

Late Origin of Glia-Restricted Progenitors in the Developing Mouse Cerebral Cortex

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In order to unravel the molecular determinants of cell fate, it is important to understand when fate restriction occurs during brain development. Lineage analysis suggested that bi- or multipotent progenitors persist into late developmental stages in some central nervous system regions, whereas most progenitor cells in the cerebral cortex appeared to be restrained to generate only a single cell type already at early stages. Here we discuss this previous work and present new data demonstrating that cortical progenitors generating exclusively glial cells appear late in development. In utero transduction of cortical progenitors at early and mid-neurogenesis using a combination of replication-defective retroviral vectors encoding different fluorescent proteins indicated that the early developing cortex is devoid of glia-restricted progenitors, although these are frequent during mid- and late neurogenesis. Clonal analyses in vitro using retroviral vectors and live cell tracking by video time-lapse microscopy confirmed these findings, revealing that the early developing cortex harbors 2 main progenitor types: neuron-restricted and bipotent (neuron–glial) progenitors. The latter are responsible for the generation of glial-restricted progenitors at mid- and late neurogenesis.

Keywords: clonal analysis, cortical development, gliogenesis, neurogenesis, video microscopy

Lineage Analysis in the Developing Cerebral Cortex

During development of the cerebral cortex, as throughout the nervous system, 3 main cell types have to be generated, namely, neurons and the 2 macroglial cell types, astrocytes and oligodendrocytes. Each of these cell types comprises a great diversity of subtypes, based on their distinct morphology, chemical properties, and functions (see e.g., Goldman and Vaysse 1991; DeFelipe 1993). This enormous diversity of cells is generated during a relatively short developmental period from progenitors located in the ventricular zone (VZ) and subventricular zone (SVZ) in both ventral and dorsal telencephalon (Götz and Huttner 2005; Miller and Gauthier 2007; Pontius et al. 2008), as well as from progenitors in the dorsal marginal zone (Costa et al. 2007). Birth-dating experiments using thymidine analogues have shown that most neurons are generated between the embryonic day (E) 12 and birth (around E20) in the rodent cerebral cortex, whereas glial cells are mostly generated at late embryogenesis (from E16 on) and first postnatal weeks (Angevine and Sidman 1961; Berry and Rogers 1965; Lent et al. 1990; Bayer and Altman 1991; Jacobson 1991). However, it is still unclear whether glial cells are generated from bipotent or multipotent progenitors present at

early stages in a sequential manner (for review, see e.g., Temple 2001; Miller and Gauthier 2007) or whether glia-restricted progenitors are set aside at early developmental stages as a distinct set of progenitors (for review, see Cameron and Rakic 1991; Richardson et al. 2006). This question is of broader relevance in regard to the question when—and ultimately of course how—glial cells become restricted to their lineage and may no longer produce a broader progeny (Laywell et al. 2000).

The progeny of individual cortical progenitor cells has so far been examined in vitro by different approaches: 1) live imaging in single-cell cultures (Temple 1989; Davis and Temple 1994; Qian et al. 1997, 1998, 2000; Shen et al. 2006; Ravin et al. 2008); 2) live imaging in slice cultures (Noctor et al. 2004, 2008); and 3) lineage analysis by viral vector-mediated genetic labeling in high-density cultures (Williams et al. 1991; Williams and Price 1995; Heins et al. 2002; Costa et al. 2007, 2008) as well as in vivo by genetic lineage analysis based on injection of retroviral vectors into the lateral ventricle of embryos in utero and analysis of transduced cells at postnatal stages (Luskin et al. 1988, 1993; Price and Thurlow 1988; Walsh and Cepko 1988, 1992, 1993; Parnavelas et al. 1991; Grove et al. 1993; Mione et al. 1994, 1997; Reid et al. 1995; McCarthy et al. 2001; Reid and Walsh 2002). Here, we will briefly review the results obtained by these previous analyses as well as presenting new in vivo and in vitro data utilizing new viral vectors and live-imaging lineage analysis in high-density cultures without any growth factor addition. We then integrate our new results with previous data into the view that the early developing cortex harbors only 2 progenitor cell types: neuron-restricted and bipotent progenitors. In contrast, glia-restricted progenitors, similar to those observed in the spinal cord (Lee et al. 2000), were observed only at later stages versus the end of neurogenesis.

Lineage Analysis of Cortical Progenitors In Vivo

The progeny of single progenitors in vivo can be identified by using retrovirus carrying genes encoding for reporter proteins, such as green fluorescent protein (GFP) or beta-galactosidase (LacZ). This clonal analysis in vivo relies on infecting a single progenitor cell into whose genome the viral vector integrates and is then passed on to its entire progeny that can be afterward identified by the expression of the reporter gene (Price 1987), revealed by means of immunohistochemistry. The progeny resulting from a progenitor infected at a given embryonic age is then analyzed at postnatal stages, when neurons and glial cells have fully developed (Bayer and Altman 1991; Cameron and Rakic 1991; Misson et al. 1991; Parnavelas 1999). Thus, no information is available about the behavior of

cells between infection and analysis, that is, to which extent some cells may die and to which extent daughter cells migrate over large distances, losing spatial relationship with their sibling cells. Hence, one major challenge of this approach is to accurately define a clone, that is, which labeled cells are indeed derived from a single progenitor.

In this regard, research has relied on the use of low titer of retroviruses and spatial definition of clones (Luskin et al. 1988; Price and Thurlow 1988; Walsh and Cepko 1988) or the use of retroviral libraries (Walsh and Cepko 1992, 1993). In both cases, the experimental design is to transduce a limited number of cortical progenitors at embryonic stages and try to identify the progenies of individual infected cells at postnatal stages, either by using spatial criteria or genetic tags (see below). However, both approaches have some disadvantages. First, the use of low titers of retroviruses carrying a single reporter gene cannot exclude the possibility that 2 neighboring progenitor cells in the VZ are infected and give rise to cells in close spatial neighborhood when the progeny is analyzed. Consequently, the progeny of 2 distinct progenitor cells would be interpreted as a single clone and result in the “lumping error,” the misinterpretation that a clone comprising, for example, glia and neurons would originate from a bipotent progenitor, even though this cell cluster actually comprises the progeny originating from 2 distinct progenitors (Fig. 1). Second, the lumping error may also be due to cell migration causing superimposition of 2 or more discrete clones. Third, cell migration can also lead to the so-called “splitting error” with the progeny of a single progenitor migrating apart from each other to settle at different positions giving rise to spatially separate clusters of neurons and glial cells that may however be derived from a single, in this example, bipotent progenitor (Fig. 1). Indeed, lateral and tangential migration of cells in the cortex appears to be particularly pronounced at the end of neurogenesis in the cerebral cortex (Walsh and Cepko 1993) and is well known for glial progenitors that cover large distances to migrate from ventral to dorsal regions (Richardson et al. 2006).

To overcome these concerns in regard to the spatial definition of clones, Walsh and Cepko established the approach to utilize an entire library of viral vectors carrying a multitude of nucleotide tags, such that each viral vector could be discriminated by polymerase chain reaction (PCR) analysis (Walsh and Cepko 1992, 1993). The rationale behind this method is that the probability of infecting 2 neighboring cells with retroviruses carrying the same tag would be extremely low. Therefore, the clonal relationship of cells expressing a reporter gene could be further confirmed by the presence of a single tag amplified by PCR. Moreover, the splitting error due to cell migration could be recognized by the presence of cells isolated from spatially separated clusters but yielding the same PCR products. Indeed, this method could show that there was a widespread dispersion of clonally related cells in the developing cortex, that is, that cells located at very distant sites shared their viral vector tag (Walsh and Cepko 1992, 1993). Thus, the first conclusion derived from lineage analysis *in vivo* using a single retrovirus, namely, that most progenitors in the cerebral cortex generate only a single cell type (Luskin et al. 1988; Grove et al. 1993), could not be substantiated given the technical concerns (Walsh and Cepko 1992). Moreover, clusters of virally infected cells were found to consist of cells with various different tags, that is, had been subject to the lumping error. Nevertheless,

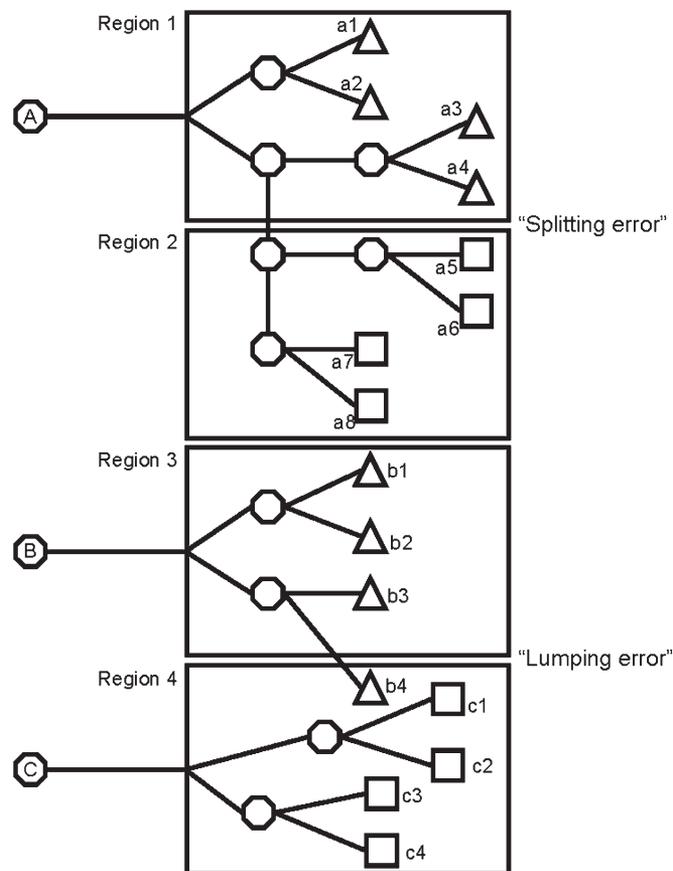


Figure 1. Splitting and lumping errors in clonal analysis. Schematic representation of neurons (triangles) and glial cells (squares) derived from 3 individual progenitors (A, B, and C) and distributed into 4 anatomically defined regions. The progeny of the cell A splits into regions 1 and 2, leading to a “splitting error”: cells a1–4 are classified as a pure neuronal clone, whereas cells a5–8 as a pure glial clone. In contrast, the progenitor B gives rise to a neuron (b4) that mixes up with glial cells (c1–4) derived from another progenitor (C). In this case, a pure glial clone would be mistakenly classified as mixed—“lumping error.” The progeny of cell B is also underestimated, as only 3 out of 4 cells are observed in region 3.

also this approach is subject to technical challenges, namely, the successful PCR from each cell identified by the expression of the reporter gene. If the PCR reaction is not successful for all virally infected cells in a brain, clonal related progeny is missed and an allegedly pure glial clone may also contain neurons that have migrated to a distant position but whose viral vector insert was not successfully amplified (for discussion, see McCarthy et al. 2001). Consequently, a combination of the above-described approaches may be promising, namely, the use of several viral vectors to minimize the likelihood of infecting neighboring cells as well as avoiding viral vector libraries that require detection via PCR.

Silencing of Viral Vector-Driven Gene Expression

Besides the clonal definition, there were additional technical concerns with the first generation of retroviral vectors as these were actually prone to silencing. Thus, some retrovirally transduced cells may shut off expression of the marker gene LacZ or GFP and hence be undetectable at the time of analysis (Gaiano et al. 1999; McCarthy et al. 2001). Such gene silencing could lead to an underestimation of clone size and, as a consequence, to a misinterpretation of the potential of cortical progenitors (e.g., neurons are particularly prone to silence long

terminal repeat (LTR)-driven gene expression such that a clone consisting only of glia would actually be mixed and contains also neurons that had turned off their marker gene expression. As a result, this would lead to the wrong assumption that a glia-restricted progenitor had been transduced at the time of retroviral infection). The LTR promoter of retroviral vectors is particularly prone to silencing, such that viral vectors utilizing different internal promoters (Gaiano et al. 1999; McCarthy et al. 2001) as well as self-inactivating the LTR promoter (van Praag et al. 2002) are less subject to gene silencing (for remaining silencing, see e.g., McCarthy et al. 2001). However, since the development of these new viral vectors, only a single study on telencephalic progenitor cell lineage has been performed (McCarthy et al. 2001). Although the authors report glia-restricted progenitors already at very early embryonic stages (E9.5), all but one clone containing glia only were found in the ventral telencephalon. As this study utilized a viral vector library requiring detection of viral vector inserts by PCR which cannot be successful in all cases, we decided here to reexamine the lineage of cortical progenitors by a combinatorial approach using viral vectors that can be easily discriminated by expression of different fluorescent proteins.

Lineage Tracing In Vivo by Analysis of Dual Vector-Injected Clones

Similar to a recently developed method (termed “mosaic analysis with double markers”) that relies on the expression of different fluorescent proteins upon the low probability Cre-mediated interchromosomal recombination joining the N- and C-terminal halves of either GFP or red fluorescent protein (RFP) in the progeny of a mitotic cell (Zong et al. 2005), we took advantage of the low probability of cotransduction when low titers of viral vectors (utilizing the CAG promoter overcoming the silencing problems, van Praag et al. 2002) containing GFP or RFP were used. This method allows detecting clonal superimposition (the lumping error) as the progeny of 2 neighboring cells can be distinguished by their differential expression of reporter proteins (GFP, RFP, and GFP and RFP). Moreover, coinfecting (yellow) cells are particularly rare if both viral vectors are used at a low titer (see Materials and methods in Supplementary Information).

To test our hypothesis, we first analyzed the brains of mice 2 days after intraventricular retroviral injection at E13. We observed an extremely low number of “yellow cells” in small radial clusters containing 2–4 cells (Table 1). These clusters spanned a limited distance in the rostrocaudal axis, with a maximal distance of 60 μm and were separated from each other by at least 200 μm , likely representing the progeny of a single cell, that is, a clone. We inferred that cell dispersion within clones (60 μm) may be largely caused by the growth of the brain between the time of injection and analysis (about 150%) and extrapolated this also for longer survival times. Accordingly, the spread of cells due to growth of the brain would be around 160 μm at the day of birth (E13–postnatal day zero [P0]: 400% growth of the brain). Indeed, more than 95% of yellow cell clusters in the P0 cortex were contained within a 150- μm diameter (data not shown). In this regard, it is also important that less than 10% of all cells in the VZ disperse for more than 150 μm (Fishell et al. 1993).

We therefore decided to use the approach of dual-color tracing with viral vectors more resistant to silencing (McCarthy et al. 2001; van Praag et al. 2002) to at least get a first idea

Table 1

Cell lineage of single cortical progenitors 2 days after infection in situ (E13–15)

Cells in the VZ	Cells in the SVZ	Cells in the IZ/CP	Frequency ^a (%)
3	0	0	2.5
3	1	2	2.5
3	0	2	5
2	0	0	5
2	2	2	2.5
2	1	0	15
2	0	1	7.5
2	0	1	7.5
2	0	2	5
2	1	1	5
1	1	0	2.5
1	2	0	2.5
0	2	0	5
0	2	1	10
0	2	2	2.5
0	1	1	2.5
0	0	2	12.5
0	0	3	2.5
0	0	4	2.5

^a n = 40 GFP-positive clones (pMXIG) and 45 “yellow clones” (CAG-GFP + CAG-dsRed). IZ, intermediate zone; CP, cortical plate.

of the progeny derived from progenitors infected at different stages of cortical development. Upon injection at E13 and examining the P0 brains, we had already observed that each clone contained neurons and some clones contained neurons and nonneuronal cells located at this time still in the VZ or SVZ (data not shown). When brains injected at E13 were analyzed at P15, we could detect GFP and RFP expression in neurons and glial cells (Supplementary Fig. 1 and data not shown). Then, we calculated that the dispersion due to growth (E13–P15: 700% growth) should be around 280 μm and classified as a clone every group of yellow cells within this limit. In 3 independent experiments, we found 20.3 ± 5.7 clones per brain and all contained at least one pyramidal neuron with obvious spines (Fig. 2). Indeed, we also observed cells derived from different infections as detectable by their red, green, or yellow fluorescence, sometimes closely intermingled (Fig. 2A–C), as previously described predominantly for the radial clusters of cells (McCarthy et al. 2001). We also observed some interneurons defined as neurons without any spines and omitted these for our definition of a clone derived from cortical progenitors as these cells originate largely in the ventral telencephalon. In fact, their long-range migration may well contribute to some of the very widespread clones observed before (Walsh and Cepko 1992, 1993). Taken together, we defined mixed clones as those containing glial cells and pyramidal neurons within 280 μm in the rostrocaudal axis. In most cases, a defined clone spanned less than 150 μm in the lateromedial axis and was separated by at least 250 μm from the next one in the rostrocaudal axis. Among these clones ($n = 63$), we observed clones containing only neurons or clones containing neurons and glial cells, but not a single clone consisted of glial cells only (Fig. 2D). As the spatial definition of clones has been shown to be subject to a low chance of lumping errors, but to a high chance of splitting error (Walsh and Cepko 1992), our analysis may underestimate the total clonal size, but it is unlikely to overestimate it. Thus, we interpret the absence of pure glial clones derived from E13 cortical progenitors as an indication that the early developing cortex may be devoid of glia-restricted progenitors.

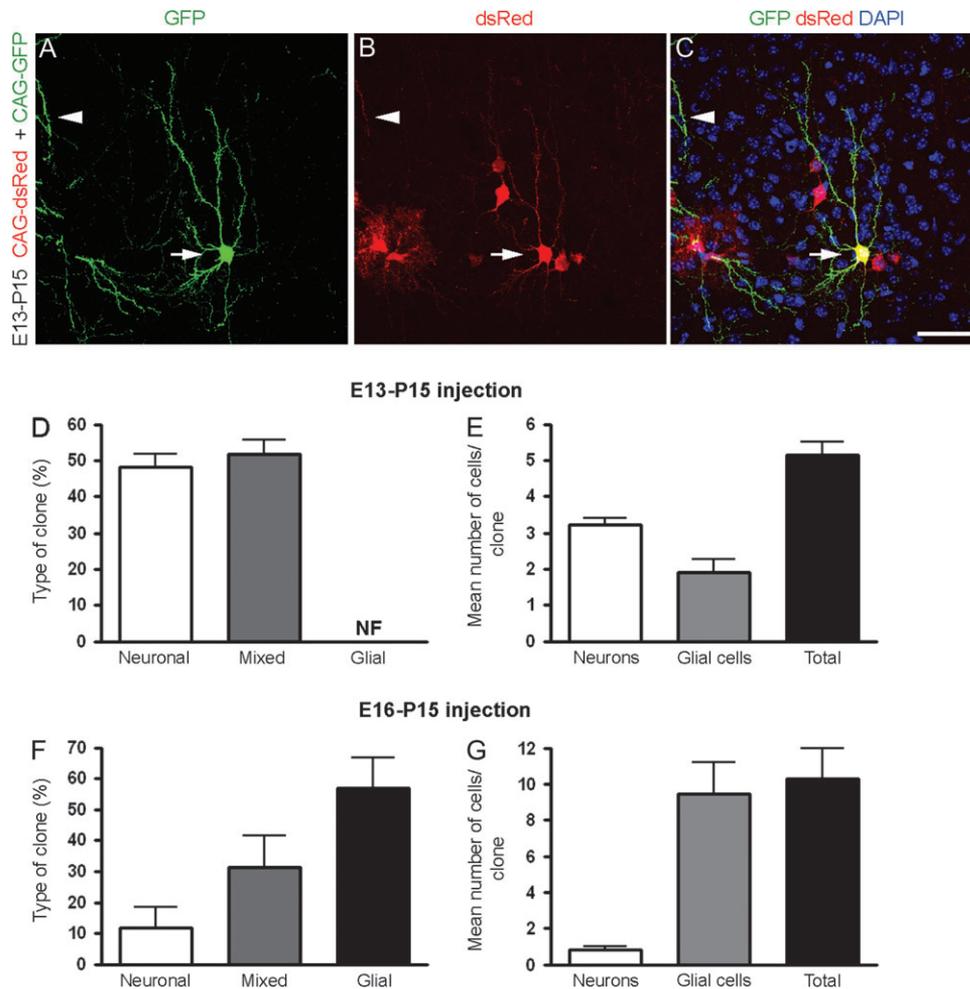


Figure 2. Absence of glia-restricted progenitors at early neurogenesis in vivo. (A–C) Coronal section of a P15 brain after injection with CAG-GFP and CAG-dsRed retroviruses at E13 in utero showing a double-labeled spiny neuron in layer 2/3 (arrow). Some processes of a sister neuron found in the adjacent section can also be visualized (arrowhead). These neurons belong to a mixed clone containing 7 cells. (D–G) Quantification of the frequency of different types of clones (D, F) and the number of cells per clone (E, G) generated from E13 ($n = 63$ clones; 3 animals) or E16 cortical progenitors ($n = 43$ clones; 3 animals). NF, not found. Calibration bar: 50 μm .

Next, we tested whether pure glial clones could be identified at P15 by similar geometric criterion upon injections at later embryonic stages (E16) when cortical gliogenesis had commenced. Indeed, upon injection of retroviruses at this stage, more than half of the “yellow clones” in the P15 cortex contained only glia (Fig. 2F), consistent with earlier data that had performed clonal analysis mostly at these later stages (Grove et al. 1993; Luskin et al. 1993). Consistent with this late stage in neurogenesis, the number of neurons per clone was small and most cells were glial (Fig. 2G). Thus, the number of neurons generated by bipotent progenitors decreases during development. These data further suggest that sometime between E13 and E16, the bipotent progenitors observed at E13 gave rise to glia-only progenitors. Given that the dispersion of cells should be equal for the cells examined all at P15 and derived either from E13 or from E16 progenitors, we interpret the data derived from our comparison as a genuine difference in the fate of a single progeny. If glia-only progenitors would migrate particularly far and hence cause the “glia-only” clones by the splitting error due to migration rather than lineage, this migration should also occur when these glia-only progenitors differentiate from the early bipotent progenitors. As we see no such glia-only clusters in

the earlier injections analyzed at the same postnatal stage, we conclude that the glia-only clones are not due to migration but due to lineage differences. Moreover, the glial progenitors that are generated at ventral positions and migrate into the dorsally located cerebral cortex (Richardson et al. 2006) would not be infected by viral vectors injected into the ventricle as they migrate at some distance from the ventricle within the parenchyma. Taken together, neither McCarthy and colleagues nor our study could observe a significant number of progenitors generating only glial cells at early stages in the developing cerebral cortex, suggesting that the early developing mouse cortex harbors only neuron-restricted and neuron-glial, but not glia-restricted, progenitors.

Lineage Analysis of Cortical Progenitors In Vitro

However, lineage analysis in vitro allows closer control of cell migration and only continuous observation of progenitors and their progeny will also reveal cell death and proliferation, important parameters influencing the composition of the final progeny (Schroeder 2008). For cerebral cortex progenitors, such lineage trees have been first analyzed in single-cell cultures that best allow following the progeny of a single progenitor cell

(Qian et al. 1998, 2000; Shen et al. 2006; Ravin et al. 2008). However, cell-cell interactions ranging from Notch signaling to growth factor-mediated signaling and cell polarity appear to be crucial for neural stem and progenitor fate and are obviously severely altered in a single-cell culture. Indeed, a tripotent lineage of a progenitor/stem cell giving rise to neurons, astrocytes, and oligodendrocytes has so far only been observed in cultures that start off with single cells and are cultured in the presence of either epidermal growth factor (EGF) or fibroblast growth factor 2 (FGF2; Gabay et al. 2003; Ravin et al. 2008). Conversely, when cortical progenitors are cultured as single cells without any growth factor addition, the majority of cells readily differentiate at a stage when most cells *in vivo* are proliferating (Qian et al. 1997). Thus, the lineage derived from single-cell cultures deviates with and without growth factors from the lineage observed *in vivo*. Conversely, in high-density dissociated cell cultures, the proportion of cells undergoing cell division is similar to that of proliferating cells *in vivo* (Dehay et al. 2001; Costa et al. 2007), possibly because no growth factors need to be added to this culture system. Indeed, previous lineage analysis using high-density rat cortex cultures reported rather similar lineages *in vitro* as observed *in vivo* in the absence of any growth factors (Williams et al. 1991; Grove et al. 1993; Williams and Price 1995). We therefore used this high-density culture system with further defined conditions (no astrocyte feeder layer; see Heins et al. 2002; Costa et al. 2007, 2008) to follow the progeny of individual progenitors utilizing a cell tracking system in live imaging that allows following the progeny of a single cell in an environment with contact to neighboring cells (Eilken et al. 2009).

In the first set of experiments *in vitro*, cells were isolated from E12 to E18 cortices, and the cell type composition of clones derived from single virally infected progenitors after 7 days (Fig. 3*A-I*) was examined in order to determine when glia-only clones may appear. Whereas the frequency of mixed clones (clusters of virally infected cells comprising neurons and glial cells) was very similar in cultures derived from cortical cells isolated from all stages, the proportion of pure neuronal and pure glial clones displayed a complementary tendency, with the first decreasing and the second increasing in cultures derived from progressively later developmental stages (Fig. 3*J*). Pure glial clones were virtually absent in E12 cortical cell cultures and peaked in E16/18 cortical cell cultures, further suggesting that early cortical progenitors generate neurons almost as a default phenotype. Interestingly, the preponderance to generate neurons that we also observed *in vivo* seems not to be present in the single-cell cultures (Ravin et al. 2008), again suggesting profound alterations in cell fate upon isolating single cells.

In general, the number of neurons generated either by neuron-restricted or by bipotent progenitors decreased during development *in vitro* (Fig. 3*K*) as well as *in vivo* (Fig. 2*E,G*). This trend is also present in neurosphere cultures (Shen et al. 2006; Naka et al. 2008) and in cultures of single isolated adherent cells (Davis and Temple 1994; Qian et al. 1997).

Besides the quantitative increase in glial cells generated in different clones, we also observed a qualitative change with only the later progenitors giving rise to glia-only clones. As discussed above, this may be due to the migration of glial-restricted progenitors from the ventral telencephalon into the cerebral cortex at around E15. To test for this possibility, we examined whether cells isolated from the cerebral cortex at early stages would also develop this feature at later stages *in vitro*. Toward this aim, in the second set of experiments *in vitro*,

we infected cells isolated from the E13 cortex immediately (2 h) or 2 or 6 days after plating (Supplementary Fig. 3). Interestingly, glia-only clones were observed when cultures were infected with viral vectors 2 or 6 days after plating, supporting the concept that glia-restricted progenitors are not present initially but then derive from bipotent progenitors *in vitro* and can therefore at least *in vitro* not be derived from other brain regions. Thus, these experiments supported the conclusion that early cortical progenitors have 2 initial choices: 1) to generate exclusively neurons and 2) to generate neurons and glial progenitors.

Noteworthy, the frequency of different clone types was not significantly changed when cells were grown in the absence of serum, but the main type of glial cells observed within the clones was affected: more astrocytes with serum and more oligodendrocytes without (Supplementary Fig. 2 and Supplementary Table 1). In line with these observations, other groups have also shown that the potential of cortical progenitors to generate astrocytes and oligodendrocytes, but not neurons, is significantly affected by culturing conditions, such as media composition and differential substrate adhesion (Williams and Price 1995; Morrow et al. 2001; Strathmann et al. 2007) and that astrocyte and oligodendrocyte lineages do not share a common precursor (see e.g., Skoff and Knapp 1991; Luskin and McDermott 1994; Costa et al. 2007). Taken together, our observations on the cortical progenitor lineage *in vivo* (Fig. 2) was fully confirmed *in vitro* (Fig. 3), and both sets of lineage analysis were well comparable as cells grown at high densities *in vitro* underwent a similar progression in fate as *in vivo* (compare Supplementary Fig. 3 with Fig. 3*J*).

With the purpose of continuously following cortical progenitors, we recently established live imaging and continuous cell tracking in high-density cortical cell cultures to determine the dispersion of cells within a clone and the mixture of different clones (Costa et al. 2007), as well as several other aspects of progenitor cells' behavior, such as the mode of cell division and cell cycle length (Costa et al. 2008).

In the last set of experiments *in vitro*, we used this approach to track 94 individual E12/13 cortical progenitors up to 7 days and assess the fate of their progeny by 2 different means: 1) by the use of mice expressing GFP under the control of the neuron-specific gene *Tau* (Tucker et al. 2001) and 2) by immunolabeling cells with antibodies against neuronal (microtubule associated protein-2 [MAP-2]) or glial (glial fibrillary acidic protein [GFAP] and O4) markers. These 2 methods complemented each other because the first allowed the identification of neurons that eventually died out, whereas the second allowed the identification of neurons and glial cells in the end of the experiment. In accordance with our previous data, we observed that glial progenitor cells were always (all 29 clones containing glia) encompassed into more complex lineages also containing neurons (Fig. 4 and Supplementary Fig. 4). These glial progenitors were most frequent after 4-5 days of observation and were distinguishable from their bipotent mother cells. Whereas bipotent progenitors moved very little and grew up quite substantially in cell size prior to each cell division, glia-restricted progenitors moved a lot and hardly grew after cell division (data not shown). As a consequence, glial progenitors generated progressively smaller cells. It would be interesting to investigate whether this phenomenon plays a role in the specification of glial cells through interference in signaling

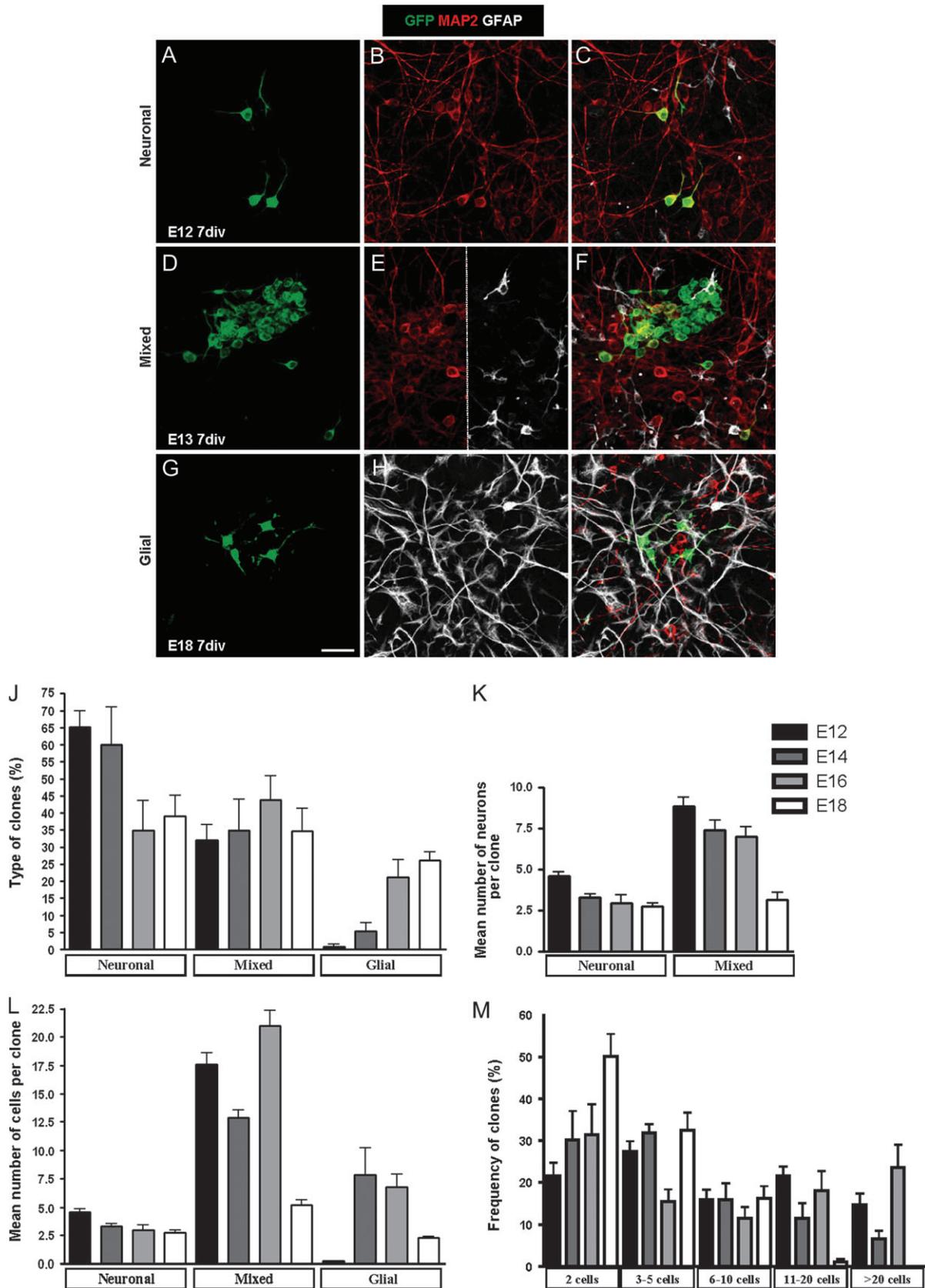


Figure 3. Clones derived from cortical progenitor cells in vitro. Pure neuronal (A–C), mixed (D–F), and pure nonneuronal or glial (G–I) clones derived from E12, E13, and E18 cortical progenitors, respectively. (J–L) The frequency of these clone types in E12–18 cortical cell cultures was plotted (J), as well as their neuronal content (K) and size (L). Note the complementary tendency between pure neuronal and nonneuronal clones. (M) Histogram showing the frequency of clones with different sizes obtained from E12 to 18 cell cultures (E12: $n = 274$ clones; E14: $n = 196$ clones; E16: $n = 154$ clones; E18: $n = 133$ clones). Calibration bar: 20 μm .

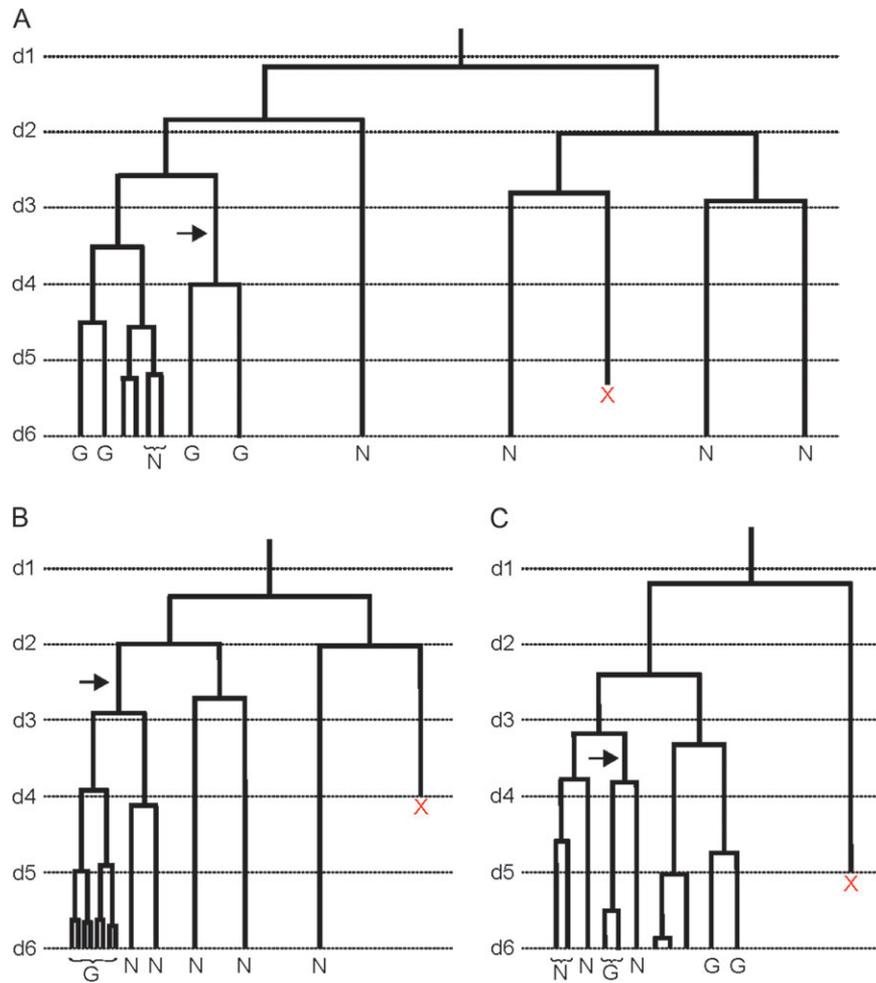


Figure 4. Glial cells are generated from bipotent progenitors. (A–C) Examples of lineage trees from E13 cortical progenitors obtained from video time-lapse microscopy images taken every 2 min for 6 days. Imaging started 1 day after plating, so that the total time of culture was 7 days. Horizontal lines indicate every 24 h of experimentation (d1, d2, d3, . . .). Note that most cells dividing in the last 48 h gave rise to glial cells and that these cells belong to a complex lineage containing many MAP-2-positive neurons (see also Supplementary Fig. 4). Observe that glial progenitors may appear before neurogenesis has come to an end into an individual lineage (arrow in A) and that some glial progenitors may arise from a true asymmetric cell division (arrow in C), whereas the classically described neuron-to-glia transition can be visualized in the lineage tree (B) (arrow). N, neurons; G, glial cells; X, cell death.

transduction within the progenitor cells (Meyers et al. 2006). Neuron-restricted progenitors were the majority of cells and displayed a similar behavior to bipotent progenitors, that is to say, both sets of progenitors grew considerably before cell division (data not shown). We could also confirm our criteria to define clones in vitro by combining retroviral infection and video time-lapse microscopy (Supplementary Fig. 4; Costa et al. 2007). Moreover, we observed some examples of clones where a single neuron was generated before glial cells, but the neuron died subsequently. These clones could explain the few cases when we observed small glial clones that would actually have been derived from a bipotent progenitor in E12 cell cultures (Fig. 3). Taken together, our data suggest that cortical progenitors at early (E12) neurogenesis are either neuron restricted or bipotent. Thus, glial cells would be generated from bipotent progenitors present in the developing cortex from early to late (E18) neurogenesis, whereas glia-only progenitors are generated sometime later, around (E14/16) mid-neurogenesis. The most parsimonious explanation for the late appearance of these glia-restricted progenitors is that they are derived from bipotent progenitors observed at

beginning of neurogenesis, and therefore, the early developing cortex would be devoid of glia-restricted progenitors.

Concluding Remarks

The existence of neuron-restricted and bipotent progenitors at early cortical development raises the key question as to the molecular determinants that specify these lineages so differently. Given the potent role of Pax6 and Neurogenins in promoting neurogenesis in the developing cerebral cortex (Nieto et al. 2001; Heins et al. 2002), one would suggest that the neuron-restricted progenitors express higher levels of these fate determinants than bipotent progenitors. Indeed, this is consistent with the higher expression of Pax6 in cortical progenitors generating mostly neurons when these had been isolated prospectively by fluorescence activated cell sorting (FACS; Pinto et al. 2008). Additional factors, such as the epigenetic status, other transcription factors, and environmental factors have also been shown to play a role in the specification of neurons and glial cells during cerebral cortex development (for a recent review, Miller and Gauthier 2007). However, it is in most cases not known whether these factors

act instructive, that is, as true fate determinants, or permissive by regulating cell proliferation or survival in a lineage-specific manner. Interestingly, recent analysis suggests that at least the gliogenic effect of ciliary neurotrophic factor (CNTF) is based on both instructive and permissive effects at least under the conditions of single-cell cultures (Ravin et al. 2008). We believe that our video time-lapse microscopy system may help to uncover the mechanisms involved in these lineage differences in a more natural environment, as we are able to observe the exact moment when the separation between neurogenesis and gliogenesis takes place in progenitors embedded within their neighbors. In the few lineages shown in Figure 4, it is already possible to envisage that this phenomenon is not stereotyped. Note, for example, the left branch in Figure 4A (arrow), where a glia-only progenitor emerges 1 day before a neurogenic division takes place, showing that even in an individual lineage neurogenesis may occur after the appearance of glia-restricted progenitors. Yet, the most frequent case is the emergence of glia-only progenitors after neurogenesis has come to an end (Fig. 4B and C). But even in these cases, bipotent progenitors may either give rise to a neuron-only and a glia-only progenitor (Fig. 4B, arrow) or a postmitotic neuron and a glia-restricted progenitor (Fig. 4C, arrow). Whether these 2 bipotent progenitors may rely on the same determinants is not known. Nevertheless, if a determinant/marker for bipotent progenitors exists, we would predict that it should be present in about 1/3 of all cortical progenitors because that is the approximate fraction of bipotent progenitors found throughout cortical development (Figs 2 and 3). Indeed, we recently succeeded to determine the transcriptome of neuron-only versus bipotent progenitors by separating these via FACS from E14 mouse cerebral cortex (Pinto et al. 2008). The next step will now be to utilize live imaging to directly correlate changes in expression with changes in lineage and cell fate.

Our results also have some broader implications in regard to the mechanisms that maintain bipotent progenitors and/or instruct gliogenesis. Notch signaling has been implicated in both the maintenance of early cortical progenitors in an undifferentiated state and in the generation of glial cells at later stages (for review, see Gaiano and Fishell 2002). Our data demonstrating the generalized neurogenic potential of early progenitors and the sole derivation of glial cells from bipotent lineages would suggest that Notch signaling in early cortical progenitors could simply increase the proportion of progenitors that will not terminally differentiate into neurons and hence could become gliogenic at later stages (which they could not have done if they differentiated into postmitotic neurons). According to this interpretation, overexpression of activated Notch1 in E9.5 telencephalic progenitors increases the frequency of neural stem cells at E14.5 (Yoon et al. 2004). Thus, molecules influencing the generation of glial cells in the developing cortex could either play a direct role on cell fate specification or simply maintain the progenitors in an undifferentiated stage, allowing them to respond to other molecules influencing their potential (for recent review, see Miller and Gauthier 2007). These data are also consistent with the presence of Notch signaling in basal progenitors (Mizutani et al. 2007) that are largely part of the bipotent lineage (Pinto et al. 2008). Thus, it is important to consider molecular pathways specifying cell fate in the context of the actual lineage, that is, incorporating the differences between progenitors generating neurons only and neurons and glia. Therefore, a systematic analysis of the role of different

molecules acting in the different cortical lineages at different developmental stages will help us to better understand how neurons and glial cells are generated in the cerebral cortex and how we may reactivate the original neurogenic bias in older cells with the aim of regeneration.

Supplementary Material

Supplementary material can be found at <http://www.cercor.oxfordjournals.org/>.

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Notes

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