Clones were expanded for up to 10 days (500–2000 cells). Differentiation of the clones was initiated by washing the plates once with HBSS and culturing in serum-free medium in the absence of bFGF. For subcloning, the clonal plates were washed and left in HBSS for 10 min. Clones of 500–2000 cells were picked in a 50- μ l volume with an adjustable pipetter while viewing through a microscope. Each clone was replated in a 10-cm plate, and single cells were marked and cultured as before.

After 6 days of differentiation, cell types in clones were analyzed by double-staining with combinations of antibodies that are cell type-specific and react with mutually exclusive cells: neurons = MAP2+, tau+, TuJ1+, NF-L+, or NF-M+; astrocytes = GFAP+; oligodendrocytes = O4+ or GalC+. Double staining was done sequentially using a commercial kit (Zymed) according to the manufacturer. The first antibody staining was developed with alkaline phosphatase reaction (blue) and the second with peroxidase reaction (red) (Zymed). For oligodendrocyte staining, cells fixed with 4% paraformaldehyde were stained first for the cell-surface antigens O4 or GalC without permeabilization.

For clonal analysis with a retrovirus, cells were dissociated from E16 rat hippocampus and plated at 1×10^5 to 5×10^5 cells per 10-cm dish. After 2 days in the presence of bFGF, cells were infected with a low-titer retrovirus encoding the *Escherichia coli lacZ* gene containing a nuclear localization signal. After an additional 3–5 days of expansion, cells were differentiated by bFGF withdrawal and analyzed 6 days later by X-gal reaction followed by triple immunofluorescence staining. Entire plates were reacted sequentially with cell type-specific antibodies against MAP2 (monoclonal IgG), GFAP (polyclonal; Chemicon), and O4 (monoclonal IgM) followed by appropriate secondary antibodies conjugated with fluorescein isothiocyanate (FITC), rhodamine, or 7-amino-4-methylcoumarin-3-acetic acid (AMCA) (Jackson Laboratory). All of the well-isolated clones with nuclear X-gal staining were individually scored for MAP2+ (neurons), GFAP+ (astrocytes), O4+ (oligodendrocytes), and unstained (unidentified) cells.

Growth factors

The mitogenic effect of various growth factors was tested by

their ability to substitute for bFGF in rapidly expanding cultures. Acidic FGF (aFGF), transforming growth factor- α (TGF- α), and EGF, each at 10 ng/ml could substitute for bFGF.

The influence of growth factors on cell-type specification was tested by adding them to the culture 2 days before the withdrawal of bFGF and during the subsequent 6 days of differentiation. Factors were added daily, and medium was changed every 2 days. At the end of the 6 days of differentiation, cell types composing individual clones were analyzed by double-immunostaining with cell type-specific antibodies as described above. Factors with significant effects were, at 10 ng/ml, PDGF-AA, PDGF-BB, PDGF-AB, CNTF, LIF, and 3 ng/ml of T3. Other factors tested with no significant effect on cell-type determination were NGF, NT-3, BDNF, aFGF, TGF- α , TGF- β 1, IL1 β , IL2-IL11, G-CSF, M-CSF, GM-CSF (Genetics Institute), oncostatin M, stem-cell factor, erythropoietin, interferon gamma, 9-*cis* and all-*trans* retinoic acid, retinyl acetate, dexamethasone, corticosterone, KCl, glutamate, and γ -amino butyric acid.

RT-PCR analysis

Cortical CNS stem cells expanded in mass culture for 8–10 days were harvested prior to differentiation or allowed to differentiate for 6 or 12 days in serum-free medium as described above. For each time point, total RNA was extracted from $\sim 2 \times 10^7$ cells using Trizol (GIBCO). cDNAs were prepared and amplified by polymerase chain reaction according to standard procedures. Primers used were β -actin (nucleotides 81–649, 569 nucleotides), 5'-ATGGATGACGATATCGCTG-3' and 5'-ATGAGGTAGTCTGTCAGGT-3'; NMDA-R1 (nucleotides 1897–2487, 591 nucleotides), 5'-ACCCTGTCTCTTGCCATGTGGTTTTC-3' and 5'-ACATTCTTGATACCGAACCCATGTC-3'; NMDA-R2B (nucleotides 3011–3504, 494 nucleotides), 5'-ATGACTGTGACAACCCACCCCTT-3' and 5'-ACTGACCGAATCTCGCTTGAAGT-3'; NMDA-R2C (nucleotides 1458–1798, 362 nucleotides), 5'-AACGGCAAACACGGCAAAG-3' and 5'-CCAATGGTGAAAGATGGTCCAC-3'; GABA α 5 (nucleotides 631–936, 306 nucleotides), 5'-GTCTCCTCTCAACAACCTTCTTG-3' and 5'-CATCTTCTGCCACCACCACAG-3'; GABA α B1 (nucleotides 1096–1547, 452 nucleotides), 5'-GAGCGAGCAAACAAGACCAGAG-3' and 5'-ACAAGCGAGGAGGAAAGGAGTC-3'; GAD δ 5 (nucleotides 713–1085, 391 nucleotides), 5'-TCTTTTCTCCTGGTGCC-3' and 5'-CCCCAAGCAGCATCCACAT-3' (Bochet et al. 1994).

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