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Timing of CNS Cell Generation: A Programmed Sequence of Neuron and Glial Cell Production from Isolated Murine Cortical Stem Cells

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Summary

Multipotent stem cells that generate both neurons and glia are widespread components of the early neuroepithelium. During CNS development, neurogenesis largely precedes gliogenesis: how is this timing achieved? Using clonal cell culture combined with long-term time-lapse video microscopy, we show that isolated stem cells from the embryonic mouse cerebral cortex exhibit a distinct order of cell-type production: neuroblasts first and glioblasts later. This is accompanied by changes in their capacity to make neurons versus glia and in their response to the mitogen EGF. Hence, multipotent stem cells alter their properties over time and undergo distinct phases of development that play a key role in scheduling production of diverse CNS cells.

Introduction

The formation of the nervous system in most invertebrate and vertebrate species involves a fundamental cell diversification—the generation of neurons and glia. For a given species, the timing of appearance of neurons and glia in the developing nervous system is highly reproducible, and a precise schedule is critical for organizing the normal cytoarchitecture. In the mammalian central nervous system (CNS), neurons are generated primarily in the embryonic period, while most glia are generated after birth (Jacobson, 1991). In the mouse or rat cerebral cortex, for example, neurogenesis commences around embryonic day 12 (E12), peaks around E15, and finishes around birth (Bayer and Altman, 1991; Jacobson, 1991). While the transient radial glia are present at early stages, macroglial production in the cortex does not start until midgestation and then only at low levels (Abney et al., 1981; Skoff, 1990; Cameron and Rakic, 1991; Misson et al., 1991; Levison et al., 1993; Parnavelas, 1999). Cortical astrocytes are first detected around E16 and oligodendrocytes around birth, but the vast majority of both cell types are produced during the first postnatal month. The temporal separation of cell production allows the neuronal population to be well established before the glial system develops. This ensures, for example, that most axons are elaborated before the appearance of the oligodendrocytes that wrap them and that the number of glial cells generated can be appropriately balanced to the extant neuronal population (Burne et al., 1996; Calver et al., 1998; Barres and Raff, 1999). Despite the fact that the separate timing of neurogenesis and gliogenesis in the CNS has been described for many years, the mechanisms involved remain largely unknown. This issue becomes particularly intriguing when we consider that CNS neurons and glia can arise from multipotent progenitor cells (Temple and Qian, 1996; McKay, 1997; Rao, 1999; Gage, 2000). We have investigated how neuron and glial cell production by multipotent cells is regulated over time by analyzing the development of stem cells derived from the embryonic mouse cerebral cortex.

The cerebral cortex arises largely from germinal cells in the cortical ventricular zone (Davis and Temple, 1994; Kilpatrick et al., 1995), with a contribution of GABAergic interneurons that migrate from basal forebrain areas (Anderson et al., 1997; Tan et al., 1998; Lavdas et al., 1999). At an early stage of development (E10), the murine cerebral cortex is a single layer of germinal neuroepithelium comprised predominantly of dividing progenitor cells. In clonal culture, most isolated E10 cortical cells make clones containing neurons only, while ~10%–20% of the cells generate large mixed clones of neurons and glia and behave like stem cells described in other systems. A minor percentage (<1%) of cells in the early murine cortex generate solely glia, and these tend to give small clones (Davis and Temple, 1994; Qian et al., 1997; Shen et al., 1998). Therefore, multipotent stem cells appear to be a major source of glia in the early cortical neuroepithelium. Consequently, the transition from neuron to glial cell generation in the cerebral cortex is likely to involve this multipotent cell. Given the widespread occurrence of multipotent, neuron-glial progenitor cells (Temple, 1989; Reynolds et al., 1992; Johe et al., 1996; Mayer-Proschel et al., 1997), this cell class is likely the focus of timing mechanisms in other parts of the mammalian CNS.

Multipotent stem cells in the cerebral cortex, like those in other areas, produce restricted progenitor cells for neurons and glia (neuroblasts and glioblasts) (Davis and Temple, 1994; Mayer-Proschel et al., 1997; Mujtaba et al., 1999). We have examined two possible models by which sequential generation of neurons and glia could be achieved. In one, stem cells produce different types of progeny in distinct phases: first neuroblasts and then glioblasts. In this case (Figure 1A), a change in stem cell behavior is critical for the timing process. In an alternative model (Figure 1B), stem cells generate neuronal and glial progenitor cells randomly, and the timing of appearance of differentiated progeny would be determined not at the stem cell level, but by regulating the restricted progenitor cells.

To test these two models, it is necessary to follow the sequence of neuron and glial cell production from a

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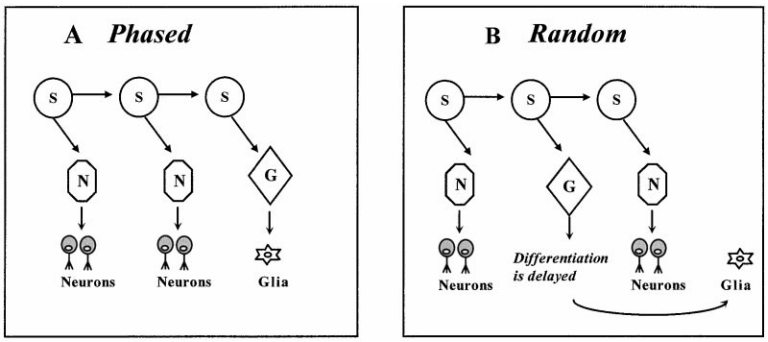

Figure 1. Models of Sequential Production of Neurons and Glia from Multipotent Cortical Stem Cells

In model (A), multipotent stem cells generate neuronal progenitor cells first and glial progenitor cells later; hence, changes in stem cell output are important to the timing process. In model (B), multipotent stem cells generate neuronal and glial progenitor cells randomly. The properties of the restricted progenitor cells and the environment of the developing cerebral cortex determine the time of appearance of neurons and glia. Note that in these models only the initial, asymmetric cell divisions via which stem cells produce restricted neuroblasts or glioblasts are illustrated. Further divisions of these restricted progenitor cells are not drawn.

single multipotent stem cell. Retroviral marking of single, dividing ventricular zone cells in vivo has allowed analysis of clone composition at specific points in development (Luskin et al., 1988; Kornack and Rakic, 1995; Reid et al., 1995; Mione et al., 1997). However, this technique cannot assess how clonal progeny are generated over time. In addition, experiments conducted in vivo do not allow segregation of cell-intrinsic and environmental mechanisms regulating ventricular zone cell development. By culturing individual ventricular zone cells at clonal density, we have been able to study these aspects of their development. Using long-term time-lapse video microscopy, we have observed how individual cortical progenitor cells, including restricted neuroblasts and glioblasts (Qian et al., 1998), and multipotent stem cells (the present study) generate progeny, identified by immunohistochemistry, over time. This allows reconstruction of the lineage trees of individual cortical progenitor cells, similar to lineage analyses conducted in invertebrates (Sulston and Horvitz, 1977; Sulston et al., 1983; Doe and Goodman, 1985; Schmid et al., 1999). Comparison of the lineage trees of many cells growing in a defined condition can reveal repeated division patterns. Furthermore, since different cells are grown at clonal density but under the same culture conditions, we can assess the intrinsic properties of the original single cells.

Our data show that cortical stem cells alter their output with time and generate neurons before glia, supporting the first model (Figure 1A). In addition, the division mode utilized by the restricted progenitor cells contributes to the timing process so that aspects of both models are important. Finally, we found that developmental changes occur in cortical stem cells that may influence the types of progeny they generate.

Interestingly, while multipotent stem cells have been isolated from a number of vertebrate tissues, such as blood, neural crest, and various CNS regions, their lineage trees have not been described. Hence, this represents the first study of the division patterns of a vertebrate multipotent stem cell. Our data illuminates a new possible mechanism for generating diverse progeny from a stem cell, by phasing the types generated. This mechanism may be used, for example, by multipotent stem cells that generate particular cell types at different times during development or during the regeneration/maintenance of a complex tissue.

Results

The Time Course of Neuron and Glial Cell Development in the Mouse Cerebral Cortex In Vivo

Previous birth-dating studies indicate that most cerebral cortical neurons are born before glia in vivo (Bayer and Altman, 1991; Jacobson, 1991). To quantify this, we examined the numbers of neurons and glia in acutely isolated cell suspensions from E10-postnatal day 7 (P7) mouse cortex, identifying cell types immunohistochemically using the markers β-tubulin III for neurons, O4 for immature oligodendrocytes, nestin for progenitor cells, and GFAP for astrocytes. The results are shown in Table 1 and the first vertical panel of Figure 2. As expected, at E10 most cortical cells stained for nestin. At this age, the only differentiated marker present was β-tubulin III, seen in ~9% of the cells. No E10 cells stained for glial markers, and macroglia were not detected for many days. The first O4-positive oligodendrocytes were seen at E17, but only 0.37% of cells labeled, and a low level was maintained through birth. The first GFAP-positive cells were detected at E17, and these too remained extremely rare, comprising <2% of cells, until after birth. These data are consistent with those obtained for the rat, indicated in italics in Table 1 (Abney et al., 1981). The first galactocerebroside (GC)-positive oligodendrocytes were found neonatally in the rat at a very low level (0.9% of the cell suspension at P3–P4; GC is expressed a few days after O4 [Gard and Pfeiffer, 1990]). GFAP expression is similarly low in the rat from midgestation through birth (0.01%–1.8% of cells). Hence, in regard to differentiated progeny, neurons dominate the environment of the embryonic cortex, while almost all glial cell differentiation occurs postnatally.

Neurons Arise before Glia in Isolated Stem Cell Clones

In previous studies we showed that cortical stem cells grown in serum-free, astrocyte-meningeal cell conditioned medium could generate neurons, astrocytes, and oligodendrocytes (Davis and Temple, 1994; Temple and Davis, 1994). Neurons appeared to be the first differentiated cells to arise, but this observation was based on morphology rather than cell-type-specific markers. To determine the sequence of cell generation in stem cell clones more precisely, isolated E10 cortical cells were

Table 1. In Vivo, Cortical Neurons Differentiate during the Embryonic Period and Glia Largely during the Postnatal Period

Antibody	E10	E11	E12	E14	E16	E17	E18	P0	P3–P4	P7
Nestin	97.9	95.46	77.58	37.97	7.87	10.67	ND	6.48	22.01	ND
β-tubulin	9.3	13.96	63.95	62.91	ND	72.95	77.67	50.35	59.34	43.73
O4/*GC*	0	0	0	0	0	0.37	0.35	0.22	0.55	5.57
								0	*0.9*	
GFAP	0	0	0	0	0	1.22	2.4	1.58	4.58	8.91
				0.01	*0.2*		*1.8*			

Acutely isolated mouse cortical cells (table shows percentages) from different ages were stained with cell-type-specific markers to indicate the extent of neuronal or glial differentiation in the population. Most of the cortical cells present during the embryonic period were progenitor cells or differentiated neurons. While some astrocytes and oligodendrocytes were detected at around E17, their incidence remained low throughout the embryonic period, similar to levels reported previously for the rat (indicated in italics, estimating mouse embryonic age as 1 day less than rat) (Abney et al., 1981).

plated at clonal density (1–5 cells per well) in poly-l-lysine-coated Terasaki wells in serum-free, conditioned medium with 10 ng/ml FGF2 added as a mitogen. After 1 hr to 14 days of culture, clones were fixed and stained. As shown in Figure 2, neurons were detected acutely and continued to develop in number and complexity throughout the culture period. Even by 6–7 days in vitro, the majority of stem cell clones contained neurons and undifferentiated cells, but no astrocytes or oligodendrocytes (Table 2). Of the 21 stem cell clones that were assessed at 6 days, only one (4.7%) contained glia (2 GFAP-positive astrocytes, but no O4-positive oligodendrocytes). By 10 days in culture (equivalent to P2), the majority of stem cell clones contained both neurons and

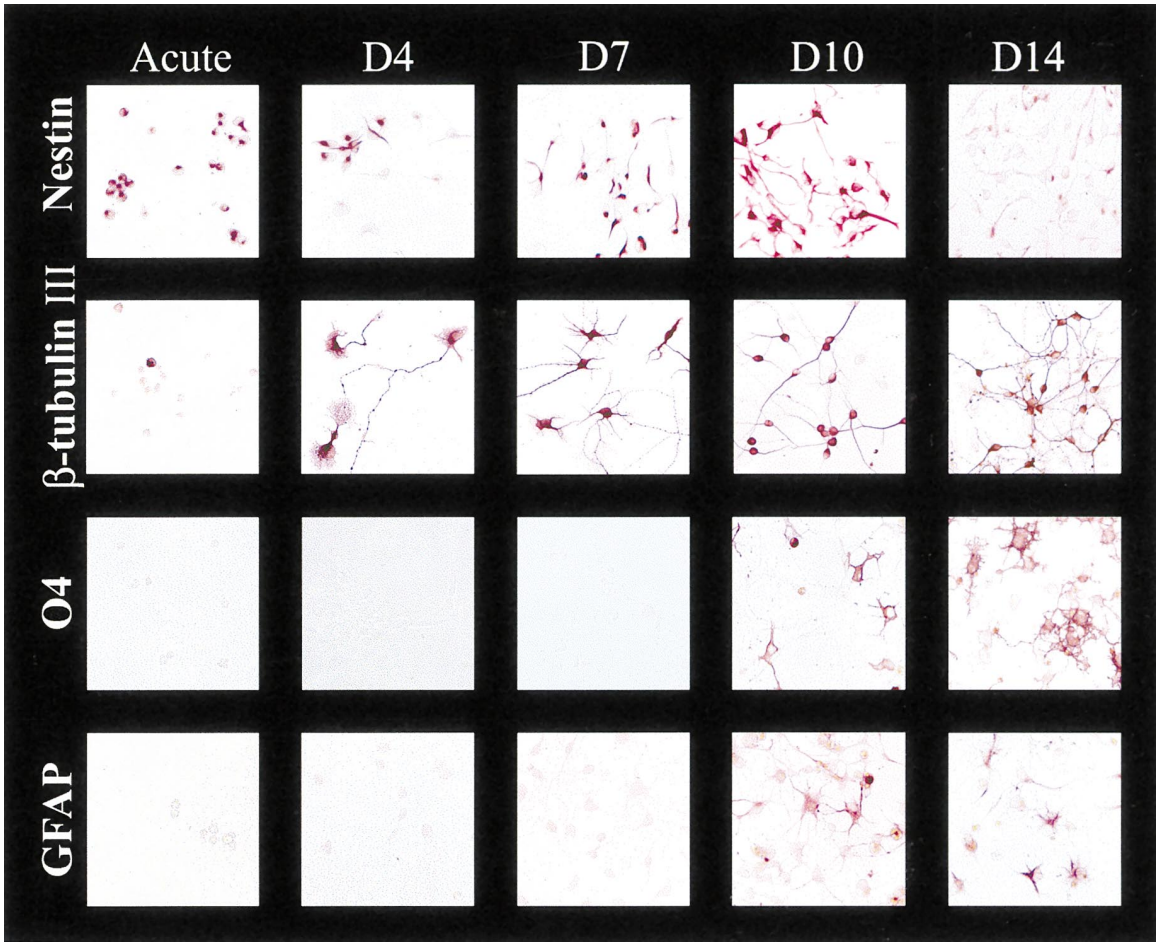

Figure 2. E10–E11 Mouse Cortical Stem Cells Growing in Clonal Culture Generate Neurons before Glia

Progenitor cell clones derived from E10–E11 mouse embryos were fixed and stained with cell-type-specific markers at different times after culture, from 1–4 hr after plating (acute) to 14 days. Neurons (indicated by β-tubulin staining) appear early, while glia (indicated by O4 for oligodendrocyte lineage cells and GFAP for astrocytes) appear days later. Hence, even when grown at clonal density, cortical stem cell clones generate morphologically and antigenically identifiable neurons days before differentiated glia. Scale: 1 cm = 87.12 μm.

Table 2. Neurons Arise before Glia in E10 Cortical Stem Cell Clones

	Total Clones	Average Clone Size	Total Number of Clones Containing		
			Neurons	Astrocytes	Oligos
Day 3	19	5	16	0	0
Day 6	21	36	21	1	0
Day 10	23	143	22	21	16

E10 stem cells were cultured in serum-free basal medium supplemented with 10 ng/ml FGF2, and astrocyte-meningeal cell conditioned medium. Under these culture conditions, cortical stem cells generate neurons, astrocytes, and oligodendrocytes (Davis and Temple, 1994). Stem cell clones were fixed and stained at day 3, 6, and 10 after plating. Morphologically and antigenically recognizable neurons were detected in stem cell clones at the day 3 time point. In contrast, glial cells appeared later—astrocytes were first detected at day 6 and oligodendrocytes at day 10.

glia—of 23 stem cell clones examined, >90% contained GFAP-positive astrocytes and >70% contained O4-positive oligodendrocyte-lineage cells. When conditioned medium was omitted and E10 cortical stem cells were grown in serum-free medium with 10 ng/ml FGF2 alone, we again found that neurons arose ~6–7 days before glia, but the first glia detected were O4-positive oligodendrocytes (data not shown). The fact that the normal temporal order of cell production—neurons before glia—occurs in clones developing from isolated cells in culture suggests that single embryonic cortical stem cells have the capacity to reconstitute the normal timing program, even outside the developing CNS.

Time-Lapse Video Recording of Multipotent Cortical Stem Cell Development Shows Stem Cells Produce Neuroblasts First and Glioblasts Later

As described in the introduction, the sequential appearance of differentiated neurons and glia could be explained by one of the two models shown in Figure 1. Determining which, if either, of these two possibilities is correct requires an understanding of how multipotent cells generate their progeny over time. E10–E11 cortical progenitor cells were plated at clonal density in Terasaki wells in serum-free medium plus 10 ng/ml FGF2. Selected clones were continuously recorded for up to 7 days using time-lapse video microscopy, and then fixed and stained with cell-type-specific markers. The lineage trees of stem cell clones were reconstructed and seven representative lineages are illustrated in Figure 3. In a previous study, we showed that neuronal progenitor cells typically used asymmetric lineage trees to generate progeny, similar to those used by invertebrate neural progenitor cells (a typical example of a mouse cortical neuroblast lineage is shown in Figure 3A). In contrast, restricted glial progenitor cells used division patterns that at least at early stages appear symmetric (Qian et al., 1998). As seen in Figures 3B–3H, multipotent cells employ a combination of these two patterns.

In Figure 3, cells that were identified as neurons using β-tubulin III staining are indicated by an "N," while cells that did not stain have a minus sign designation. All the stem cell lineage trees we reconstructed had a similar pattern of development, with neurons being made first. For example, in Figure 3F, the first division of the stem cell generates a neuroblast that gives two neurons and the second division generates another neuroblast that gives four neurons. At the third division, the stem cell divides asymmetrically again to yield one daughter that produces eight, mostly neuronal progeny, and another that makes eight β-tubulin III–negative cells. Of the β-tubulin III–negative cells that appeared later in stem cell clones, most had the appearance of glial progenitor cells: undergoing initially symmetric divisions, having a more rapid cell division rate, and being highly motile. The majority of cells identified as glial progenitors in these clones stain with the early glial marker NG2, which does not label neurons (Levine and Nishiyama, 1996). This is illustrated in Figure 4—a stem cell produced two neurons and then a large number of typical glial progenitor cells that are β-tubulin III negative but positive for NG2, as well as a small number of β-tubulin III–negative, NG2-negative progenitor cells (arrowheads). The distinct division patterns of stem cells is seen by comparing a typical neuroblast lineage (Figure 3A) to stem cell lineages (Figures 3B–3H). Note that late-born cells in the neuroblast lineage are β-tubulin III positive, whereas cells in a similar position in stem cell lineages are negative. Note also that in neuroblast lineage trees the number of progeny made diminishes with time, while the stem cell lineage trees expand at late stages as glioblasts begin to proliferate. The neuroblast shown in Figure 3A makes many more neurons than the stem cells illustrated. Some E10 stem cells are capable of producing more neurons (as described later), but for this study we focused on stem cells that made both neurons and glia within the recording period, and these made fewer neurons. It is possible therefore that neuroblasts making large clones are offshoots of early stem cells making many neurons. Alternatively, they may represent separate lineages from the outset. Nevertheless, the example chosen illustrates that neuroblasts growing for a similar culture period generate neurons at late points in the lineage, unlike the stem cells we followed, strengthening our conclusion that the late cells in stem cell lineages are glial rather than undifferentiated neurons. Because of the large numbers of glial progenitor cells and their highly motile nature, it was impossible to follow them to their final differentiated state using the time-lapse system. However, in sister clones these cells continued to divide for days and differentiated into numerous oligodendrocytes and astrocytes. Most (93.2% at 10 days in vitro) of the astrocytes generated were of the flattened, A2B5-negative, type 1 morphology (Figure 2; Raff, 1989).

In some cases, a stem cell generated just one neuroblast before making glia (Figure 3C); here neurons and glial cells diverge at the first asymmetric division. In

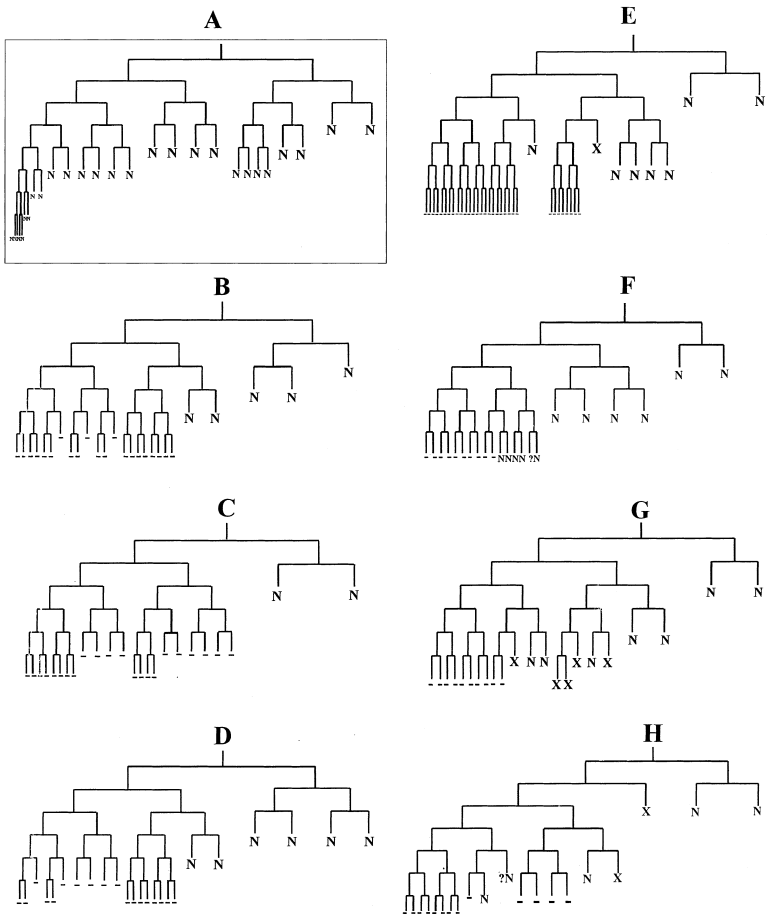

Figure 3. Time-Lapse Lineage Trees Show that Multipotent Cortical Stem Cells Generate Neuronal Progenitor Cells before Glial Progenitor Cells

E10–E11 mouse cortical stem cells were grown in serum-free, basal medium with 10 ng/ml FGF2. Clonal development was continuously recorded using time-lapse video microscopy for up to 7 days. At the end of the recording period, clones were stained using cell-type-specific antibodies, and the lineage trees were reconstructed from the recorded images. In all the stem cell clones recorded, including the examples shown in (B)–(H), neurons were generated before glia. The complete lineage trees could not be reconstructed because stem cells generate hundreds of progeny. However, the initial divisions of the glioblasts appeared symmetric. The boxed lineage shown in (A) is that of a restricted neuroblast. N = neuron, ?N = cell with neuronal features that were lost or died before staining was complete, minus sign = cell that did not stain for β-tubulin III and had the characteristics of a glial progenitor cell, x = cell that died.

other cases, as in Figure 3E, the divergence of neurons and glia occurs at multiple points in the lineage tree. As described above, the change from neuron to glial cell production is accompanied by an alteration in division pattern from an asymmetric mode to one that is more proliferative. Although the early cell divisions in the gliogenic phase appear overtly symmetric, we know that some asymmetric cell divisions occur because the stem cell self-renews during this period (see later section) and because both oligodendrocytes and astrocytes arise.

Hence, in cortical stem cell clones, neurons not only differentiate early, they are born early. These data support the model shown in Figure 1A, in which the stem cell alters the types of progeny it produces over time. Figure 1A only shows the initial asymmetric divisions of the stem cell that produce neuroblasts or glioblasts; in

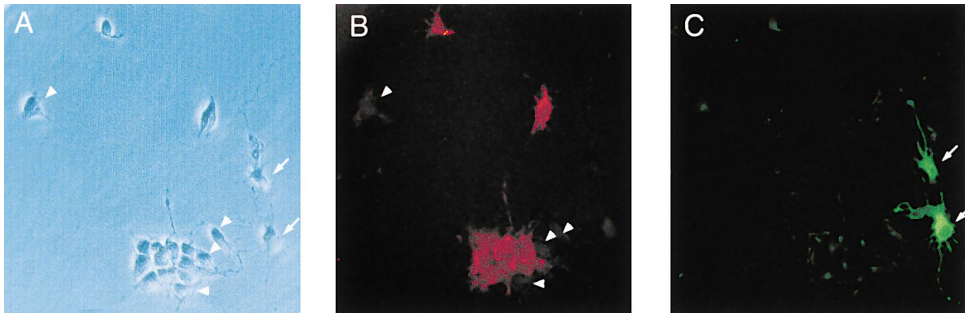

Figure 4. Identification of Neurons and Glial Lineage Cells within Stem Cell Clones

After time-lapse analysis, clones were fixed and stained with cell-type-specific markers. This clone made two neurons before switching over to glial cell generation.

(A) Phase image of the clone.

(B) The glial progenitor cells stain with NG2 antibody, which stains glial lineage cells (Levine and Nishiyama, 1996). A small number of clonal progeny do not stain for NG2 or β-tubulin III (arrowheads).

(C) Staining for β-tubulin III reveals the two neuronal daughter cells of the clone (arrows). Scale: 1 cm = 53.82 μm.

our recorded lineages, these restricted progeny are then able to divide further, generating neurons or glia. Even in cases where a stem cell produced just one neuroblast before generating glioblasts (e.g., Figure 3C), neurons still appeared days before differentiated glia because of the different division patterns and mitotic potentials of the neuronal and glial progenitor cells. Neuroblasts arise first and undergo just a few divisions before generating differentiated progeny. Glial cells arise later because glioblasts are generated after the majority of neuroblasts and undergo a series of proliferative divisions that effectively delay the appearance of differentiated progeny. Thus, both the order of cell production from stem cells and the division mode of individual progenitor cells contribute to the early appearance of neurons and the later appearance of glia in stem cell clones.

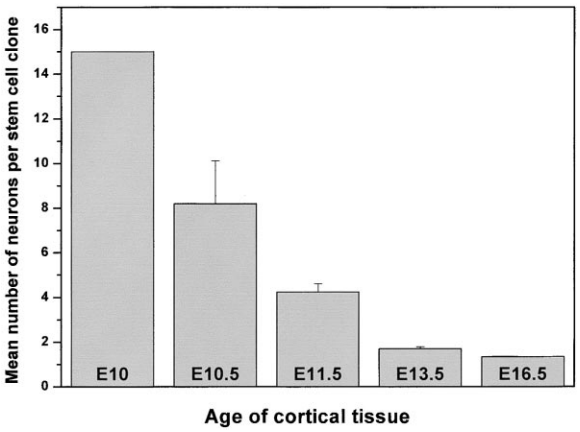

Figure 5. Cortical Stem Cells Produce Fewer Neurons with Age

Cortical stem cells from E10–E16.5 were plated at clonal density under identical conditions: serum-free basal medium containing 10 ng/ml FGF2 in poly-l-lysine-coated Terasaki plates. The cells were allowed to grow for 7 days, and then the clones were fixed and stained for neurons and glia using cell-specific markers. At E10, the number of neurons made per stem cell clone varies widely from 1 to ~100, with an average of around 15. The average number of neurons made by stem cells declines with age to 1.7 per clone at E16.5. Note that glia-only clones were not included in this figure.

The Neurogenic and Gliogenic Capacities of Cortical Progenitor Cells Change with Time

The observed behavior of cortical stem cells could be explained by two different mechanisms. In one, the stem cell changes as the clone develops so that it initially has a high probability of generating neurons, which reduces with development, and a low probability of generating glia, which increases with development. Environmental changes, cell-intrinsic changes, or a combination of both could alter the neurogenic and gliogenic potential of the stem cell. Alternatively, the potential of the stem cell to generate neurons and glia could remain unchanged during development, and the sequence of cell production could be purely environmentally determined. These two mechanisms can be distinguished by exposing stem cells from different ages to the same environmental conditions. In the first case, we predict that stem cells from early embryonic ages will make more neurons and have a lower propensity to make glia than those derived from later ages. In the second case, stem cells from different ages will make the same number of neurons and glia generation should be unchanged.

To examine their neurogenic potential, we compared stem cells from E10–E16.5 cerebral cortices growing in a standardized environment: serum-free medium containing 10 ng/ml FGF2. As shown in Figure 5, the number of neurons generated per stem cell clone differed dramatically with age. The average number of neurons made by E10 cells is 15, whereas at E16.5 the average is 1.7. Interestingly, a similar result was obtained when restricted neuroblasts rather than stem cells were examined. In one experiment, E10 neuroblasts made an average of 12 neurons in 10 ng/ml FGF2, whereas E12 neuroblasts produced on average only 5 neurons under identical culture conditions.

To examine the gliogenic potential of stem cells, we compared the composition of E10 and E12 cortical progenitor cell clones that were exposed to different concentrations of FGF2 during the culture period. We showed previously that changes in the concentration of FGF2 could regulate the appearance of glia from E10 cortical stem cells (Qian et al., 1997). When grown in 0.1 ng/ml FGF2, E10 stem cells generate neurons, but most do not generate glia. In contrast, in 1 or 10 ng/ml FGF2, E10 stem cells generate the same number of neurons as in 0.1 ng/ml FGF2, but also generate glia. As shown in Figure 6, in each FGF2 concentration tested, the percentage of clones containing glia is higher for E12 cells than for E10 cells. Moreover, the percentage of glia-only clones increased dramatically with age. E10 cells grown in 0.1 ng/ml FGF2 had 0% glia-only clones. This increased to 15% when clones were grown in 10 ng/ml FGF2. In contrast, the percentage of glia-only clones seen in cultures made from E12 cells was significantly higher—20% in 0.1 ng/ml FGF2 and 75% in 10 ng/ml FGF2. This suggests that more cells are committed to making solely glia in the E12 cortex than in the E10 cortex. Why the incidence of glia-only clones increases with increasing FGF2 concentration is not clear. Perhaps there is a subpopulation of glial progenitor cells that requires a higher level of FGF2 to divide. Alternatively, perhaps some of the cells that at low concentrations of FGF2 give neurons as well as glia are switched to generate solely glia at the higher FGF2 levels. If this were the case, then these data suggest that E12 cells can be pushed more easily to produce solely glia than E10 cells.

Hence, cortical ventricular zone cells vary significantly with developmental age. E10 cells make more neurons than those of older ages, which have a greater tendency to make glia than younger cells do. These results indicate that cortical progenitor cells, including the stem cell population, change as development proceeds so that the descendents of the early cortical cells differ from their parents. An alternative explanation for these findings is that the stem cells we examined from older cortices do not represent the descendants of the E10 stem cells but are lineally separate. In this case, early stem cells with high neurogenic potential and low gliogenic potential would die out or become undetectable, while a separate population of stem cells with lower neurogenic potential and high gliogenic potential would

emerge. To examine this issue, we allowed early stem cell clones to grow for 3–4 days until they reached the 20–30 cell stage and then subcloned them as single cells into microwells. The stem clones that emerged contained on average only two neurons per clone. This demonstrates that the descendants of early stem cells have a reduced neurogenic potential. Hence, while it is still possible that changes in the proportions of different stem cell and progenitor populations occur in vivo, our data indicate that cortical stem cells change with time, an explanation that is consistent with our time-lapse data.

After Making Neurons and then Glia, Early Embryonic Cortical Stem Cells Acquire Characteristics of Postnatal Stem Cells

In invertebrates, the CNS progenitor cells, called neuroblasts, are asymmetrically dividing stem cells (Lin and Schagat, 1997; Fuerstenberg et al., 1998). After CNS development grasshopper neuroblasts die out (Bate, 1976). It is possible that embryonic stem cells in the cortex are similarly short lived and that after a period of neuron and glial cell production they are extinguished. However, stem cells have been detected around the forebrain lateral ventricles in adult mammals, and new neurons have been seen in the adult primate cerebral cortex (Gould, 1999; Temple and Alvarez-Buylla, 1999). Some of the adult forebrain stem cells may arise from embryonic cortical stem cells that continue dividing into adulthood. Adult stem cells differ from those in the embryo in a number of ways. For example, while E10 cortical or striatal stem cells do not divide readily in response to epidermal growth factor (EGF), this growth factor is highly mitogenic for forebrain stem cells at later embryonic through adult stages (Marmur et al., 1998; Tropepe et al., 1999; Zhu et al., 1999). To examine whether embryonic cortical stem cells self-renew through the phases of neurogenesis and gliogenesis and whether they acquire mature properties, we plated E10.5–E11 cerebral cortical stem cells at clonal density and allowed them to develop for 10 days, giving neurons, astrocytes, and oligodendrocytes (Figure 7A). The cells were then subcloned into bacteriological culture dishes containing serum-free medium with either 20 ng/ml EGF or 20 ng/ml FGF2—culture conditions that allow the development of neurospheres (an assay for stem cell function [Reynolds and Weiss, 1996]) from late embryonic to postnatal animals. After a further 7 days of culture, abundant neurosphere development was found in dishes containing either EGF (Figure 7B) or FGF2. This experiment indicates that cortical stem cells continue to maintain the capacity for self-renewal throughout the period of clone development and that they acquire a mature response to EGF. At 12 days, the neurospheres were dissociated, replated into poly-l-lysine-coated Terasaki wells, and stimulated to differentiate by mitogen withdrawal. All three types of CNS cells were found—neurons, astrocytes, and oligodendrocytes, as illustrated in Figures 7C and 7D. Hence, the neurosphere-generating cells that developed later in clonal cultures were still capable of producing neurons, even though this capacity was not revealed until they were removed from clonal culture and grown as neurospheres. Currently, the identity of the neurosphere generating cells is not known. The neurospheres may have been derived from NG2-negative, β-tubulin III–negative cells seen at late stages of clone development (Figure 4) or from a subpopulation of NG2-positive cells that might retain stem cell characteristics. It is also possible that they developed from glial-restricted cells that are able to reacquire neurogenic capacity under the conditions of neurosphere culture.

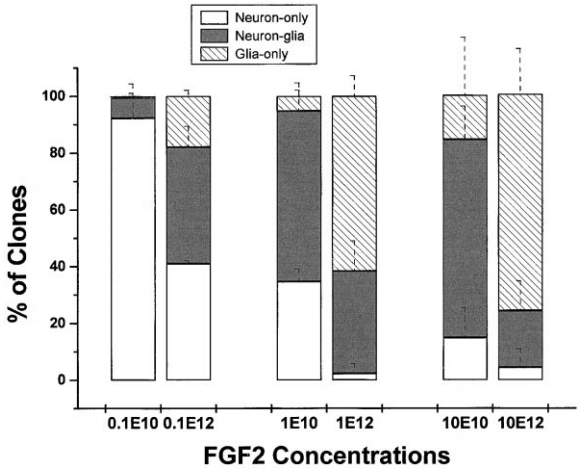

Figure 6. Cortical Progenitor Cells Increase their Tendency to Make Glia with Age

E10 and E12 ventricular zone cells were grown in serum-free medium with 0.1, 1, or 10 ng/ml FGF2 for 12 days, and then stained with cell-type-specific markers for neurons, astrocytes, and oligodendrocytes. The clones were divided into three groups: neuron-only, mixed neuron-glia, and glia-only. The percentage of each type of clone in each condition is presented; data represent the mean ± SEM for three independent experiments. More than 50 clones were analyzed in each condition. Note that more cortical progenitor cells at E12 than at E10 generate glia in the FGF2 concentrations tested.

Discussion

Clonal analysis and time-lapse video microscopy were used to gain insights into the timing of neuron and glial cell production from multipotent cortical stem cells, which are a major source of glia in the developing cerebral cortex. Our data demonstrate that cortical stem cells generate neuroblasts before glioblasts, accompanied by a switch from asymmetric to initially symmetric division patterns. We present evidence that cortical stem cells change during development, decreasing their neurogenic potential, increasing their gliogenic potential, and acquiring a mature response to the mitogen EGF. Our data indicate that the cortical stem cell plays a central role in timing neuron and glial cell production.

Programmed Timing Information within Cortical Stem Cells

Single multipotent cortical stem cells growing in a relatively sparse culture environment—serum-free medium on a poly-l-lysine-coated substrate—recapitulate the normal order of cell generation: neurons before glia. This was the case in all the stem cell clones analyzed—we did not find clones that produced astrocytes or oligo-

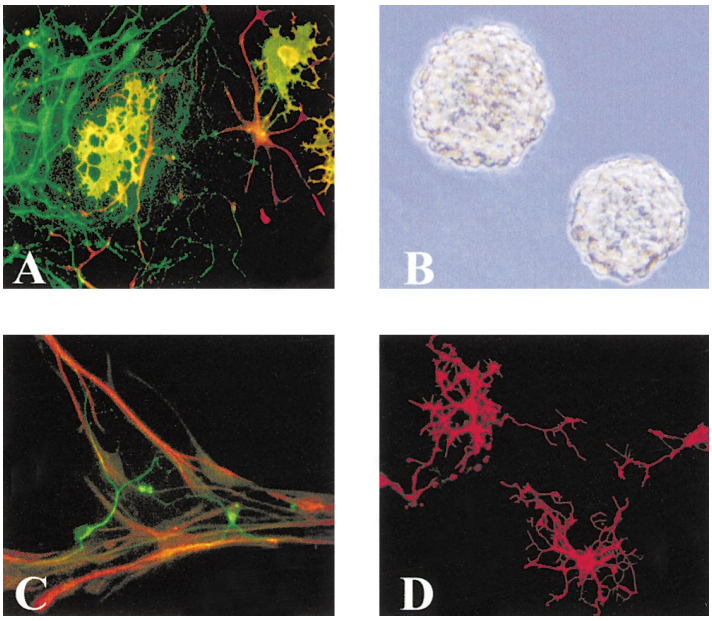

Figure 7. E10 Cortical Stem Cells make Neurons and then Glia, and Develop into Mature Stem Cells that Readily Make Neurospheres in Response to EGF

To examine whether E10–E11 cortical stem cell clones that had undergone neurogenesis and gliogenesis still contained active stem cells, they were allowed to develop for 10 days, subcloned, and assessed for neurosphere development. (A) A typical stem cell clone at the 10 day time point contains neurons (green), oligodendrocytes (yellow), and astrocytes (red). (B) The subcloned cells readily generated neurospheres in EGF. Dissociated neurosphere cells generated all three major CNS cell types as shown in (C) β-tubulin-positive neurons (green), GFAP-positive astrocytes (red), and (D) O4-positive oligodendrocytes. This demonstrates that cortical stem cells are maintained through late stages of clonal development and that they develop into a more mature form capable of responding to EGF. Scale: (A and B) 1 cm = 63 μm, (C) 1 cm = 56 μm, (D) 1 cm = 70 μm.

dendrocytes before neurons, or clones that generated alternating waves, e.g., neurons, then glia, then neurons, over the time course of the experiments. Hence, the information for the normal order of cell-type generation is programmed within single stem cells or by emerging interactions among their progeny, at very early stages. Furthermore, stem cells need not be present within the complex milieu of the developing brain in order for the timing schedule to be realized. This does not mean, however, that the environment is inconsequential to the timing mechanism: signals present within the CNS are likely to modulate programmed timing events.

Given the prevalence of multipotent stem cells in other regions of the developing CNS and the general order of early neuron and late glial cell production, a similar scheduling mechanism might operate elsewhere in the nervous system. For example, in the spinal cord, motor neurons and oligodendrocytes are stimulated to develop in the ventral region by the signaling molecule sonic hedgehog (Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996). Retroviral lineage data point to a common progenitor cell for these two cell types (Leber et al., 1990). Given that motor neurons are born before oligodendroglia in vivo, it has been suggested that the progenitor exhibits an order to cell generation. A hypothetical model for the schedule of motor neuron and oligodendrocyte production is very similar to the lineage trees we have observed for cortical stem cells (Richardson et al., 1997). Hence, these data on cortical stem cells may well be applicable to the behavior of stem cells in other regions of the developing CNS, such as the spinal cord.

The behavior of vertebrate cortical stem cells is reminiscent of that of invertebrate progenitor cells that can make both neurons and glia. For example, *Drosophila* CNS neuroblast 1.1 first produces a ganglion mother cell that makes neurons and generates glia later (Bossing et al., 1996). The midline neural progenitors in grasshopper also undergo temporal phases of neuron and glial cell production, in waves of neuron then glia then neuron generation (Condron and Zinn, 1994). These similarities highlight the possible conservation of underlying molecular mechanisms for phasing the generation of diverse cell types by stem cells, from invertebrates to mammals.

What Regulates the Switch from Neuron to Glial Cell Generation?

Stem cell behavior changes dramatically at the switch point, the asymmetric division at which it stops making neurons and starts making glia. How might the switch be regulated? FGF2, from neurons produced earlier in development (Qian et al., 1997), might act back on stem cells to promote the switch. Neural stem cells in vitro respond to a number of growth factors, including EGF, CNTF, PDGF, GGF2, and BMPs, by enhancing glial cell development (Kilpatrick et al., 1995; Cameron et al., 1998). It is likely that these and other as yet unidentified factors present in the complex in vivo environment will influence whether stem cells transit through the switch point or when the switch occurs.

The molecular mechanisms underlying the switch might involve genes important in specifying the glial fate and suppressing the neuronal fate, analogous to the actions of *gcm* and *tramtrack* in regulating neuron to glial cell production in *Drosophila* (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997). It will be interesting to examine how expression of the recently described basic helix-loop-helix proteins oligo-1 and oligo-2, which are initiated early in oligodendrocyte formation, relates to the switch point (Lu et al., 2000; Zhou et al., 2000).

Cortical Stem Cells Change during Development

One of the defining features of stem cells is self-renewal, the ability to make copies of themselves (Morrison, et al. 1997). This concept was established largely through studies on adults, in which the key function of stem cells is homeostasis, and maintaining their potential to make

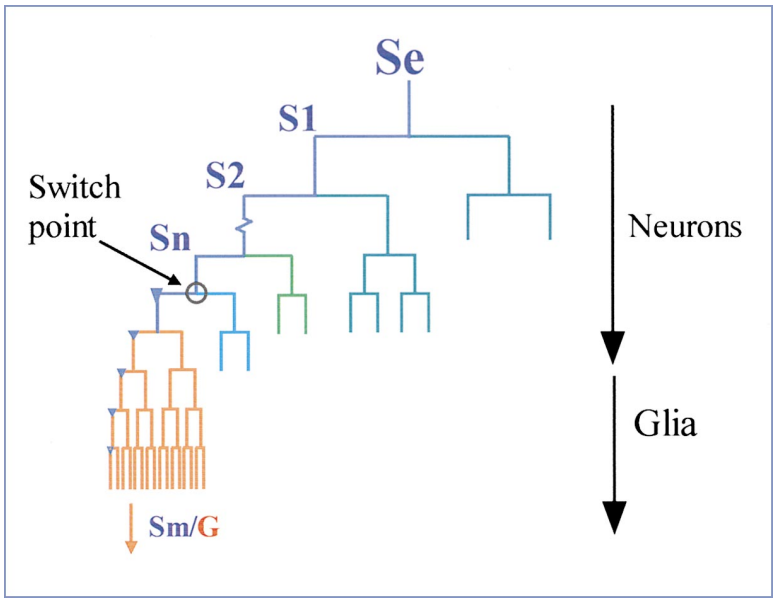

Figure 8. Model of Neuron-Glia Generation from Multipotent Cortical Stem Cells

Diagram illustrating our current working model of how cortical stem cells generate neurons and glia over time. As it develops, the embryonic stem cell (Se) changes (S1, S2…Sn), altering the progenitor cell types it makes with time. At early stages, it generates neuroblasts. Later, it undergoes a specific asymmetric division (the "switch point") at which it changes from making neurons to making glia. This can occur more than once in a stem cell lineage, although only one is shown in the model. Neurons arise first in stem cell clones because neuroblasts are generated before glioblasts and because neuroblasts undergo only a small number of divisions before they generate differentiated progeny, whereas glial progenitors undergo a large number of proliferative divisions before differentiating. Finally, the stem cell matures to a form (Sm) with properties appropriate to the postnatal environment. An alternative model is that at the switch point the stem cell becomes a glioblast that produces glial progeny, some of which (G) have the capacity to behave like stem cells in neurosphere-generating conditions.

a specific range of cell types makes sense. The major function of stem cells in developing systems, however, is not homeostasis, but cell production, and in the CNS, it is diverse cell production at defined times. Consequently, stem cells in developing systems may not be adapted to make exactly the same range of progeny over their sequence of divisions. In invertebrates, neuroblasts change as they divide, helping them produce a series of diverse ganglion mother cells (Cui and Doe, 1992). In vertebrates, there are few studies of stem cells in development. However, evidence indicates that stem cells in the developing blood system are distinct from adult blood stem cells (Lansdorp, 1995). Our data suggest that stem cells in the developing nervous system also change over time. Acutely isolated stem cells at various embryonic ages differ in their neurogenic and gliogenic capacities even when grown under identical conditions, indicating changes occur during development. Perhaps changes in growth factor receptor systems seen in the cortical ventricular zone (Temple and Qian, 1995; Burrows et al., 1997) occur within the stem cell population. Indeed, early embryonic stem cells allowed to develop in culture eventually acquire the EGF responsiveness characteristic of late embryonic and postnatal stem cells. These data indicate that cortical stem cells are not immutable and that, as suggested for invertebrate neuroblasts, changes in their properties may have important consequences for normal CNS development and lead eventually to a form adapted to the adult.

Given that the cerebral cortex produces an array of distinct types of neuronal cells in a predictable temporal order, it is tempting to speculate that changes in stem cells are involved in this process. Neurons arise from asymmetrically dividing neuroblasts and from stem cells making neurons and glia, both of which are isolated from E10 cortex. Whether these progenitor cells are lineally related via an earlier stem cell or represent discrete cell types is not clear at this stage. However, mechanisms of generating diverse neuronal types by altering the properties of an asymmetrically dividing progenitor might well operate in both progenitor types. Changes in stem cell behavior may involve both cell-intrinsic events and environmental signals. Feedback from early-born cortical cells influences the fate of later-born cells, perhaps acting through a multipotent progenitor population (McConnell, 1995). However, older cells are unable to generate as many types of neurons as younger cells (Frantz and McConnell, 1996), a restriction that may occur at the stem cell level.

In summary, we propose a model incorporating our observations and current hypotheses to explain the role of stem cells in timing cortical development (Figure 8). The early embryonic cortical stem cell (Se) undergoes asymmetric cell divisions, each generating a different restricted neuroblast and another, asymmetrically dividing stem cell (S1, S2, S3…Sn). The neurogenic potential of the stem cell declines so that it generates fewer neurons with age. The neurogenic potential may be enhanced or decreased by environmental factors, but at a certain point (the switch point), the stem cell stops making neurons and produces a highly proliferative progenitor cell that makes glial cells, provided that a high level of FGF2 or another suitable environmental signal is present. During this process, the stem cell matures to a form (Sm) that has a different response to growth factors than the early embryonic stem cell. An alternative model consistent with our current data is that at the switch point the stem cell changes into a glioblast that generates glial progeny, some of which have the potential to revert to a mature stem cell phenotype. Our observation that isolated stem cells make neurons before glia focuses our attention on these cells for continued exploration of cellular and molecular mechanisms that

control the stereotyped schedule of neural cell generation in the developing CNS.

Experimental Procedures

Preparation of Single-Cell Suspension of Cortical Ventricular Zone Cells

Mouse cerebral cortices were dissected, harvested in hibernation medium, and dissociated either manually, as described previously (Qian et al., 1997, 1998), or using papain (MacLeish and Townes-Anderson, 1988). For enzymatic dissociation, embryonic cerebral cortices were placed into 5 ml of prewarmed, filtered dissociation solution: 1 mM glutamine, 1 mM sodium pyruvate, 1 mM N-acetyl-cysteine (Sigma), 7 units/ml papain (Worthington), and 15 μl of 4 mg/ml DNase (Sigma) in DMEM (GIBCO). The suspension was bubbled with 95% O_2 and 5% CO_2 for 30 s. The tube was placed on a shaker at a 30° angle at low speed (15 rpm/min) for 30 min at room temperature. Tissues were rinsed three times with DMEM and gently dissociated by trituration. After settling 15 min, the top fraction, containing ~95% single cells, was collected.

Cell Culture

Dissociated cortical ventricular zone cells were plated at clonal density (1–10 cells/well) into poly-l-lysine-coated microwells in Terasaki plates. Each well contained 12 μl of basal serum-free culture medium—DMEM, B27, N2, Na pyruvate, glutamine (GIBCO), and 1 mM N-acetyl-cysteine (Sigma)—with 0.1, 1, or 10 ng/ml FGF2 (GIBCO). For some experiments, 50% serum-free conditioned medium from a neonatal astrocyte-meningeal cell culture (Temple and Davis, 1994) was added to basal medium with 10 mg/ml FGF2.

For subcloning experiments, cells were plated at clonal density into basal medium with 10 ng/ml FGF2 in poly-l-lysine-coated 35 mm tissue culture dishes and allowed to generate clonal progeny for 3–10 days. Clones were removed by trypsinization with 0.5 ml 0.05% trypsin-EDTA (GIBCO) at 37°C. Trypsin inhibitor was added, cells were dislodged by gentle pipetting, and collected by centrifugation at 150 g for 10 min. After resuspension in DMEM containing B27, N2, NAC, and 20 ng/ml bFGF or 20 ng/ml EGF (GIBCO), cells were plated. For subcloning at the 3–4 day stage, cells were replated into single wells by micromanipulation using a pulled glass micropipette, as described previously (Davis and Temple, 1994). For subcloning at 10 days to make neurospheres, cells in suspension were plated into uncoated 35 mm petri dishes at 500 cells per dish. Cultures were maintained in a tissue culture incubator at 35°C with 6% CO_2 and 100% humidity.

Time-Lapse Video Recording and Analysis of Isolated Cortical Ventricular Zone Cells

Time-lapse analysis was conducted as described (Qian et al., 1998). After recording, clones were fixed and stained with cell-type-specific markers: β-tubulin III (Sigma) for neurons, GFAP (Dako) for astrocytes, O4 and O1 for oligodendrocyte lineage cells, and NG2 for early glial lineage cells (Dr. Joel Levine).

Immunostaining of Acutely Isolated and Clonally Derived Cortical Neural Cells

Immunostaining for O4, O1, GFAP, and β-tubulin III was carried out as described previously (Qian et al., 1997, 1998), using Cappell fluorescent–conjugated secondary antibodies or a biotinylated secondary antibody followed by the ABC kit (Vector) with VIP as substrate. For NG2 staining, fixed cells were incubated in NG2 at 1:400 dilution for 1 hr at room temperature and then visualized with cy3-conjugated secondary antibody (Jackson).

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