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Intrinsic differences distinguish transiently neurogenic progenitors from neural stem cells in the early postnatal brain

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Abstract

Recent reports of stem cell plasticity have led to the suggestion that there are few intrinsic differences between precursor cells, and that environment dictates fundamental cellular properties such as differentiation potential. This suggestion has been buoyed by other work suggesting that apparent in vivo differences between neural precursor cells are lost when placed in a culture environment. We sought to further test this hypothesis by comparing neural precursors present in various neural tissues during the early postnatal period. Precursors from three postnatal actively neurogenic regions and three postneurogenic regions (cerebral cortex, lateral striatum, and optic nerve) were assayed at postnatal day 1, day 10, and adulthood, and compared to well-characterized ventricular subependymal neural stem cells. In contrast to stem cells that remain multipotential throughout life, the progenitor cells become restricted in a time- and region-dependent manner to an exclusively glial-producing phenotype, a phenomenon that occurs both in vitro and in vivo. Transcription factors associated with neural precursor identity are expressed regardless of brain region of origin or time in vitro. Environmental coculture manipulations are only able to rescue neurogenesis in olfactory bulb precursors but not other restricted progenitors. Thus, in contrast to the views that the in vitro environment has a homogenizing effect on distinct neural precursors, our data suggest that robust intrinsic differences with respect to self-renewal and continued neuron production exist between neural precursors from different brain regions. These differences are evident in vitro and in vivo.

Keywords: Progenitor; Stem cell; Neuron; Neurogenesis; Postnatal; Optic nerve; Intrinsic

Introduction

It has been suggested that there are no true intrinsic differences between cells, but rather that "a stem cell most accurately refers to a biological function that can be induced in many distinct types of cells, even differentiated cells" (Blau et al., 2001). In a similar vein, others have suggested that many cells possess a latent neurogenic or multilineage program and that a stem cell-like phenotype can be induced in restricted neural cells (Doetsch et al., 2002; Gabay et al., 2003; Kondo and Raff, 2000; Markakis et al., 2004; Palmer et al., 1999). These findings are in contrast to other recent reports that neural precursors maintain fundamental properties including number of divisions, lineage potential, and even the order in which various cellular progenies are generated irrespective of the in vitro environment (Cayouette et al., 2003; Seaberg and van der Kooy, 2002). Moreover, neural stem cells have been shown to maintain a precise region-specific gene expression profile representative of their neural region of origin, even after being passaged over time in vitro (Hitoshi et al., 2002a). We sought to test the hypothesis that intrinsic hard-wired differences exist between early postnatal neural precursors that are resistant to in vitro manipulations, as has been recently demonstrated for retinal precursors (Cayouette et al., 2003) and subgranular zone progenitors (Seaberg and van der Kooy, 2002).

Neurogenesis occurs in vivo in only three early postnatal brain regions: the cerebellum (Altman, 1969a, 1972; Miale

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and Sidman, 1961), olfactory bulb, and dentate gyrus (Altman, 1969b; Altman and Das, 1966, 1965). This developmental period is ideal for the identification and comparative study of precursor cells indigenous to neurogenic and non-neurogenic regions to determine whether intrinsic, culture-resistant differences exist between these cell types. The postnatally non-neurogenic regions investigated in this study included the cerebral cortex (Angevine and Sidman, 1961; Bayer and Altman, 1991), striatum (Fentress et al., 1981; van Vulpen and van der Kooy, 1998), and the optic nerve, which consists mostly of oligodendrocytes, astrocytes, and their glial-restricted precursors but no neuronal cell bodies (Raff et al., 1983). Thus, the optic nerve is not a neurogenic tissue at any time in development.

In contrast to the idea that the environment determines the functional properties of neural precursor cells, we find intrinsic, culture-resistant differences between precursors isolated from different postnatal brain regions. Over time, neural progenitors from postnatal neurogenic and non-neurogenic regions lose the ability to generate neurons while retaining the capacity to produce glial cells. This phenomenon takes place in both a time- and region-dependent manner, occurs both in vitro and in vivo, and is not associated with changes in neural precursor transcription factor expression pattern. Manipulations of the cellular environment are not able to rescue the neurogenic ability of these cells, further indicating that the observed transient neurogenesis is an intrinsic property of these postnatal neural progenitors and is not easily altered by the cellular environment. Thus, these progenitors are clearly different from ventricular subependymal stem cells, which remain multipotential throughout long-term culture and indeed throughout life. These findings suggest that the in vitro environment does not homogenize cells (Doetsch et al., 2002; Gabay et al., 2003), but rather provides a system that enables the investigation of hard-wired, intrinsic cellular differences that are reflective of the cells' fundamental properties in vivo.

Materials and methods

Dissection and tissue preparation

Pregnant female and young adult male CD1 mice (6–8 weeks) were obtained from Charles River Laboratories (Charles River, St. Constant, Quebec, Canada). The day of birth was counted as postnatal day 0, and early postnatal experiments were performed on PND1 or PND10. Pups were killed by decapitation and the brains removed under a dissecting microscope. Each optic nerve was dissected rostral to, but not including, the optic chiasm. Olfactory bulbs and cerebellums were removed, and the remaining whole brains were prepared for sectioning on a Vibratome (Vibratome Tissue Sectioning System 100, Pelco International) as previously described (Seaberg and van der Kooy, 2002).

Under a Zeiss dissecting microscope, various brain regions from each section were precisely removed with fine surgical instruments. From the rostral sections, a strip of tissue containing both ependyma and subependyma was removed from the striatal (lateral) aspect of the anterior lateral ventricles. Although there are a small number of neurons born in the striatum at PND2, this is confined to the medially located striatal matrix, while our experiments examined the lateral striatum. Thus, we consider our striatal dissection to be of a region that is non-neurogenic postnatally. For all analyses of anterior ventricular tissue, only subependymal spheres were counted, passaged, and differentiated (Chiasson et al., 1999). Additionally, a small block of cerebral cortical tissue was removed dorsal to the corpus callosum, and a block of non-ventricular striatal tissue was removed from the lateral striatum. Importantly, the cerebral cortical tissue block did not include the cortical germinal zone at any age. From more caudal sections containing hippocampus, a block of tissue was removed containing both blades of the DG (perhaps more appropriately termed the "DG anlage" at PND1) and the entire subgranular cell layer but excluding its most medial, third ventricle-adjacent tip and any lateral ventricular tissue surrounding the hippocampus. This strategy includes the entire subgranular zone but effectively excludes adjacent subependymal stem cells (Seaberg and van der Kooy, 2002). Details of the dissection strategy can be found in Supplementary Fig. 1. Dissected tissue from each region was pooled, minced into small pieces, and transferred into chemically defined serum-free media (SFM) as previously described (Reynolds and Weiss, 1992; Tropepe et al., 1999). The tissue was then either treated using a Papain Dissociation System according to kit directions (Worthington Biochemical Corporation, Lakewood, NJ) or mechanically dissociated into a cell suspension without enzyme treatment using a small-bore, fire-polished Pasteur pipette.

Adult mice were killed by cervical dislocation and dissected in the same manner as the postnatal mice, except that the tissue was dissected in artificial cerebrospinal fluid treated either with papain or with an alternate enzyme solution (trypsin, hyalurondiase, and kynurenic acid) as previously described (Seaberg and van der Kooy, 2002).

In vitro cell culture

To determine whether there are intrinsic differences between neural precursors from different regions, environmental conditions must be standardized and held constant to enable such differences to emerge. A clonal colony-forming neurosphere assay (Reynolds and Weiss, 1992) was employed to investigate the properties of primary, freshly isolated precursor cells present in both neurogenic and non-neurogenic brain regions over a range of postnatal ages: PND1, PND10, and adulthood. The neurosphere assay is a powerful tool in this regard, because both neural stem cells

(Chiasson et al., 1999; Tropepe et al., 2000) and some types of more restricted progenitor cells (Seaberg and van der Kooy, 2002; Tropepe et al., 2000; Zhang et al., 1998) are capable of forming clonal colonies in vitro. These clonal colonies can then be further manipulated to investigate the fundamental aspects of their biology: proliferation, multipotentiality, self-renewal, and longevity (longevity is defined as the maintenance of these first three properties over time). It has been shown previously that neurosphere and adherent monolayer culture conditions yield identical results when investigating fundamental properties of stem and progenitor cells (Seaberg and van der Kooy, 2002), but the advantage of neurosphere cultures is the relative ease with which clonality can be demonstrated (Tropepe et al., 2000). Moreover, clonal colony-forming assays are commonly used to investigate the properties of stem and progenitors cells in other tissue systems as well, such as the hematopoietic system (Akashi et al., 2000).

Growth factors were used in SFM at final concentrations of 10 ng/ml FGF2 (human recombinant; Sigma), 2 μg/ml heparin (Sigma), 20 ng/ml EGF (mouse submaxillary; Sigma), unless otherwise noted. Cell viability was assessed just prior to plating using trypan blue exclusion (0.4%, Sigma) and was greater than 85% regardless of primary tissue region dissected or dissociation conditions utilized. Primary tissue was plated at 20 viable cells/μl (10,000 cells/well) in uncoated 24-well culture plates (Nunc, Naperville, IL). It has been previously demonstrated by mixing marked and unmarked cells that culturing cells at this density (or lower densities) will result in clonal neurosphere colonies, as form in single-cell cultures, and that neurospheres do no arise as a result of cell aggregation at the cell culture densities employed here (Tropepe et al., 2000). The total number of spheres that formed in each well was counted after 7 days in vitro unless otherwise specified. Where indicated, this protocol was modified by first exposing the primary cell isolates to FCS for 3 days, then rinsing the cells and transferring them to the mitogen-containing SFM as described. To determine the self-renewal capacity of cells isolated from each region derived from stem cells, we assayed for self-renewal by dissociating spheres in bulk and reculturing single cells at a constant density of 20 cells/μl. This was repeated up to a maximum of 10 passages; previous reports suggest that neurospheres formed by neural stem cells passaged up to 10 times are virtually identical to primary spheres (Reynolds and Weiss, 1996), but beyond this time in vitro sphere colonies have been shown to undergo a variety of genetic and epigenetic transformations (Morshead et al., 2002).

This culture procedure was modified in the following instances: additional experiments were performed in which adult optic nerve cell isolates were first exposed to 10% FCS for 24 h and then transferred to mitogen-containing SFM for 4 weeks of culture. Additional experiments were performed on PND1 and PND10 optic nerve cell isolates in which the cells were exposed to 15% FCS for 3 days and then transferred to regular serum-free mitogen-containing media for 7 days.

For some experiments, conditioned media (CM) taken from either postnatal or adult subependymal sphere cultures were used. To generate this CM, ventricular subependymal cultures were initiated as described and the supernatant was collected and filtered to eliminate the possibility of cell transfer. CM was applied to primary cultures of progenitor cells and represented 50% of the total volume, with the remaining volume being comprised of fresh SFM containing mitogens as outlined above. These conditions were maintained over multiple passages in vitro.

Mixing strategies were also employed in some studies. This procedure involved using both CD1 tissue and tissue from transgenic mice expressing enhanced-GFP constitutively under the control of an actin promoter (Jackson Laboratories, Bar Harbor, ME, USA) so that cells originating from different regions could be followed in vitro. Progenitor cells from optic nerve or olfactory bulb were mixed with either postnatal or adult subependymal tissue in the following proportions: (1) 1 cell/μl of progenitor cells with 9 cells/μl of subependymal cells for a final density of 10 cells/μl, at which neurospheres have been shown to arise clonally in the present work and elsewhere (Tropepe et al., 2000), or (2) 1 cell/μl of progenitor cells with 39 cells/μl of subependymal cells for a final density of 40 cells/μl. At 40 cells/μl, a large proportion of spheres does not arise clonally and indeed contain both CD1 and GFP^+ cells. This density was chosen to facilitate cell–cell contact between stem cells and progenitor cells in vitro. These mixed cultures were maintained over multiple passages, as described for the preceding experiments. However, for the mixed cultures that were maintained at 40 cells/μl, the final passage was performed at 10 cells/μl to once again derive clonal spheres for the differentiation assay and subsequent immunocytochemistry. To control for potential effects of strain differences, these experiments were performed such that some cultures involved CD1 tissue as the source of subependymal cells and others GFP^+ tissue.

Immunocytochemistry, cell quantification, and statistical analysis

Multipotentiality of single spheres was assayed by differentiation in 1% FCS and subsequent immunocytochemistry as described (Seaberg and van der Kooy, 2002). The quantification of cells expressing neural precursor markers was carried out using neurosphere colonies that were acutely dissociated and plated on Matrigel. The following antibodies were used: anti-β-tubulin isotype III mouse monoclonal (IgG) (1:500, Sigma), anti-GFAP rabbit polyclonal (IgG) (1:400; Chemicon, Temecula, CA), anti-Mash1 rabbit polyclonal (IgG) (1:300; kind gift from Dr. Johnson), anti-Musashi1 rabbit polyclonal (IgG) (1:200; Chemicon), anti-NeuroD rabbit polyclonal (IgG) (1:400; Chemicon), anti-O4 mouse monoclonal (IgM)

(1:100; Chemicon), anti-Olig1 rabbit polyclonoal (IgG) (1:200; Chemicon), anti-Olig2 rabbit polyclonal (1:10,000; kind gift from Dr. Rowitch), anti-Sox1 rabbit polyclonal (IgG) (1:150; Chemicon), anti-Sox2 rabbit polyclonal (IgG) (1:300; Chemicon), Alexa 488 goat anti-rabbit (1:350; Molecular Probes), Alexa 488 goat anti-mouse (1:350; Molecular Probes), Alexa 568 goat anti-rabbit (1:350; Molecular Probes), Alexa 568 goat anti-mouse (1:350; Molecular Probes), FITC goat anti-rabbit (1:200; Jackson ImmunoResearch, West Grove, PA), TRITC goat anti-mouse (1:200; Jackson ImmunoResearch). The Hoechst 33258 nuclear stain (0.015 mg/ml stock solution diluted to 0.001 mg/ml; Roche) was used to stain the nuclei of all of the cells. Secondary-only wells were processed simultaneously using the identical protocol except solutions did not contain primary antibodies. All secondary-only controls were negative for staining. The numbers of neurons, astrocytes, or oligodendrocytes generated per neurosphere were determined by counting the numbers of β-tubulin isotype III$^+$, GFAP$^+$, or O4$^+$cells, respectively, as a percentage of Hoescht-positive nuclei in at least 40 random fields of differentiated cells. Fluorescence was visualized using a Nikon diaphot-inverted microscope, and images were recorded with a Nikon COOLPIX digital camera.

For quantification of cells positive for various neural precursor markers, the numbers of antibody$^+$ cells were counted as a percentage of Hoescht-positive nuclei in at least 12 fields of undifferentiated cells. The absolute number of cells counted per condition to determine the percentages (Fig. 4) was at least 1000 cells.

Data are expressed as means ± SEM unless specified otherwise. Statistical comparisons between two groups were performed using a Student's *t* test where appropriate or by one-way analysis of variance (ANOVA) with the Bonferroni post-test for comparisons between groups where an acceptable level of significance was considered at $P < 0.05$.

RT-PCR

Total RNA was extracted from sphere colonies using an RNeasy extraction kit (Qiagen). Reverse transcription and PCR were carried out using a One-Step RT-PCR kit (Qiagen) in a GeneAmp PCR System 9700 (Applied Biosystems) according to kit instructions. PCR reactions were performed for 30 cycles. All samples were treated with DNAse to avoid contamination with genomic DNA. Only sphere colony RNA isolates that were found to express β-actin were considered for further analysis. Forward and reverse primers (5′–3′), annealing temperature, and expected product size are as follows: β-actin, ATC ATG TTT GAG ACC TTC AA and TCT GCG CAA GTT AGG TTT TGT C (825 bp, 56°C); Mash1, AAG AAG ATG AGC AAG GTG GAG ACG and CAG AAC CAG TTG GTA AAG TCC AGC (257 bp, 55°C); Msi1h (Musashi-1 homolog), CTA AGA TGG TCA CTC GGA CGA and GAG AGC CTG TCC CTC GAA CTA (560 bp, 58°C); Nestin, ATA CAG GAC TCT GCT GGA GG and AGG ACA CCA GTA GAA CTG GG (410 bp, 56°C); NeuroD, CTG ATC TGG TCT CCT TCG TAC AG and GAT GCG AAT GGC TAT CGA AAG (540 bp, 58°C); Ngn1, TGC ATC TCT GAT CTC GAC TGC and AGA TGT AGT TGT AGG CGA AGC G (406 bp, 58°C); Ngn2, AAG CTC ACG AAG ATC GAG ACG and TGC CAG TAG TCC ACG TCT GAC (290 bp, 58°C); Olig1, ATC CTC ATC CTC TTC CAC CG and AGC AGC AGG ATG TAG TTG CG (317 bp, 58°C); Olig2, GTG TCT AGT CGC CCA TCG TC and TCT TTC TTG GTG GAA GAC GTG (250 bp, 58°C); Sox1, AAT CCC CTC TCA GAC GGT G and TTG ATG CAT TTT GGG GGT A (224 bp 58°C); Sox2, GGA GTG GAA ACT TTT GTC CGA and TTC ATG TAG GTC TGC GAG CTG (420 bp, 58°C); and Sox3, AGA CTG AAC TCA AGA ACC CCG and GTC CTT CTT GAG CAG CGT CTT (435 bp, 58°C). Neural tube tissue isolated from 12.5 dpc embryos was used as positive control.

Results

The population of proliferative progenitors in diverse early postnatal brain regions decreases significantly between PND1 and PND10 but the ventricular subependymal stem cell population is maintained

Four neural precursor characteristics were studied: proliferation, multipotentiality, self-renewal, and longevity. Here, proliferation is operationally defined as the ability to generate a floating clonal colony in response to EGF and FGF2 (Reynolds and Weiss, 1992), multipotentiality as the ability of an individual cell to generate three main neural cell lineages (neurons, astrocytes, and oligodendrocytes), self-renewal as the ability of cells from dissociated colonies to generate secondary colonies, and longevity as both the in vitro maintenance of the first three characteristics over an extended time and as in vivo maintenance of that cell throughout the lifetime of the organism. The term stem cell refers to a single cell that exhibits all four of these properties, progenitor to a cell that exhibits at least one, two, or three of these properties, and the term precursor refers more generally to either a mixed population or one in which the cells' properties are unknown (Weiss et al., 1996). The distinction between stem cells and progenitors in this context is not trivial; surely, there are fundamental differences between the biologies of a cell that can persist throughout the lifetime of an organism (and extended culture periods) and retain its ability to proliferate, self-renew, and generate all three major neural lineages and of cells that can exhibit these properties for only a short time in vitro or in vivo (Seaberg and van der Kooy, 2003).

Proliferative ability was assessed by plating cells isolated from microdissections of early postnatal (PND1 and PND10) neurogenic (olfactory bulb, anterior lateral ven-

tricle, dentate gyrus, cerebellum) and non-neurogenic (optic nerve, cerebral cortex, striatum) regions in EGF- and FGF2-containing serum-free media (SFM) (Reynolds and Weiss, 1992). It has been shown previously that after mid-gestation, there is no significant difference in the number of neurospheres formed in EGF or FGF2 (Tropepe et al., 1999). It is important to note that the cortical germinal zone was not included in the cerebral cortex dissections at any age (see Materials and methods and Supplementary Fig. 1 for further details). Neural stem cells from the ventricular subependyma were cultured in the same experiments to provide a point of comparison.

All regions assayed contained subpopulations of cells that proliferated and formed spherical colonies in response to these mitogens (Fig. 1A). Interestingly, the frequency of these proliferative cells decreased significantly ($P < 0.05$) in isolates from each region over the first 10 days of postnatal development in vivo, indicating that these subpopulations may be present for only a discrete postnatal period. The only exception was the proliferative subpopulation present in the anterior lateral ventricle dissection, which did not change over time ($P > 0.05$). This region included the subependyma, which has previously been shown to contain a population of neural stem cells that are proliferative, multipotential, self-renewing (Chiasson et al., 1999; Seaberg and van der Kooy, 2002), and persist into senescence (Tropepe et al., 1997).

To establish that the absolute number of sphere-forming progenitor cells was declining between PND1 and PND10, and to control for the possibility that progenitor density was changing over these times in vivo and confounding the results, we determined the absolute total number of sphere-forming cells contained within a number of different postnatal brain structures at PND1 and PND10. Because

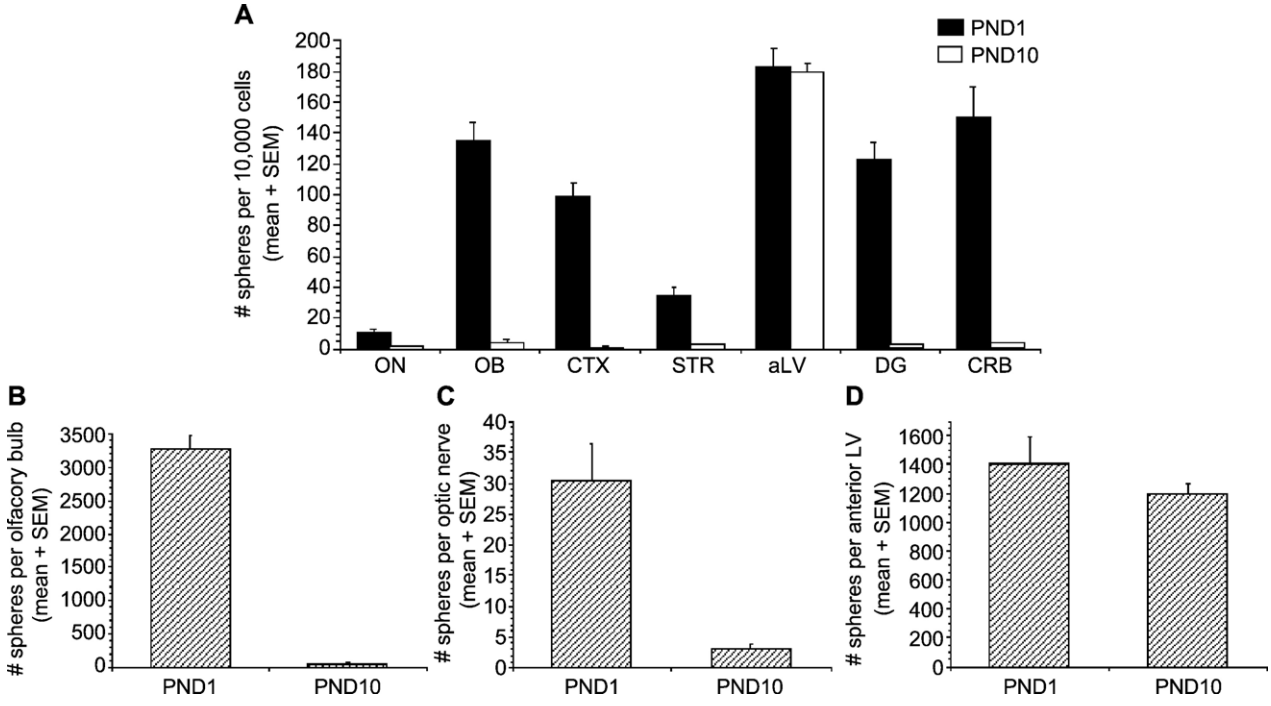


Fig. 1. Comparison of the numbers of primary spheres generated from different mouse neurogenic and non-neurogenic regions at PND1 and PND10. This represents the number of cells present in each isolate capable of proliferating in vitro in response to EGF and FGF2. (A) There is a significant decline in the frequency of sphere-forming cells from all non-subependymal brain regions between PND1 and PND10. The data are expressed as the mean (+SEM) number of spheres generated per 10,000 cells plated (i.e., per well; cell density = 20 cells/μl) and *n* is a minimum of three independent experiments. For all regions except the anterior lateral ventricle (aLV) dissection, the number of spheres formed at PND1 is significantly greater than the number formed at PND10 ($P < 0.05$), suggesting a limited capacity for self-renewal in vivo. ON, optic nerve; OB, olfactory bulbs; CTX, cerebral cortex; aLV, anterior lateral ventricular subependyma; DG, dentate gyrus; CRB, cerebellum. (B and C) There is a significant decline in the total absolute number of sphere-forming cells from the olfactory bulb and optic nerve between PND1 and PND10. The data are expressed as the mean (±SEM) absolute number of spheres that are generated per olfactory bulb (B) or optic nerve (C), and *n* represents a minimum of seven separate experiments. Note that between PND1 and PND10, there is a 64-fold decline in the total number of sphere-forming cells present in a single olfactory bulb and a 10-fold decline in the total number of sphere-forming cells present in a single optic nerve. These large changes in absolute number are important because they suggest that the sphere-forming cells are genuinely decreasing in number and are not simply becoming more widely dispersed throughout the tissue region assayed. These comparisons of absolute number are only fair for dissections that include whole structures (olfactory bulb and optic nerve) and so data generated from dissections of partial structures (e.g., cortex, striatum) were not analyzed in this manner. (D) There is no significant decline in the number of sphere-forming stem cells in the ventricular subependyma between PND1 and PND10. The data are expressed as the mean (+SEM) absolute number of spheres that are generated per ventricular subependyma dissection (per hemisphere) and *n* represents a minimum of seven separate experiments. Note that the 1.2-fold decline in sphere number is not statistically significant. These data provide evidence that, in contrast to postnatal progenitor cells from other regions, the absolute number of stem cells in the subependyma does not decline between PND1 and PND10.

this analysis is only relevant to dissections that involve the dissociation of an entire neural structure, we determined the absolute total number of sphere-forming progenitor cells derived from single whole olfactory bulbs (Fig. 1B), optic nerves (Fig. 1C), and ventricular subependyma dissections for a single hemisphere (Fig. 1D). It was determined that the absolute number of progenitors per olfactory bulb declines 64-fold between PND1 (3267 ± 216) and PND10 (51 ± 10) ($P < 0.05$); indeed, this large decline is underestimated by examination of the frequency data alone that suggest a 30-fold decline over the same time period in vivo. Similarly, it was determined that the absolute number of progenitors per optic nerve declines 10-fold between PND1 (30.4 ± 6) and PND10 (3.1 ± 0.9) ($P < 0.05$), a greater decline than the 5.5-fold decrease suggested by the frequency data. Moreover, as predicted by the frequency data, there was no significant decline ($P > 0.05$) in the absolute total number of subependymal sphere-forming stem cells from anterior lateral ventricle dissections between PND1 (1403 ± 191) and PND10 (1197 ± 70) (Fig. 1D).

Table 1
Single-cell analysis of postnatal brain regions reveals that primary multipotential sphere colonies arise clonally

Region	No. of single cells assayed	No. of spheres	Frequency	Frequency at 20 cells/μl
aLV	2762	73	1/38	1/54
OB	2665	29	1/92	1/74
ON	2783	3	1/928	1/962

Single-cell analyses were performed for PND1 anterior lateral ventricular (aLV) subependymal cells, olfactory bulb (OB) cells, and optic nerve (ON) cells. These regions were chosen for closer study because two of them (aLV and OB) generate large numbers of primary spheres at 20 cells/μl and ON generates the smallest number of primary spheres at this density. Single cells were individually plated in 96-well plates. Within 8 h of plating, wells were assayed and scored for the presence of single cells, and subsequently followed for 7 days in vitro. The frequency of sphere formation was similar at 1 cell/well (0.005 cells/μl) as at 4000 cells/well (20 cells/μl), that is, the frequency of sphere formation is comparable despite the 4000-fold difference in cell density. This provides strong evidence that these sphere colonies arise clonally from single cells even at 20 cells/μl. All of the single primary spheres from this study that were assayed either for self-renewal or multipotentiality met the criterion for which they were tested.

Clonal analysis reveals that sphere colonies arise from single precursor cells

It has been shown previously that when marked (e.g., with a constitutive GFP transgene) and unmarked sphere-forming cells are mixed together in equal proportions at or below 20 cells/μl, virtually all of the spheres generated by 7 days in vitro are comprised entirely of either marked or unmarked cells (Tropepe et al., 2000). This strongly suggests that sphere colonies arise from the proliferation of single cells rather than as a result of cellular aggregation. Thus, we posited that postnatal progenitor spheres generated from neural tissues in this study were also the result of single-cell proliferation rather than aggregation.

To establish more convincingly that the sphere colonies generated by the diverse neural tissues investigated in this study were indeed the result of clonal proliferation of single cells, we performed a rigorous, large-scale, single-cell clonal analysis. Cells were diluted to a density of 1 cell per 200 μl (1 cell/well of a 96-well plate) and plated in the same mitogen-containing media used for the aforementioned experiments. Within 8 h of plating, wells were scored for the presence of a single cell and followed for 7 days in vitro. Approximately 2700 single cells were analyzed for each dissected region (Table 1). As expected, the frequency of sphere generation from wells containing single cells was similar to the frequency of sphere generation at 20 cells/μl (Fig. 1). This is important because there is a 4000-fold difference in density between these two paradigms. These data provide strong evidence that spheres from postnatal brain regions arise clonally from the proliferation of single cells. Primary spheres generated by this clonal analysis study were tested either for self-renewal (by dissociation and replating) or for multipotentiality (see below).

Early postnatal progenitor cells are intrinsically multipotential

To determine whether the sphere-initiating cells isolated from each of these regions were capable of generating multiple neural lineages, individual spheres were withdrawn from the mitogens, differentiated in the presence of 1% FCS, and processed using immunocytochemistry. Primary spheres from all of the PND1 and PND10 regions assayed were capable of generating βIII-tubulin$^+$ neurons, glial fibrillary acidic protein$^+$ (GFAP$^+$) astrocytes, and O4$^+$ oligodendrocytes (Fig. 2). A similar multipotential progenitor has been isolated from PND2 rat cerebral cortex (Marmur et al., 1998). Multipotential sphere colonies were isolated from neurogenic and non-neurogenic regions directly after a relatively short culture period (7 days); cell isolates were not pretreated with putative extracellular reprogramming factors, such as serum, before the clonal proliferation assay was initiated. Furthermore, when cell isolates were pre-exposed to 3 days of culture in 15% serum, we observed no difference in the number of multipotential sphere colonies that arose after transfer into the neurosphere assay. Thus, these sphere colonies were not dependent on serum pre-exposure and a priori differentiation into type 2 astrocytes, as has been reported previously for cells isolated from postnatal cerebral cortex, cerebellum (Laywell et al., 2000), and optic nerve (Kondo and Raff, 2000). As demonstrated previously for retinal precursors and subgranular zone progenitors (Cayouette et al., 2003; Seaberg and van der Kooy, 2002), early postnatal neural precursors were resistant to such culture manipulations. Indeed, even dramatic transformation events that alter the cells' gene expression, adhesion properties, proliferation characteristics, and confer the faculty to generate solid tumors

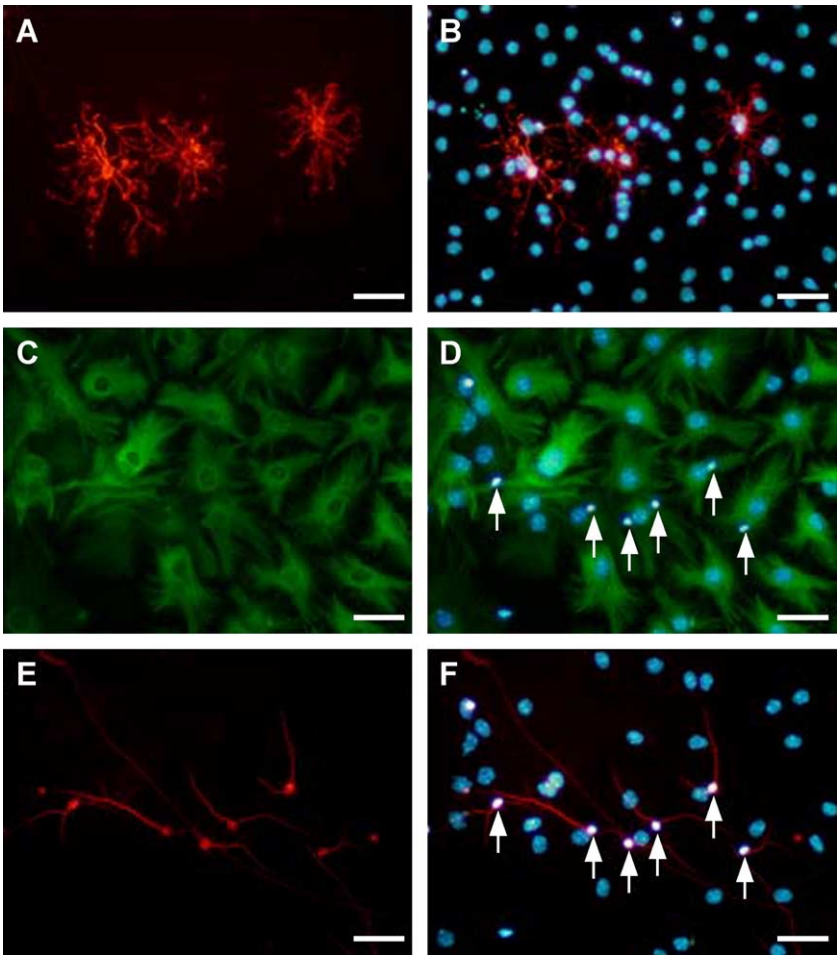


Fig. 2. Representative images of immunolabeled neural progeny generated from single primary sphere colonies, as seen for all neurogenic and non-neurogenic regions at PND1 and PND10. All three types of neural progeny were generated by 100% of the primary spheres assayed from three independent experiments. (A and B) The same field of $O4^+$ oligodendrocytes is shown alone in A and overlaid with the corresponding $Hoescht^+$ nuclei in B. (C–F) The same field of cells is shown in these four micrographs. C illustrates $GFAP^+$ astrocytes and D illustrates the overlay with the corresponding $Hoescht^+$ nuclei; E illustrates βIII-$tubulin^+$ neurons, while F illustrates the overlay with corresponding $Hoescht^+$ nuclei. Note that the nuclei illustrated in D and F represent entirely separate populations of neurons (arrows) and astrocytes. Scale bars, 50 μm.

(Morshead et al., 2002) do not affect the multipotency of clonally derived neurosphere cells.

Multipotential progenitors from neurogenic and non-neurogenic regions become restricted to an exclusively glial-producing phenotype in vitro and in vivo

Self-renewal capacity was investigated by dissociating spheres from each region and replating the cells in identical media conditions over serial passages. Sphere-forming cells from all PND1 regions were capable of extended self-renewal in vitro, as illustrated by the formation of secondary and tertiary sphere colonies, and so on up to 10 passages. Spheres from all PND10 regions exhibited self-renewal, with two exceptions: although PND1 cortical and striatal spheres were passageable, secondary cortical and striatal spheres were never observed when the primary sphere-forming cells were isolated at PND10.

To examine whether these cells isolated from diverse regions at early postnatal ages exhibited in vitro longevity (i.e., whether they maintained their multipotentiality over continued passages), a sample of sphere colonies was removed from culture with mitogens at each passage, differentiated, and processed using immunocytochemistry to detect neurons, astrocytes, and oligodendrocytes. All PND1 (Fig. 3A) and PND10 (Fig. 3B) regions exhibited a decline in the generation of neurons with continued passaging, and all regions except the subependyma-containing anterior lateral ventricle exhausted their capacity to generate neurons completely and irrevocably by passage 6 (7° spheres). To rule out the possibility that neurons were being generated but dying during the period of differentiation culture, passaged sphere colonies were assayed within 12 h of transfer to differentiation medium. Again, only the ventricular subependymal passaged spheres contained cells that were β_{III}-$tubulin^+$. It has been shown previously that a slightly higher percentage of

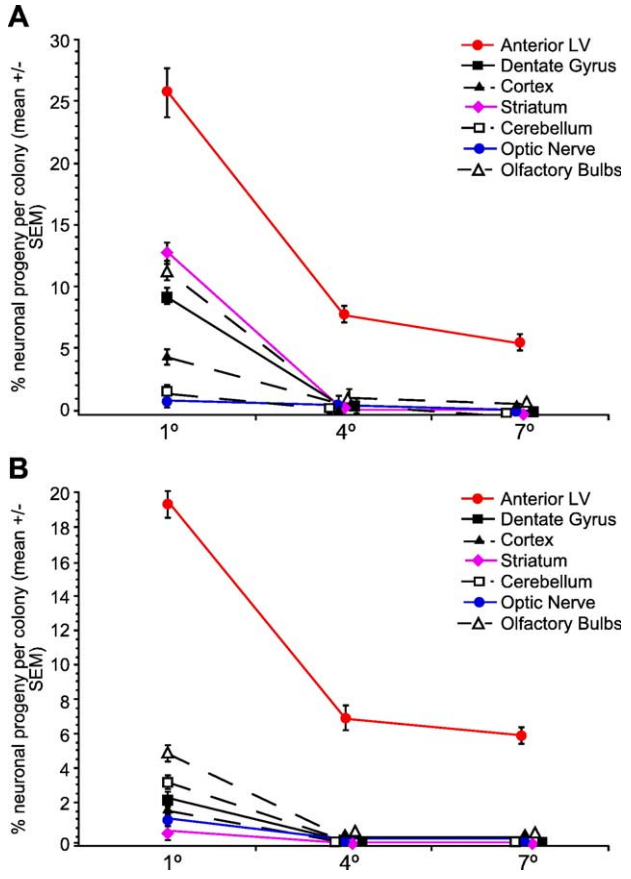


Fig. 3. Comparison of the percentage of neuronal progeny generated from individual-passaged sphere colonies derived from neurogenic and non-neurogenic regions at PND1 and PND10. (A and B) The data for PND1 (A) and PND10 (B) are expressed as the mean percentage of Hoescht-positive cells that were also immunopositive for the neuron-specific marker βIII-tubulin per colony (±SEM), and are representative of at least 40 random fields of differentiated cells from a minimum of three independent experiments. One hundred percent of assayed colonies from all regions at PND1 and PND10 remained capable of generating both astrocytes and oligodendrocytes. Note that while all other PND1 (A) and PND10 (B) neurogenic and non-neurogenic regions ultimately exhaust their capacity to generate neurons, the sphere forming stem cells isolated from the anterior lateral ventricular subependyma (red) do not. 1°, primary sphere colonies; 4°, quaternary sphere colonies (i.e., colonies formed after passage 3), and so forth. Anterior LV, anterior lateral ventricular subependyma.

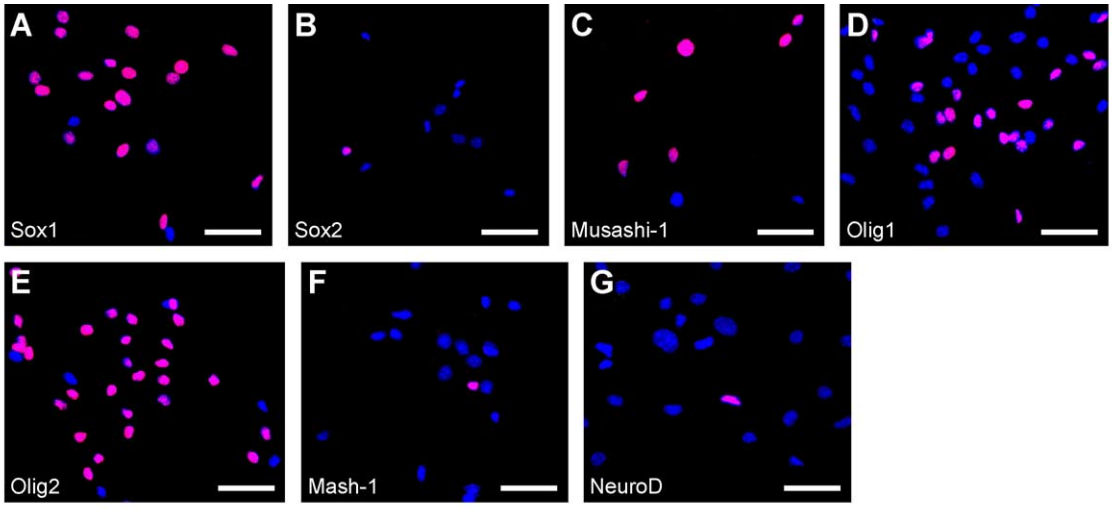
neuronal progeny is generated from primary embryonic neural stem cell spheres grown in FGF2 (Tropepe et al., 1999). However, experiments in which each early postnatal region was cultured and passaged separately in either EGF alone or FGF2 alone, or EGF and FGF2 in combination revealed that FGF2 does not prolong transient neurogenesis in early postnatal progenitors (Supplementary Table 1). Furthermore, in contrast to a recent study suggesting that multipotential clones arise only by a mechanism whereby FGF2 artificially forces putative bipotential oligodendrocyte/neuron precursors to make astrocytes (Gabay et al., 2003), these data demonstrate that multipotential clones can be isolated in the absence of FGF2 (i.e., in EGF alone). The percentage of neurons generated from PND1 and PND10 neural stem cell spheres decreases but reaches a stable level; interestingly, this stable level corresponds to the percentage of neurons generated from adult subependymal neurospheres (Seaberg and van der Kooy, 2002). Passaged spheres from all PND1 and PND10 regions continued to generate similar numbers of both astrocytes and oligodendrocytes. It should be emphasized that the proliferative ability remained unchanged over passages; the only property that was altered was specifically the capacity to generate neuronal cells. That the progenitors isolated from the PND1 and PND10 olfactory bulbs also displayed this loss of neuronal differentiation phenotype was surprising in light of a study reporting the presence of neural stem cells in the adult olfactory bulb (Gritti et al., 2002). Perhaps the early postnatal ages of the olfactory bulbs assayed here precede the acquisition of a mature, stable neural stem cell phenotype in this region.

To determine whether early multipotential postnatal progenitors from neurogenic and non-neurogenic regions are maintained in vivo into adulthood, the same seven regions investigated at PND1 and PND10 were also dissected and assayed in adult mice. Interestingly, all regions except the adult optic nerve still contained at least a small subpopulation of cells that were capable of proliferation and colony formation in vitro. As has been reported previously, non-subependymal adult spheres were significantly smaller than those generated by neural stem cells (Seaberg and van der Kooy, 2002). Only the adult anterior lateral ventricle (which still contains a large population of neurosphere-forming neural stem cells, 95.7 ± 12.3 spheres per 10,000 viable cells) and olfactory bulb (4.4 ± 0.2 spheres per 10,000 viable cells) dissections yielded spheres that were multipotential and self-renewing (Chiasson et al., 1999; Gritti et al., 2002; Seaberg and van der Kooy, 2002). The few spheres that formed from adult cerebral cortex, striatum, dentate gyrus, and cerebellum did not passage or generate neurons, although small proliferative, entirely neuronal clones have been isolated from adult dentate gyrus (Seaberg and van der Kooy, 2002).

To address the possibility that exposure to serum and long-term culture in FGF2 would facilitate the proliferation of a neuron-producing cell from the adult optic nerve (Palmer et al., 1999), cells were exposed to 10% FCS for 24 h and then transferred to serum-free media containing FGF2. Even after culture for 4 weeks, adult optic nerve isolates did not yield sphere colonies. These data, together with the finding that the progenitor population in the optic nerve decreases dramatically between PND1 and PND10 (Figs. 1A and 1C), support the idea that this population exists only transiently. However, it remains formally possible that this progenitor population is maintained in the adult optic nerve but it undergoes such a dramatic change in phenotype and mitogen responsiveness that the culture conditions utilized for early postnatal tissue are no longer appropriate for their proliferation and identification.

Multiple transcription factors associated with neural and neuronal precursor identity are expressed by both primary and passaged sphere colonies

To determine whether changes in neurogenic capacity were associated with changes in neural precursor transcription factor expression, primary and passaged spheres from anterior lateral ventricular subependyma and cortex were analyzed by immunocytochemistry (Figs. 4A–G) and RT-PCR (Fig. 4H). Neural precursor markers included nestin (Lendahl et al., 1990), Sox1–3 (Bylund et al., 2003), Ngn1–2 (Sommer et al., 1996), Olig1–2 (Liu and Rao, 2004), and Musashi-1 (Kaneko et al., 2000). Mash-1 and NeuroD, transcription factors known to play an important role specifically in neurogenesis (Guillemot and Joyner, 1993; Schwab et al., 2000), were also analyzed.



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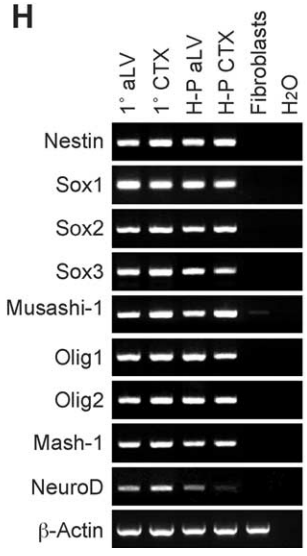

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	1º aLV	1º CTX	H-P aLV	H-P CTX
Sox1	94.7±1.9	95.4±0.8	93.6±1.2	93.3±1.1
Sox2	2.1±0.6	1.6±0.6	2.2±1.1	1.1±0.4
Musashi-1	77.9±4.0	83.0±3.1	39.7±6.7‡	76.3±4.9*
Olig1	37.2±4.3	27.5±4.7	13.3±2.5‡	15.9±1.8‡
Olig2	80.1±3.5	83.7±2.4	44.0±6.3‡	75.4±2.9*
Mash-1	<1%	<1%	<1%	<1%
NeuroD	<1%	<1%	<1%	<1%

Fig. 4. Multiple transcription factors associated with neural and neuronal precursor identity are expressed by both primary and passaged sphere colonies. (A–G) Neural precursor markers (red) Sox1 (A), Sox2 (B), Musashi-1 (C), Olig1 (D), Olig2 (E), and neuronal precursor markers Mash-1 (F) and NeuroD (G) are expressed in single cells from both primary and highly passaged dissociated sphere colonies by immunocytochemistry (see also I). Note that the nuclei in these fluorescence micrographs are labeled with both Hoescht (blue) and the respective neural precursor markers (red), giving positive nuclei a pink appearance. Fluorescence micrographs of cells from dissociated primary ventricular subependymal sphere colonies are pictured and the appearance of positive cells is representative of those from other brain regions and passages. Scale bars, 50 μm. (H) RT-PCR analysis of genes associated with neural (Nestin, Sox1–3, Musashi-1, Olig1, Olig2) and neuronal precursor (Mash-1, NeuroD) identity. Note that all neural and neuronal precursor markers are expressed in both primary (1°) and high passage (H-P) sphere colonies from all regions tested. (I) Percentages of single cells from individual acutely dissociated undifferentiated sphere colonies from both primary and high passage (H-P) conditions. The "*" represents a significant difference relative to the aLV value for the respective passage (primary or H-P), $P < 0.01$. The "‡" represents a significant difference relative to the value of the primary sphere colonies for the same tissue, $P < 0.05$. Note that although there were no differences in Mash-1 or NeuroD expression (neuronal precursor markers) in terms of percentage of positive cells, the RT-PCR results in H are suggestive of lower levels of NeuroD expression in high-passaged CTX spheres. Further, although there were some significant differences in the numbers of cells expressing various transcription factors between primary and high passage conditions (as noted in the table), in no cases was gene expression abolished. aLV, anterior lateral ventricle; CTX, cerebral cortex.

All genes analyzed were expressed by both immunocytochemistry and RT-PCR in primary and high passaged sphere colonies from both tissue regions, except for Ngn1 and Ngn2, which were not expressed in any condition.

The percentage of cells expressing each transcription factor from both individual primary and passaged undifferentiated colonies from each region was determined (Fig. 4I). There were no significant differences between primary and passaged spheres from ventricular subependyma and cortex in terms of Sox1, Sox2, Mash-1, or NeuroD expression. However, although there were no differences in Mash-1 or NeuroD expression (neuronal precursor markers) in terms of percentage of positive cells, the RT-PCR results (Fig. 4H) are suggestive of lower levels of NeuroD expression in high-passaged cortex spheres. There were some differences in the percentages of cells expressing Musashi-1, Olig1, and Olig2 among the various conditions as noted in Fig. 4I, but in all conditions there were a subpopulation of cells positive for each transcription factor. Thus, there does not appear to be an obvious relationship between changes in transcription factor expression and loss of neurogenic capacity.

Restriction to a glial-specific phenotype occurs independently of the cellular environment

Our findings to this point suggest that postnatal progenitor cells isolated from diverse neural tissues lose the ability to generate neurons irrespective of their environment. There are three pieces of evidence to support this idea. The first is that we maintain the same culture conditions throughout our passaging procedure and the cells' capacity to generate neurons changes over time. Clearly, for the first few passages in vitro, cells can maintain their neurogenic property. This characteristic is lost over the next few passages in vitro despite the constant, unchanging in vitro environment. Second, this same phenomenon is observed whether the neurosphere colonies are generated in EGF alone, FGF2 alone, or EGF and FGF2 in combination (Supplementary Table 1). Third, over a similar time course, the loss of neurogenesis occurs both in vitro over multiple cell passages and in vivo if cells are allowed to remain in situ until early adulthood. This also argues for a cell-autonomous mechanism of neurogenic restriction, because even in two vastly different environments, the same restriction is observed. These data suggest not that these cells change intrinsically over time, but rather that they are progenitors that had only limited neurogenic potential at the outset.

To test this idea further, the culture environment was manipulated in a number of different ways. We reasoned that if there was a critical environmental, non-cell autonomous signal that was positively mediating the cells' capacity to generate neurons, this signal would need to be present in regions that contain stem cells, since stem cells maintain the capacity to generate neurons throughout the lifetime of the organism. Perhaps if these diverse postnatal progenitors were maintained in a stem cell environment or niche, they would continue to generate neurons in addition to astrocytes and oligodendrocytes. So, rather than testing the effect of randomly chosen mitogens or cytokines on the cells' capacity to generate neurons, the influence of a stem cell environment was investigated.

Three different approaches were employed to address the possibility that something in the stem cell environment influences the capacity of progenitor cells to generate neurons: (1) adding stem cell conditioned media to progenitor cell cultures, (2) initiating mixed cultures containing a small proportion (10%) of progenitor cells in the presence of a large proportion of stem cells (90%) at a density that will generate clonal sphere colonies (10 cells/μl), and (3) initiating mixed cultures containing a small proportion (2.5%) of progenitor cells in the presence of a large proportion of stem cells (97.5%) at high density (40 cells/μl) to facilitate cell–cell contact (Table 2). For the mixing experiments, cells were isolated from wild-type CD1 mice and from transgenic mice that constitutively express GFP in every cell (Hadjantonakis et al., 1998). Experiments were performed such that in some cases the CD1 cells were the source of progenitor cells, and in some cases GFP cells were the source of progenitors in order to control for potential strain differences in cell behavior. As well, some experiments used early postnatal subependyma as the source of stem cells and others used adult subependyma. These conditions were maintained over multiple passages as described for the preceding experiments. In all cases, the final passage was performed at 10 cells/μl so that clonally derived sphere colonies could be generated, differentiated, and assayed with immunocytochemistry for markers of neurons, astrocytes, and oligodendrocytes. For controls, we passaged unmixed cells from anterior lateral ventricular subependyma as well as unmixed cells from optic nerve and olfactory bulb. As well, subependymal stem cell spheres that were generated from the mixing experiments were assayed for multipotentiality at the same time as the progenitor spheres with which they had been cultured.

A summary of the results for experiments involving the optic nerve and olfactory bulb is illustrated in Table 2. In all conditions, unmixed 7° sphere populations behaved as predicted, that is, progenitors from olfactory bulb and optic nerve lost neurogenic potential (0/12 tested spheres generated neurons for each region) and anterior lateral ventricular subependymal tissue did not (12/12 tested spheres did generate neurons). Conditioned media had no effect on the neurogenic capacity of progenitor cells, that is, with continued passaging these cells lost the ability to generate neurons (0/12 spheres with neuronal progeny) but still generated astrocytes and oligodendrocytes as in the previous experiments. Similarly, coculture with postnatal subependymal stem cells at both clonal and non-clonal densities did not affect the loss of neurogenesis. Importantly, subependymal spheres taken from these coculture

Table 2
The cellular environment does not influence the transient neurogenic capacity of postnatal progenitor cells

Culture strategy	Postnatal region (No. 7° spheres that generated neurons)		
	Optic nerve	Olfactory Bulb	aLV
a. Regular culture conditions (each region passaged alone)	0/12	0/12	12/12
b. Conditioned media	0/12	0/12	–
c. Mixed with 9× postnatal aLV (clonal density)	0/12	0/12	12/12
d. Mixed with 40× postnatal aLV (non-clonal density)	0/12	0/12	12/12
e. Mixed with 9× adult aLV (clonal density)	0/12	12/12	12/12

Summary description of the culture strategies utilized to determine whether the loss of neurogenic capacity can be rescued. These strategies were applied over multiple passages in vitro; passaging was carried out as described for the preceding experiments. Two types of controls were included: progenitors that were passaged alone as described, and anterior lateral ventricular subependymal (aLV) spheres that were generated alongside progenitor spheres in the coculture experiments. Conditioned media from adult subependymal neurospheres did not induce either optic nerve or olfactory bulb precursor cells to continue to generate neurons over passages in vitro (note that conditioned media from adult subependymal neurospheres were not reapplied to similar cultures, as indicated in the table). Thus, mixing strategies were employed. A small proportion (10% or 2.5%) of either optic nerve or olfactory bulb cells were mixed with a large proportion (90% or 97.5%) of postnatal aLV subependymal cells for a total density of either 10 cells/μl or 40 cells/μl, respectively. At 10 cells/μl, spheres arise clonally and thus it can be inferred that there is relatively little cell–cell contact between mixed regions. At 40 cells/μl, many spheres contain both wild type and GFP^+ cells, thus permitting contact between cells from different regions. For this latter strategy, dissociated spheres were passaged at 10 cells/μl for the final passage to obtain 7° clonally derived spheres for differentiation. For each region and condition, 12 randomly chosen sphere colonies were tested under differentiation conditions. Coculture with postnatal subependymal cells at 10 or 40 cells/μl did not rescue the neurogenic capacity of either optic nerve or olfactory bulb precursors. When cocultures were performed with adult aLV subependymal cells, neurons continued to be generated over passages in vitro from the olfactory bulb precursors (see also Fig. 4), but not from the optic nerve cells.

experiments continued to generate neurons (12/12 spheres tested), indicating that their ability to generate neurons was not negatively affected by being cultured with cells that become glial-restricted progenitors. Finally, there was no effect of coculture with adult subependymal stem cells on most progenitor cells (Table 2). However, we did notice a small but significant effect of adult stem cell coculture on olfactory bulb progenitor spheres. Although the other culture manipulations had no effect on neurogenic capacity, coculture with adult subependymal spheres produced a modest rescue of neurogenesis from these cells; 1.5 ± 0.2% of the differentiated cells were neurons from 12/12 sphere colonies tested (Table 2 and Fig. 5). These data support the aforementioned idea that perhaps the early postnatal olfactory bulb precursors have not acquired a stable stem cell phenotype, and require a constant stem cell environment in order to show their true potential.

In summary, the results presented here suggest that there are at least three types of intrinsically multipotential precursors present in the early postnatal mouse brain. At PND1, a distinction can be made between neural stem cells and progenitor cells from all other (neurogenic and non-neurogenic) brain regions: all of the initially multipotential progenitor cells become restricted over time in vitro and in vivo to an exclusively glial-producing phenotype that is not altered by the environment (except for the olfactory bulb progenitor), whereas neural stem cells retain multipotentiality indefinitely. It is possible that the olfactory bulb precursors fit into this category, since in vivo a small number are maintained into adulthood (Gritti et al., 2002). Other differences emerge at PND10, at which time three populations can be described. These include the multipotential, self-renewing neural stem cell population that resides in the subependyma around the lateral ventricles, as has been described in the adult brain (Chiasson et al., 1999), and two populations of transiently multipotential progenitors: some that exhibit limited self-renewal in vitro (optic nerve, cerebellum, dentate gyrus) and some that do not (cortex and striatum).

Discussion

At PND1, both neurogenic and non-neurogenic regions of the murine brain contain subpopulations of cells that

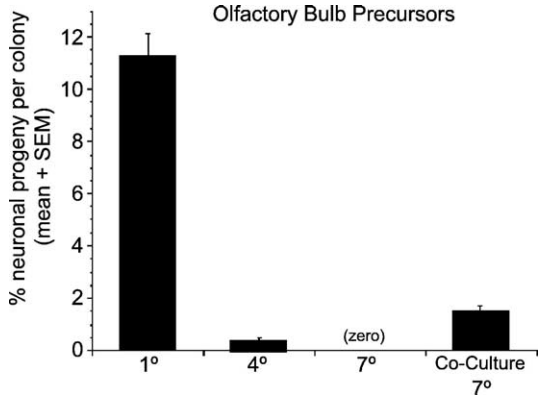

Fig. 5. Coculture with a large proportion of adult subependymal stem cells over multiple passages in vitro elicits a modest rescue of postnatal olfactory precursor cell neurogenesis. The data are expressed as the percentage of $Hoescht^+$ cells that were also immunopositive for the neuron-specific marker βIII-tubulin per colony (+SEM). Note that olfactory bulb cells were the only ones that exhibited any response to the stem cell environment; precursor cells from the other regions were unaffected by stem cell coculture. Interestingly, only the adult (and not the postnatal) subependymal environment permitted the retention of neurogenic capacity (see Table 3) in 7° spheres. 1°, primary sphere colonies; 4°, quaternary sphere colonies (i.e., colonies formed after passage 3), and so forth.

are capable of proliferating to form clonal sphere colonies, generating all three neural cell lineages, and passaging to form new colonies. They do not exhibit in vitro or in vivo longevity (maintenance of proliferation, self-renewal, and multipotentiality), but instead neurogenesis is a transient property of these progenitor cells. Interestingly, this transient neurogenesis is also an intrinsic property of these progenitor cells in that it is exhibited immediately after primary sphere formation in culture and is not dependent on prior exposure to reprogramming signals such as serum. Indeed, when cells are exposed to reprogramming signals such as serum, there is no change in the frequencies of multipotential progenitor cell isolation. Other neural cell types, retinal progenitors, and distinct adult neuronal and glial progenitors have also been shown recently to be resistant to such culture manipulations (Cayouette et al., 2003; Seaberg and van der Kooy, 2002). Neural stem cells isolated at PND1 from the ventricular subependyma exhibit in vitro longevity in that they maintain their proliferative, multipotential, and self-renewing ability over many passages in culture, and exhibit in vivo longevity as they are maintained in this niche into adulthood (see below) and senescence (Tropepe et al., 1997).

Although this is the first study to suggest that there are regional differences in early postnatal neural precursor cells in terms of intrinsic neurogenic and self-renewal potential, other studies have demonstrated regional differences between neural precursors. For example, Hitoshi et al. (2002a) demonstrated that there are regional differences in transcription factor expression in embryonic neural precursors. In addition, transplantation experiments have demonstrated that the host brain region can have profound effects on the cell types produced when neurogenic cells are transplanted into non-neurogenic regions. For example, spinal cord precursor cells that can generate neurons in vitro do not generate neurons in the spinal cord in vivo (Shihabuddin et al., 2000), and hippocampal precursors that can generate neurons in neurogenic regions cannot generate neurons in non-neurogenic regions such as the cerebellum (Suhonen et al., 1996). In contrast to the present study, these past studies showed that regional differences could be altered by the cellular environment. Here, we demonstrate that there are hard-wired intrinsic differences between cells in terms of neurogenic potential and self-renewal that become apparent whether the cells experience an in vivo or in vitro environment (i.e., that are independent and unchangeable by the environment). Because the sphere colonies that we study are clonally derived from single stem or progenitor cells and because these stem or progenitor cells from different regions are grown in identical culture conditions, these clonal colony-forming cells must have been different at the outset of the culture period. The conclusions from our study are very different: that there are intrinsically different types of cells present in different regions of the postnatal brain, not that the environment is simply altering the properties of a single neural precursor that is common to all regions.

A recent study demonstrated that neurosphere culture conditions induced changes in *Olig2* gene expression patterns of embryonic spinal cord progenitors and suggested that cell culture artificially induced multipotentiality in these "fate-restricted" cells (Gabay et al., 2003). This study would seem to undermine the utility of the neurosphere assay for predicting the in vivo properties of neural precursors. However, the key assumption of this study, that *Olig2* gene expression is confined to restricted bipotential neuron/oligodendrocyte progenitors in vivo, has more recently been demonstrated to be false. Indeed, it has been shown that *Olig2* is expressed not only in astrocytic cells but also in neural stem cells, thus precluding its use as a specific marker for "fate-restricted" neuron/oligodendrocyte progenitors (Liu and Rao, 2004). Based on the incorrect assumption that *Olig2* is never expressed in astrocyte progenitors or neural stem cells, this study (Gabay et al., 2003) provides little insight into the effects of culture on the fundamental properties of neural precursors.

Another recent study suggested that transit-amplifying C cells could be reprogrammed to a stem cell phenotype when exposed to EGF in vivo (Doetsch et al., 2002). However, there are a number of unresolved issues concerning this study. For example, Doetsch et al. utilized Dlx2 as a marker of rapidly proliferating progenitors, but only 1% of the Dlx2-positive proliferative cells generated neurospheres. This small population is more consistent with the known parameters of the stem cell population than the transit-amplifying population. Moreover, Dlx2 is not specific to the transit-amplifying population but may also be expressed by the stem cell population (Hitoshi et al., 2002a). Most importantly, an earlier paper (Morshead et al., 1994) established that when the rapidly proliferating transit-amplifying progenitor cells were selectively killed in vivo, there was no effect on the number of neurospheres that formed in vitro, illustrating that it is the relatively quiescent stem cell population and not the transit-amplifying population that forms neurospheres. The present study found no difference in the number of neurosphere colonies generated from any region, regardless of whether EGF (or serum) was included in the culture media or not. Further, the results obtained from these studies do not rely on the culture technique utilized; it has been shown that for the investigation of cellular properties such as potentiality and self-renewal, the identical results are obtained regardless of whether adherent (monolayer) or non-adherent (neurosphere) conditions are utilized (Seaberg and van der Kooy, 2002), or whether cultures include serum or are serum-free (Cayouette et al., 2003; Seaberg and van der Kooy, 2002).

Previously, it was reported that oligodendrocyte precursor cells were able to generate only two cell types: type 2 astrocytes and oligodendrocytes (Raff et al., 1983). To some extent, our findings regarding the intrinsic properties of

proliferative early postnatal optic nerve cells contrast with a more recent study (Kondo and Raff, 2000), which reported that oligodendrocyte precursor cells needed to be exposed to various extracellular signals in a regimented, stepwise culture protocol to "reprogram" the cells to an astrocytic phenotype capable of exhibiting multipotentiality and self-renewal. Here, by assaying the characteristics of cells present in primary tissue isolates, we provide evidence that this reprogramming and long-term culture is unnecessary, and suggest instead that a population of multipotential cells exists normally in the unpurified, unmanipulated early postnatal optic nerve.

Similar to the PND1 and PND10 subependymal neural stem cells isolated from around the lateral ventricles, adult cells from this region include a subset that displays proliferation, multipotentiality, and self-renewal as well as in vitro longevity (Morshead et al., 2002) and in vivo longevity (Tropepe et al., 1997). Interestingly, the initially multipotent early postnatal precursors from most other neurogenic and non-neurogenic regions become restricted in vitro to an exclusively glial-producing phenotype, which is similar to the phenotype that is seen when proliferative cells are isolated directly from the adult. This suggests that similar processes occur in vitro and in vivo to restrict the lineage potential of neural progenitor cells.

Because the in vitro and in vivo environments are profoundly different, perhaps transient neurogenesis is an intrinsic property of many postnatal progenitors and is not a product of the cellular environment. Abundant evidence in the present paper supports this idea. First, in spite of the maintenance of culture conditions throughout the passaging procedure, the progenitor cells' capacity to generate neurons changes over time. For the first passages, in vitro cells can maintain their neurogenic capacity, but this characteristic is lost over the next few passages despite the constant in vitro environment. Second, over a similar time course, the loss of neurogenesis occurs both in vitro over multiple cell passages and in vivo if the cells are allowed to remain in situ until adulthood. This also argues for a cell-autonomous mechanism of neurogenic restriction, because even in two diverse environments, the same restriction of progenitors is observed. While we provide no direct evidence that we are sampling from continuous cell lineages at these different ages (early postnatal and adult), this remains a provocative correlation. Third, markers of both neural precursors (Sox1, Sox2, Sox3, Musashi-1, Olig1, and Olig2) and neuronal precursors (Mash-1, and NeuroD) are expressed in undifferentiated sphere colonies from all brain regions tested, regardless of whether they were from primary or high passage cultures. Finally, direct manipulation of the culture environment using conditioned media and stem cell coculture experiments allowed the investigation of whether there was a non-cell autonomous signal that was positively mediating neurogenic capacity in multipotential cells. The majority of postnatal progenitors were unaltered by these procedures, although there was a modest effect on precursors from the olfactory bulb. This result served as a positive control for our manipulations and also suggests that perhaps a stable stem cell phenotype is not established in the early postnatal olfactory bulb, but that these cells are at a stage where they require a stem cell environment to maintain their potentiality. This stable phenotype is acquired by adulthood as shown in the present study and elsewhere (Gritti et al., 2002). However, it is also possible that bona fide neural stem cells simply do not migrate into the olfactory bulb until a later stage of development than the early postnatal ages assayed in the present study, and that the only proliferative cells available for assay at PND1 and PND10 are progenitors.

What emerges from these experiments is a more comprehensive picture of the fundamental cellular characteristics of diverse postnatal progenitors. In contrast to subependymal stem cells, these novel progenitors from other postnatal brain regions exhibit stem-like properties that gradually become restricted in a time- and brain region-dependent manner, both in vitro and in vivo (Table 3). At least three intrinsically multipotential subpopulations exist in the early postnatal brain; these can be differentiated clearly at PND10. A population of neural stem cells resides in the ventricular subependyma and exhibits long-term maintenance of proliferative ability, multipotentiality, and self-renewal, and two populations of transiently multipotential progenitors exist in other regions: populations that exhibit some self-renewal in vitro (optic nerve, cerebellum,

Table 3
Three types of intrinsically multipotential precursor cells can be isolated from the early postnatal (PND10) mouse brain

	1° Sphere multipotentiality	Longevity	
		Maintenance of multipotentiality	Passageability
Neural stem cells ventricular subependyma	+	+	+
Self-renewing progenitors cerebellum, optic nerve, dentate gyrus, olfactory bulb	+	–	+
Non-self-renewing progenitors cortex, striatum	+	–	–

Summary description of the characteristics of cells isolated from neurogenic and non-neurogenic early postnatal brain regions. Data from ventricular subependymal stem cells are shown for comparison. Note that a distinction can be drawn between neural stem cells and the transiently multipotential progenitors present in both neurogenic and non-neurogenic regions at PND1 and PND10, but it is only at PND10 that differences among these transiently multipotential progenitors emerge. A simplified version of the results is illustrated where "+" represents the presence of the characteristic in the subpopulations of cells isolated from each tissue dissection.

olfactory bulb, dentate gyrus) and populations that do not (cortex and striatum). This information regarding cellular properties that become evident in vitro may prove valuable when considering among neural precursors for cell replacement strategies.

Further investigation into the cellular and molecular mechanisms that enable the long-term maintenance of stem cell properties in ventricular subependymal cells but not in other brain regions is warranted and will doubtlessly provide significant insights into neural stem and progenitor cell biology. There are some hints in the current literature as to potential signaling pathways that may mediate the maintenance of stem cell properties. For example, it has recently been demonstrated that molecules in the Notch signaling pathway are critical for the long-term maintenance of neural stem cells in vivo and in vitro (Hitoshi et al., 2002b). It has also been suggested that BMP antagonist molecules such as Noggin play a role in maintaining an environment conducive to neurogenesis in the adult subependyma (Lim et al., 2000). Perhaps changes in these pathways also mediate the variable maintenance and neurogenic capacity of postnatal progenitors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.10.017.

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