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Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage

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SUMMARY

The developing central nervous system of vertebrates contains an abundant cell type designated radial glial cells. These cells are known as guiding cables for migrating neurons, while their role as precursor cells is less clear. Since radial glial cells express a variety of astroglial characteristics and differentiate as astrocytes after completing their guidance function, they have been considered as part of the glial lineage. Using fluorescence-activated cell sorting, we show here that radial glial cells also are neuronal precursors and only later, after neurogenesis, do they shift towards an exclusive generation of astrocytes. These results thus demonstrate a novel function for radial glial cells, namely their ability to generate two major cell types found in the nervous system, neurons and astrocytes.

Key words: Cerebral cortex, Radial glial cells, Neuronal precursors, Glial precursors, Glutamate astrocyte-specific transporter (GLAST), GFP, Human GFAP-promoter, Clonal analysis

INTRODUCTION

The diversity of cell types that constitute the adult nervous system is generated during development by a series of precursor cells. Early, multipotential precursors give rise to progenitors with a more restricted neuronal or glial progeny (Anderson, 1989; Lillien, 1997). This is best examined in the developing cerebral cortex, where cell lineage experiments revealed the co-existence of diverse sets of precursor cells specified towards the generation of single cell types (Luskin et al., 1988; Williams et al., 1991; Grove et al., 1993; Reid et al., 1995; Williams and Price, 1995; Qian et al., 1998). The morphological identity of these precursor subtypes is, however, not known.

Morphological subsets of precursor cells are observed throughout the CNS and have been implicated in the generation of different cell types. Cells with long radial processes and glial expression patterns were identified at the onset of neurogenesis as guides for migrating neurons, and were named radial glial cells (Rakic, 1972; Levitt and Rakic, 1980). Similar to astrocytes, radial glial cells contain glycogen granules (Choi, 1981) and express glial fibrillary acidic protein (GFAP) in primate cortex (Levitt and Rakic, 1980). Rodent radial glial cells express hardly any detectable levels of GFAP mRNA (Sancho-Tello et al., 1995), but contain the astrocyte-specific glutamate transporter (GLAST; Shibata et al., 1997) and brain-lipid-binding protein (BLBP, Feng et al., 1994; Kurtz et al., 1994), both of which are later restricted to astrocytes (Ullensvang et al., 1997). Moreover, radial glial cells transform into astrocytes after neurogenesis and neuronal migration (Voigt, 1989; Culican et al., 1990) and have therefore been considered as glial progenitor cells (Cameron and Rakic, 1991). The progeny of radial glial cells during neurogenesis, however, has never been examined, even though pulse labeling with S-phase markers (Schmechel and Rakic, 1979; Misson et al., 1988) and retroviral experiments (Gray and Sanes, 1992; Halliday and Cepko, 1992; Gaiano et al., 2000) have shown that radial glial cells divide throughout development, including the phase of neurogenesis.

In contrast to mammals, radial glial cells persist into the mature brain in most other classes of vertebrates (Margotta and Morelli, 1997). Interestingly, cells with many characteristics of radial glial cells seem to act as neuronal precursors in the forebrain of adult songbirds (Alvarez-Buylla et al., 1990). Moreover, recent evidence suggest that cells with astrocyte characteristics are stem cells in the adult brain of mammals, thereby demonstrating a neurogenic potential of at least some astrocyte subsets (Doetsch et al., 1999). These results emphasize the importance to determine the progeny of radial glial cells in the developing brain (Barres, 1999).

To this end, we have fluorescently labeled radial glial cells by two independent techniques and isolated them via fluorescence-activated cell sorting (FACS). First, we used a transgenic mouse line expressing the green fluorescent protein (GFP) under the human GFAP promoter (Zhuo et al., 1997) to select precursor cells by means of their astroglial expression patterns. Second, precursor cells with long radial processes were traced from the pial surface with fluorescent dyes (Katz et al., 1984; Voigt, 1989). Both labeling techniques were also combined to ensure the isolation of radial glial cells as precursor cells with long radial processes and astroglial traits. These experiments gave the surprising result that radial glial

cells are a heterogeneous population of precursor cells, consisting mainly of neuronal and astroglial precursor cells, and only few multipotential precursor cells. During later stages of development, radial glial cells lose the neuronal lineage and become restricted to the generation of astrocytes.

MATERIALS AND METHODS

Animals

Cortical cells were isolated from time-mated Wistar rats (day of sperm detection was E1), C57/Bl6 mice (day of plug was E0; tracing experiments) and the 94-4 transgenic mouse line (Zhuo et al., 1997) that was generously given to us by A. Messing, University of Wisconsin, Madison, USA.

Cell culture

Cortices were dissected from mouse or rat at embryonic day 13-18, dissociated with trypsin and cultured on poly-D-lysine-coated glass coverslips in chemically defined medium (SATO) as described previously (Williams and Price, 1995; Götz et al., 1998). Rat cortical cells were plated at a density of $4\times10^5/cm^2$ less than 2 hours prior to plating the cells sorted from mouse cortex. To detect dividing cells, 5-bromo-2′-deoxyuridine (BrdU; Sigma) was added to the culture medium at a final concentration of 10 μM.

Fluorescence-activated cell sorting (FACS)

Fluorescent cells were isolated using a FACSort or FACStar (Becton Dickinson) in the single cell mode at the appropriate sort rate (e.g. below 100 cells per second with the FACSort). A negative control of non-fluorescent cells (see Figs 2, 4) was used to determine the background fluorescence such that less than 1% of non-fluorescent cells were included in the sort gate. In the case of double-labeled cells (red fluorescent beads and green fluorescent protein) compensation was determined using cells with only one staining.

The composition of sorted cells was examined as soon as the sorted cells were adherent (after 1-2 hours). Cells were either analyzed for their DiI-, beads or GFP-fluorescence and/or stained with cell-type-specific antibodies (see Figs 2, 3, 5). This analysis revealed about 10% contamination by non-fluorescent cells. Cell death of the sorted cells was examined by counting the total number of M2M6-positive cells from 1 hour to 7 days after plating. This revealed a survival of 69±15% cells sorted from E14 or E18 cortex for 1 week in vitro.

Tracing

The fluorescent dye DiI (Molecular Probes) was applied either as small crystals or in ethanol/sucrose suspension onto the surface of cerebral hemispheres after careful removal of the pial membrane as described previously (Voigt, 1989; Götz et al., 1998). Red fluorescent latex microsphere beads (Lumafluor, 50 nm; Katz et al., 1984) were diluted 1:1 in Hanks-balanced salt solution (HBSS, Gibco) and 5 μl of this solution were used per well (24-well plate, Nunc) for three brains that were oriented with the pial surface into the beads solution. Brains were incubated for 7 minutes, transferred to fresh HBSS and incubated for further 15 minutes before dissection. Each hemisphere was carefully inspected for fluorescent label on the ventricular side and hemispheres with ventricular contamination were discarded. The labeling was further evaluated by sectioning the brains directly after labeling (Fig. 5B), by immunostaining of the cells immediately after sorting (see above, Fig. 5C) and by injuring some control brains with the forceps to mimic potential damage inflicted during dissection (see Results). Three sets of experiments were performed to control for the spread of DiI or beads during the dissociation procedure. First, DiI crystals were put in the dissociation solution of unlabeled cells. No cells had incorporated the label after dissociation. Second, bead-labeled hemispheres were dissociated with unlabeled hemispheres from rat, from green mice (Okabe et al., 1997) or with an increasing number of unlabeled hemispheres from mice. The mixtures with rat and transgenic mouse cells allowed the direct determination of the proportion of unlabeled cells that had taken up the label (3%, see Results). The serial dilution confirmed the low amount of dye spreading. 28% cells were fluorescent in a cell suspension prepared from one labeled brain. Only 16% cells were fluorescent when one labeled and one unlabeled brain were dissociated together and only 7% fluorescent cells were detected in a cell suspension prepared from one labeled and two unlabeled brains. The results demonstrate at a quantitative level by three independent approaches that neither DiI nor beads are passed on in serious quantities to unlabeled cells during the dissociation. Since the results obtained by DiI- and bead-labeling were very similar, in both the proportion of labeled cell types (see Fig. 5) and the progeny generated by the labeled precursors in vitro (DiI, 51±13%; beads, 52±5% neuron-containing clones), these data are pooled throughout the results section.

Clonal analysis

The probability of clonal superimposition was calculated as in retroviral experiments (Williams et al., 1991). This value (7%, maximum 65 clones/well) was identical to a value obtained by computer simulation (Malatesta, unpublished observations). In addition, we ensured that the main precursor types survive the sorting procedure by analyzing the progeny of all cells (healthy cells indicated by the gate in dot plots in Fig. 2 and 4). After 1 week in vitro, all cell types (neurons, astrocytes and oligodendrocytes) were detected in a quantitatively similar composition as observed in retroviral or time-lapse cell lineage experiments (Williams et al., 1991; Grove et al., 1993; Reid et al., 1995; Williams and Price, 1995; Götz et al., 1998; Qian et al., 1998). Similarly, the mean clonal size observed in our cultures (94-4 GFP-positive cells: 3.1±0.2, n=241 at E13 to 2.1±0.1, n=527 at E18; pial traced clones, E14: 2.7±0.1, n=56; GFP-beads-double-label, E14: 2.1±0.6, n=46) was in agreement with previous retroviral cell lineage experiments (Williams et al., 1991; Grove et al., 1993; Reid et al., 1995; Williams and Price, 1995; Götz et al., 1998). Data were derived from at least two different experimental batches with 2-16 coverslips analyzed. The mean was calculated from the values obtained per coverslip, n indicates the total number of clones analyzed ±s.e.m.

Immunostaining

Cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at room temperature and processed for antibody staining as described previously (Götz et al., 1998). Primary antibodies (M2M6, C. Lagenaur, rat) 1:200; anti-β-tubulin-III (Sigma, mouse IgG2b), 1:100; anti-GFAP (Sigma, mouse IgG1) 1:200; anti-GFP (Clonetech, rabbit), 1:1000; anti-nestin (Dev. Hybridoma Bank, mouse IgG1) 1:4; RC2 (P. Leprince, mouse IgM) 1:100; O4 (J. Price, mouse IgM) 1:100) were incubated overnight at 4°C and detected by subclass specific secondary antibodies labeled with FITC, TRITC or biotin. The latter was then detected by AMCA-coupled streptavidin.

RESULTS

FACS analysis of cortical precursors expressing astroglial markers

The human GFAP promoter has been shown to direct transgene expression to astrocytes in the mature brain (Zhuo et al., 1997) and to ventricular zone cells in the developing cortex (Brenner et al., 1994). Since GFAP is expressed in radial glial cells in primate cortex, we examined whether the ventricular zone cells expressing the transgene might be radial glial cells. Indeed, GFP was contained in cells with long radial processes (Fig. 1A)

in sections of the cortex from E14-E18. Since, however, the GFP signal was rather weak, it was difficult to decide whether GFP was also in cells with short processes.

Next, we determined the subsets of cortical cells that express GFP using different precursor markers that had previously been detected in radial glial cells, such as RC2 (Misson et al., 1988; Edwards et al., 1990), GLAST (Shibata et al., 1997) and BLBP (Feng et al., 1994; Kurtz et al., 1994; Anton et al., 1997). While rodent radial glial cells do not contain detectable protein levels of GFAP (Sancho-Tello et al., 1995), radial glial cells in the spinal cord have been reported to contain the astrocyte-specific glutamate transporter GLAST, another molecule later restricted to astrocytes (Shibata et al., 1997; Ullensvang et al., 1997). Indeed, GLAST is also contained in radial glial cells of the developing cerebral cortex from E13 until early postnatal stages (Hartfuss et al., 2000; Fig. 1B). Since GFP was detected with a similar time of onset (E13) in the developing cortex of hGFAP-GFP transgenic mice, we performed a co-localization analysis using acutely dissociated cells to evaluate co-expression at the single cell level. Indeed, most GFP-positive cells also contained GLAST (Fig. 1C,D).

Since the low GFP signal is best detected by FACS and our aim was to analyze the progeny of the sorted cells, we further quantified the composition of GFP-positive cells immediately after their isolation by fluorescence-activated cell sorting. GFP-positive cells were sorted from E14-E18 cortex (sort profiles in Fig. 2A-C) and analyzed by immunocytochemistry as soon as they were adherent (after about 2 hours). This analysis was performed on sorted cells plated with or without a rat cortical feeder layer that was used for the clonal analysis described below. No differences were detected in the composition of sorted cells after two hours on these two substrates. When cells were isolated by their green fluorescence from E14 cortex and analyzed directly after the sort, we detected 80% precursor cells as marked by Ki67 (Gerdes et al., 1997) and nestin (Frederiksen and McKay, 1988) immunoreactivity, and the absence of staining with β-tubulin-III-antiserum (Lee et al., 1990). Almost all of these cells were RC2 and GLAST positive (78±4% RC2-positive cells, *n*=350; 70±7% GLAST-positive cells, *n*=600; Fig. 2A'). The remaining 20±2% (*n*=450) were β-tubulin-III-positive postmitotic neurons. Since this percentage exceeds the normal contamination in the range of 5-10% determined for the FACSort (see Materials and Methods), there appears to be a weak GFP signal in some neurons. It is also important to emphasize that in our stainings no co-localization was observed between Ki67-/nestin-positive and β-tubulin-III-immunoreactive cells (see, however, Menezes and Luskin, 1994). Thus, the β-tubulin-III-immunoreactive cells included in the sort were postmitotic. A similar composition of cells isolated by their GFP content was observed at E16 and E18 with about 20% neurons and 80% precursor cells (Fig. 2B',C'). The precursor cells were at all ages almost exclusively GLAST immunoreactive (90% of the precursors isolated from E14 cortex and 100% of the precursors isolated from E16 and E18 cortex). We noted, however, a change in the proportion of RC2- and BLBP-positive cells among the GLAST-positive precursors isolated from E14-E18 cortex. While at E14 all sorted precursors contained RC2, only half of them were RC2 immunoreactive at E18 (Fig. 2A'-C'). In contrast, 70% of all precursors (56% amongst 80%, Fig. 2A') isolated from E14 cortex contained BLBP and this proportion increased to 100% at E18 (Fig. 2C'). Thus, while some antigens change in the precursors isolated by their GFP content, almost all of them contain GLAST throughout development. Sorting of GFP-positive cells from the cortex of hGFAP-GFP transgenic mice therefore selectively enriches for precursor cells expressing GLAST, a molecule later characteristic for astrocytes.

Progeny of cortical precursors expressing astroglial markers

Sorted cells were then cultured for 7 days and their progeny determined by the use of cell-type specific antibodies. Neurons were labeled with anti-β-tubulin-III (Lee et al., 1990), astrocytes with anti-GFAP (Bignami et al., 1972) and oligodendrocyte precursor cells with the O4-antiserum (Sommer and Schachner, 1981). When GFP-sorted cells were isolated from mouse cortex at E13-E16, i.e. during neurogenesis, the predominant cell type observed after 1 week in vitro was neuronal (Fig. 3A,B), but we also detected astrocytes and oligodendrocytes (data not shown). We confirmed that the neuronal cells had arisen from dividing precursor cells by addition of 5-bromo-2′-deoxyuridine (BrdU), a DNA-base analogue that is incorporated into the DNA of dividing cells (Nowakowski et al., 1989), to the culture medium (Fig. 3B). Thus, despite their astroglial expression pattern, these precursors seem to generate neurons.

Cortical precursor cells have a characteristic cell-type specific lineage. Retroviral labeling experiments and imaging of isolated cortical precursors in vitro demonstrated that the progeny of individual precursor cells is small and mostly composed of a single cell type (e.g. Grove et al., 1993; Reid et al., 1995; Williams and Price, 1995; Qian et al., 1998). We therefore devised a method for cell lineage analysis to determine the potential of the sorted precursor cells using a mouse-rat co-culture system. Less than 100 cells sorted from mouse cortex were cultured with an excess of rat cortical cells (see Materials and Methods), allowing the identification of individual sorted cells and their progeny by the mouse-specific antibodies M2M6 (Fig. 3C; Lagenaur et al., 1981; Lund et al., 1985; Reh, 1992). This co-culture system provides a cortical environment for the sorted cells that is similar to that which they would encounter during normal development, without the addition of specific grow factors that may bias the analysis by selecting for particular cell types (Lillien et al., 1997; Qian et al., 1998; Williams et al., 1991; Hajihosseini and Dickson, 1999). To validate this novel system for clonal analysis we first ensured that all cells from mouse cortex were labeled by the M2M6 antisera (data not shown). Second, we examined the amount of cell clusters immediately after sorting and found only 3% M2M6-immunoreactive cell-duplets (*n*=2020), i.e. 97% of the sorted cells are initially plated as single cells. These results validate the definition of distinct M2M6-positive cell clusters after 1 week in vitro as clones, i.e. derivatives of individual sorted precursor cells (Fig. 3C,D). Triple immunostaining with cell-type specific antisera served to identify the cellular composition of the M2M6-immunoreactive clusters (Fig. 3E-J).

Most of the M2M6-positive cell clusters after 1 week in vitro consisted of a single cell type (Fig. 3E-J). For example, 96% of the M2M6-positive clusters from E14 GFP-sorted cells were pure and contained either only neurons or only non-neuronal

cells (Fig. 2A'). More than half of all clones (67±5%, *n*=331) contained only neurons and only a third of all clusters (28±3%) consisted exclusively of nestin-immunoreactive precursor cells and/or GFAP-positive astrocytes. This composition corresponds closely to the mostly restricted lineage observed for cortical precursors by other techniques in vivo and in vitro (Grove et al., 1993; Reid et al., 1995; Williams and Price, 1995; Qian et al., 1998; see also Fig. 4 and below).

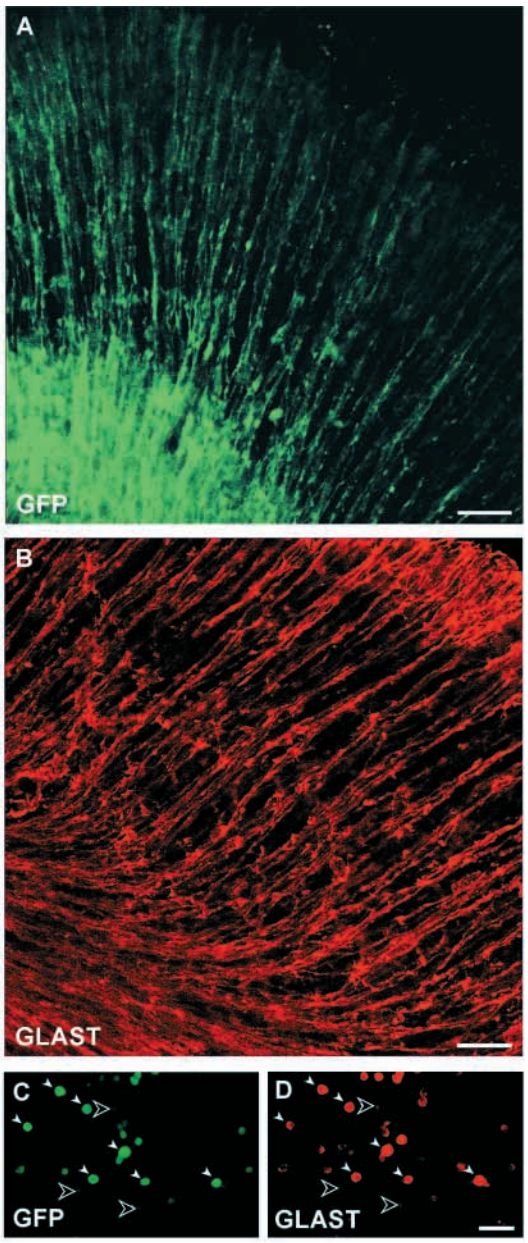

Fig. 1. Green fluorescent radial glial cells of 94-4 transgenic mice, in which the human GFAP-promoter drives expression of the S65T-mutant green fluorescent protein (GFP) (Zhuo et al., 1997). (A) GFP-immunostained and (B) GLAST-immunoreactive radial glial cells in frontal sections from E16 (A) and E14 (B) mouse cortex (pial surface upwards in A and towards the upper right corner in B). (C) depicts GFP-immunostained cells from acutely dissociated E14 cortex double stained with GLAST antiserum (D). Filled arrowheads depict double-positive cells and open arrowheads double-negative cells in the corresponding fluorescent micrographs. This shows that GFP is localized in GLAST-containing precursor cells with radial glial morphology. Scale bars: 25 μm.

To allow a direct comparison of these data with the composition of the sorted cells 2 hours after the sort, the immunochemical analyses depicted in Fig. 2A'-C' were performed identically after 2 hours and 7 days. In both cases, sorted cells were mixed at a clonal density with the rat cortical cells, identified by their M2M6 immunoreactivity and triple stained with other cell type markers. As described above, clusters after 2 hours consist in 97% of a single cell, while the clusters after 5-7 days in vitro consist mostly of two to four cells (mean clone size was 3.1, see Materials and Methods), indicating the proliferation and differentiation of sorted precursor cells. The proportion of pure neuronal clusters detected after 7 days in vitro (E14, 67%; E16, 66%, *n*=297) exceeded by far the proportion detected immediately after the sort (E14, 20%; E16, 18%), suggesting that about 50% of the sorted precursors gave rise to pure neuronal clones. This large difference can also not be explained by selective cell death, since the survival rate of sorted cells is about 70% (see Materials and Methods). In contrast, when GFP-positive cells were isolated from E18 cortex, only few neuronal clusters were detected (29%, *n*=527) and their number hardly exceeded the value of neurons determined directly after the sort (22%, *n*=500). We also noted that the non-neuronal clones generated by GFP-positive cells sorted from E18 cortex were further differentiated compared with the non-neuronal clones derived from E14 cortex precursors. Non-neuronal clones from E18 precursors were composed exclusively of GFAP-positive cells (Fig. 3I,J), while those from E14 cortex contained in most cases some GFAP- and some nestin-positive cells (Fig. 3G,H). Taken together, these results suggest that the majority of precursor cells sorted by their green fluorescence from the hGFAP-GFP transgenic mice at E14 and E16 differentiated into neurons, while the majority of cells isolated from E18 cortex differentiated into GFAP-positive astrocytes (Fig. 2).

To examine the progeny of GFP-positive precursor cells further, we also analyzed exclusively cells that had divided in vitro, as assessed by BrdU incorporation (Fig. 4A, see Materials and Methods). This analysis was performed to exclude the contamination of 20% postmitotic neurons included in the sort. Most clones (95%) derived from dividing precursors isolated from the cortex of hGFAP-GFP transgenic mice at E14 were composed of a single cell type (Fig. 4A). Again, half of all clones generated by GFP-positive precursor cells contained only neurons (Fig. 4A) and similar results were obtained at E16 (data not shown). Since 90-100% of the sorted precursors were GLAST immunoreactive, these results demonstrate that precursors expressing astroglial markers contribute to the generation of neurons during neurogenesis. Similar results were obtained when cells were isolated from the developing spinal cord (data not shown) suggesting that the neuronal progeny of precursors containing GLAST is not a peculiar feature of the developing cortex.

Morphological tracing of precursors with long radial processes

In the transgenic mice expressing GFP under the control of the hGFAP promoter, GFP was localized mostly in precursor cells with a glial expression pattern (GLAST immunoreactive), some of which clearly had long radial processes. However, as

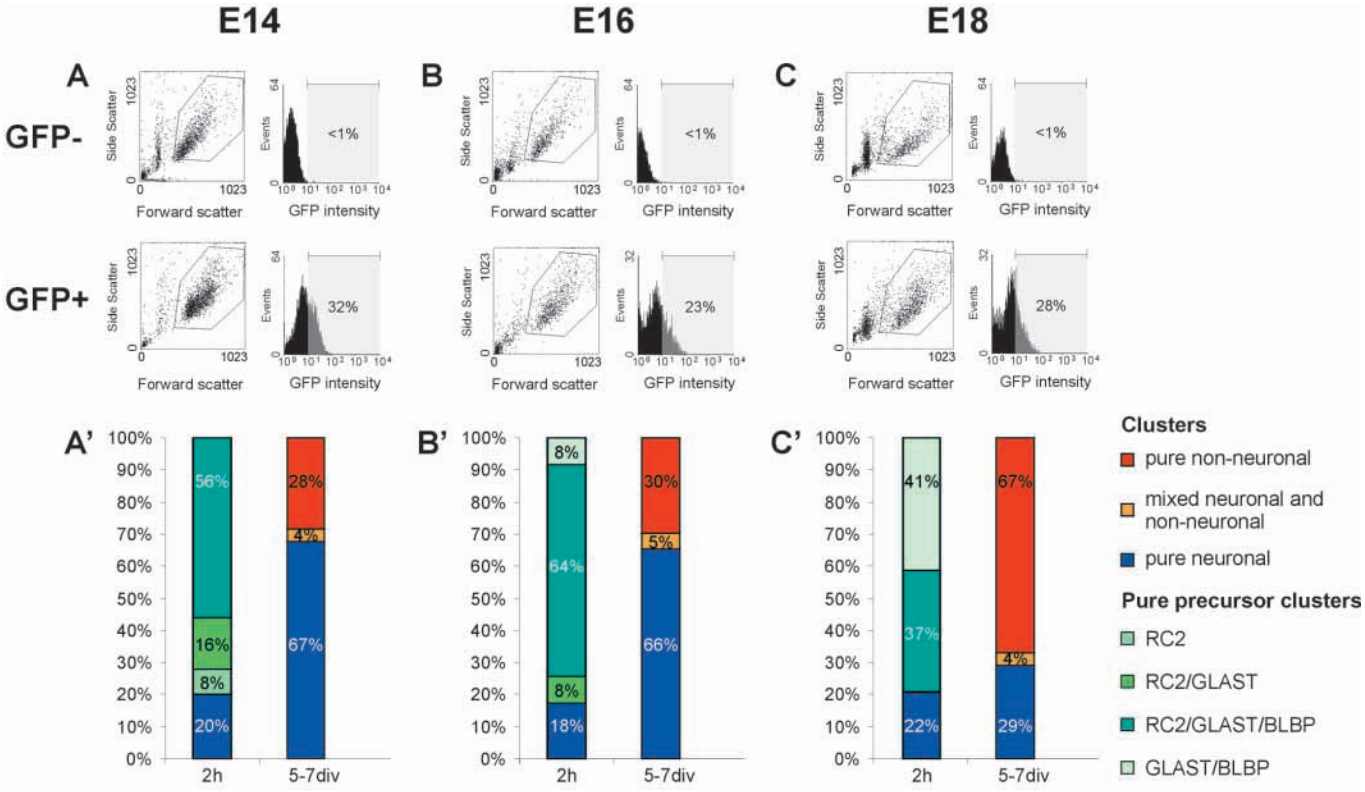

Fig. 2. Characterization of green fluorescent cells isolated from the cortex of hGFAP-GFP transgenic mice and their progeny. Green fluorescent cells were isolated from the transgenic mouse line 94-4 expressing GFP under the control of the human GFAP promoter (Zhuo et al., 1997; Fig. 1). (A-C) depict examples of sort profiles of cells from E14 (A), E16 (B) and E18 (C) cortex. The left columns in (A-C) show the dot plots of cells in forward scatter (FSC) and side scatter (SSC) with a polygon indicating the gate selecting the healthy cells. The histograms in the right columns in (A-C) show the number of cells ('events', y-axis) with a fluorescent intensity indicated on the *x*-axis of wild-type controls (upper panels) and hGFAP-GFP transgenic littermates (lower panels). The percentage of fluorescent cells in the sort gate (grey shading, A-C) is indicated. (A'-C') depict the composition of cells 2 hours (2h) or 5-7 days after the sort (days in vitro, div). A few cells sorted from mouse cortex were plated on a rat cortex feeder layer and identified by their M2M6 immunoreactivity, as depicted in Fig. 3. Clusters of M2M6-positive cells after 2 hours were mostly (97%) single cells and were double stained with antiserum directed against β-tubulin-III as a neuronal and RC2, GLAST, BLBP, Ki67 or nestin as precursor markers. Neurons are depicted in blue, precursor cells in green, indicating their differential RC2, RC2/GLAST, RC2/GLAST/BLBP or GLAST/BLBP immunoreactivity as depicted in the figure. Note that almost all sorted precursor cells are GLAST immunoreactive. Clusters after 7 div contained, in most cases, several cells and were interpreted as clones derived from single plated precursor cells. Clusters were classified as pure neuronal when all cells of a cluster were β-tubulin-III immunoreactive (see example in Fig. 3E,F) and their proportion is depicted in blue, as pure non-neuronal when no cell of a clone was β-tubulin-III immunoreactive (depicted in red), but cells were nestin or GFAP positive (see examples in Fig. 3G-J) and as mixed neuronal and non-neuronal when clones contained β-tubulin-III-positive and -negative cells (depicted in orange). Note the large increase in the number of neuronal clusters at E14 and E16 (A',B'), suggesting that most GLAST-positive precursors generate neurons at this stage. In contrast, the progeny were mostly astrocytes when cells were isolated from an E18 cortex (C').

mentioned above, it is difficult to exclude the possibility that GFP is also contained in precursor cells with a different morphology, which might act as neuronal precursors. We therefore proceeded to select precursor cells based on their morphology. As previously described (Voigt, 1989; Götz et al., 1998), application of the fluorescent tracer DiI on the surface of the cortex labels cells in contact with this surface, namely neurons and radial glial cells (Fig. 5A; see Materials and Methods). This morphological tracing allows the selective labeling of those precursor cells with a process reaching the pial surface, because the somata of precursor cells are located in the ventricular and subventricular zones. No dividing cells have been detected close to the pial surface in the developing cortex (see, for example, Bayer and Altman, 1991; Götz and Bolz, 1992; Götz et al., 1998).

This tracing method therefore depends crucially on the limited spread of the tracer to label exclusively cells reaching close to the pial surface. To ensure this, in most of our experiments we used fluorescent microsphere beads that are taken up by endocytosis and hence do not diffuse (Katz et al., 1984). Fig. 5B,B' depicts the restriction of the tracer to the place of application. The limited spread of tracer also depends crucially on an intact telencephalon. To evaluate this parameter in our experiments, we mimicked potential damage inflicted during the dissection and analyzed the proportion of neurons and precursor cells labeled from the pial surface of an intact or severed cortex (see Materials and Methods). Tracer application onto an intact cortex resulted in a large proportion of labeled postmitotic neurons and only 15±2% of the labeled cells were precursor cells containing nestin or Ki67 (Fig. 5C; n=974). In contrast, the proportion of labeled precursor cells was increased (38±7%, n=594), when we had pinched small holes

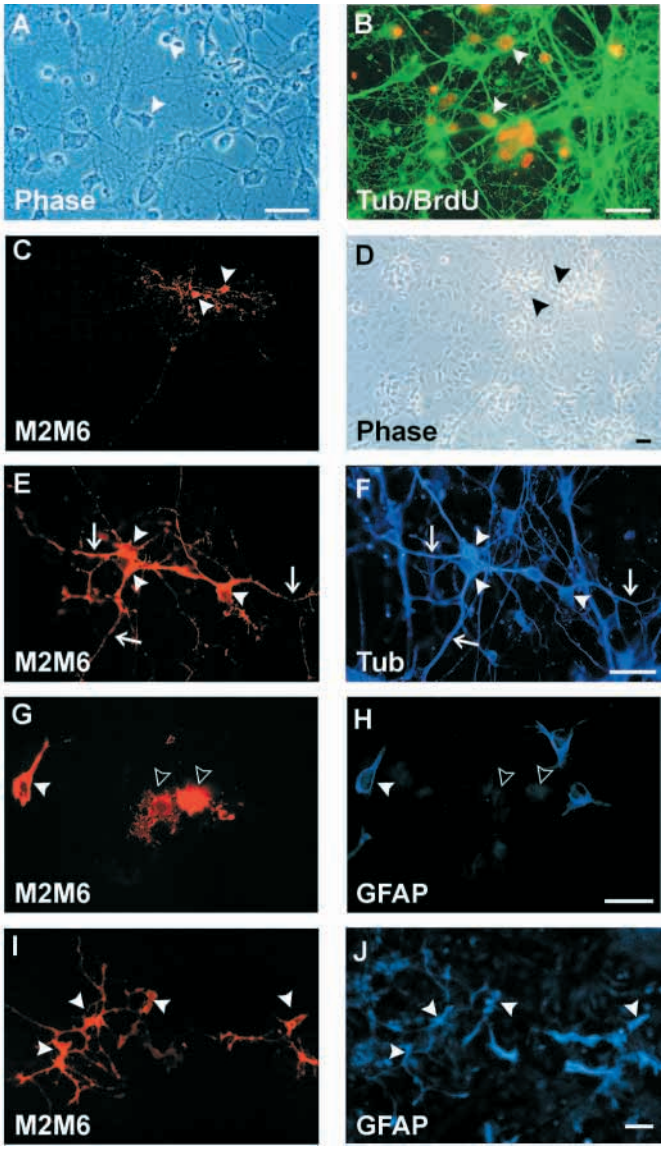

Fig. 3. Examples of the progeny of hGFAP-GFP- and GLAST-positive precursor cells isolated by fluorescence-activated cell sorting. Cells were sorted from E14 (A-H) and E18 (I,J) mouse cortex by green fluorescent protein content driven from the human GFAP promoter (Zhuo et al., 1997; see Fig.1, sort gates as in Fig. 2). The sorted cells were cultured for 5-7 days. In C-J, sorted cells were cultured on a rat cortex feeder layer of the corresponding age and identified by the mouse-specific antibody M2M6 (Lagenaur and Schachner, 1981; Lund et al., 1985) (C,E,G,I). Clusters of labeled cells were considered as clones derived from a single sorted precursor cell, as illustrated in the overview in C,D. Cell-type-specific antibodies were used as indicated in the panels to identify the composition of the clones. Pure neuronal clones were composed exclusively of β-tubulin-III-positive cells extending neurites marked by arrows (E,F). Neurons were generated in vitro and incorporated BrdU (red in B). An example of a non-neuronal clone generated from E14 precursors containing a GFAP-positive cell (filled arrowhead) is depicted in G,H. (I,J) A non-neuronal clone composed exclusively of GFAP-positive astrocytes generated by cells sorted from E18 cortex. Filled arrowheads indicate double-labeled cells, open arrowheads indicate single-labeled cells in corresponding micrographs. Note that GLAST-positive precursor cells generate neurons and astrocytes in two separate lineages. Scale bars: 25μm.

in the cortex and the tracer had access to the ventricle. This confirmed the restriction of the fluorescent dyes to the pial surface of an intact cortex, thereby labeling neurons and only a subpopulation of precursors, namely those with radial processes reaching the pial surface. Besides visual inspection of the ventricular surface under the fluorescence microscope, we always analyzed the composition of sorted cells immediately after the sort. We could thereby use the reproducibly small proportion of precursor cells labeled from the pial surface of an intact hemisphere to monitor that no tracer had entered the ventricle. Last, we tested a potential spread of dye from labeled to unlabeled cells during the dissociation procedure. Hemispheres from mouse cortex labeled from the pial surface were dissociated together with unlabeled hemispheres from rat. M2M6 immunoreactivity was then used to discriminate mouse from rat cells and revealed that only 3% of bead-labeled cells were M2M6-negative rat cells (n=400). Similar results were obtained with DiI and in experiments using green mice instead of rats (Okabe et al., 1997; see Materials and Methods). Thus, there is a negligible spread of the dye during dissociation. Taken together, these controls demonstrate the specific labeling of a subset of precursor cells from the pial surface.

Further immunochemical analysis of the sorted precursor cells, identified by their Ki67 or nestin immunoreactivity and the absence of β-tubulin-III immunoreactivity, proved their similarity to the GFP-sorted precursor cells (Fig. 5C). As for the GFP-sorted precursors, the precursor cells labeled by fluorescent tracing from the pial surface were all RC2 immunoreactive and about 90% were GLAST immunoreactive. Also the proportion of BLBP-immunoreactive precursors among the morphologically traced precursors (65%) closely resembled the value obtained from the GFP-positive precursors isolated from E14 cortex (70%). These results suggest that both methods label the same precursor population, namely cells with long radial processes and glial expression, i.e. radial glial cells.

Progeny of cortical precursors with long radial processes

To analyze the progeny of the morphologically traced precursor cells, we examined exclusively clones that incorporated BrdU during the time in vitro (Fig. 6A,B), thereby excluding the high proportion of postmitotic neurons included in the sort. At E14, half of the progeny of sorted precursor cells (BrdU-positive) were neuronal (Fig. 4C, D; 51%: 45±5% pure neuronal, 6±3% mixed neuronal clones, n=56) and similar results were obtained at E16 (53%: 41±17% pure neuronal, 13±5% mixed clones, n=25). Thus, at mid-neurogenesis half of all precursors with long radial processes give rise to neurons and most of these neurogenic precursors generate exclusively neurons. Note that these data are very similar to the BrdU-containing clones obtained with GFP-sorted cells from the 94-4 mouse line (Fig. 4A,B: 52%: 45±8% pure neuronal, 7±4% mixed clones, n=45).

The implication of this similarity is that both methods seem to isolate largely the same population of cells, namely radial glial cells. This is further supported by the similarity of results obtained when both labeling techniques were combined and GFP-positive precursors with long radial processes labeled by bead application on the pial surface were isolated from E14

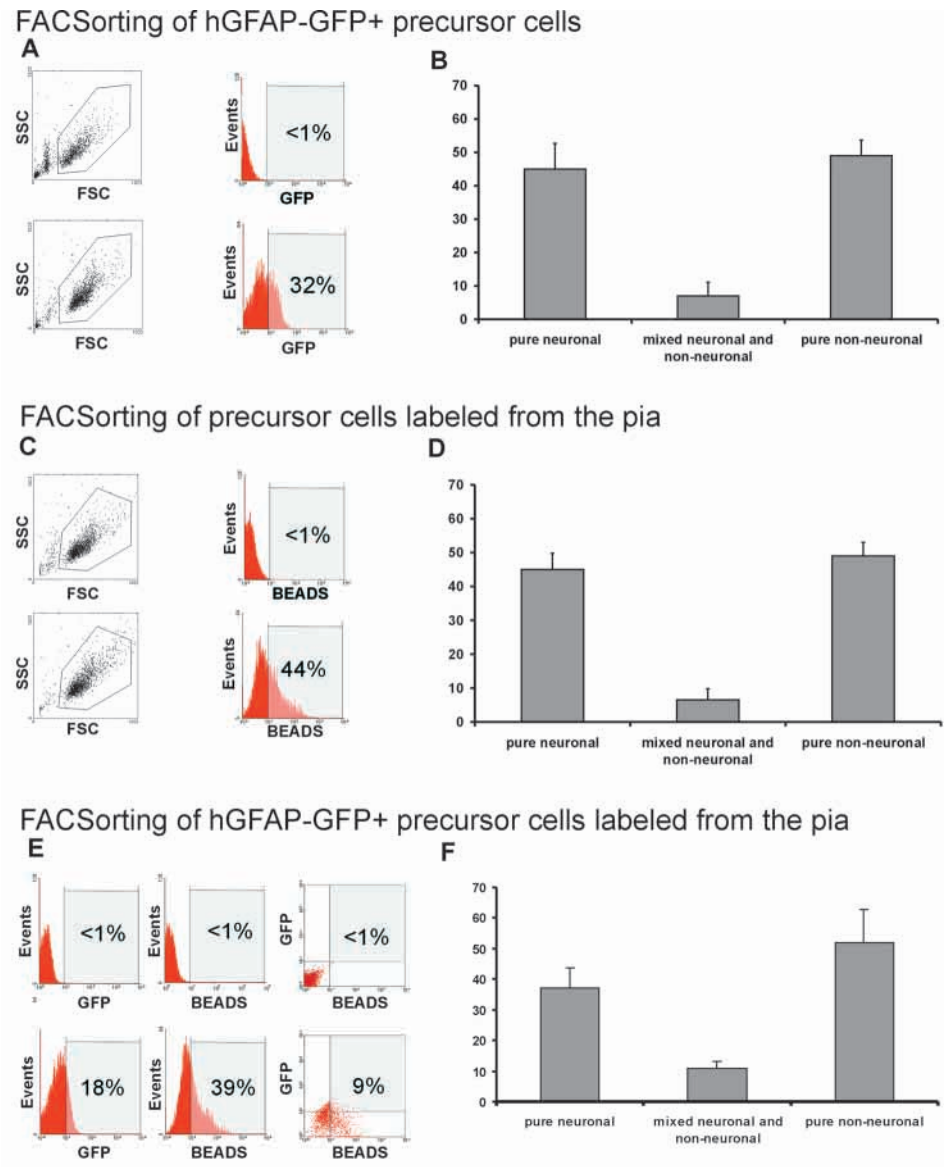

Fig. 4. Fluorescence-activated cell sorting of radial glial cells from E14 cortex. Cells were fluorescently labeled by GFP using the transgenic mouse line 94-4 (Zhuo et al., 1997) (A,B), by red fluorescent tracers from the pial surface (C,D) and by both (E,F). The left column in A,C shows the dot plots of cells and the histograms of distribution of the unlabeled (upper panels) and labeled cells (lower panels) versus their fluorescence intensity (see Fig. 2 for description). The percentage of fluorescent cells in the sort gate (gray shading, A,C) is indicated. In E, the percentages of green and red fluorescent cells are depicted in two separate histograms and the sort gate of the double-fluorescent cells is shown as gray shading in the dot blot. (B,D,F) The progeny of dividing sorted precursor cells (labeled by M2M6 and BrdU, see Fig. 6) after 5-7 days in vitro obtained with the respective sort indicated in the left column. Clones were classified as described in Fig. 2. The clonal composition of the progeny generated by precursor cells sorted by glial marker expression (GFP expression under the control of the hGFAP promoter, GLAST positive) (B); by precursors with long radial processes (D) and by double-labeled precursor cells (F). Note that the progeny is almost identical in the three sort paradigms indicating that all label radial glial cells. Radial glial cells generate neurons and glial cells in equal amounts in two distinct lineages.

hGFAP-GFP transgenic mice (Fig. 4E; see Materials and Methods). Note that bead application on the pial surface labels stochastically some, but not all cells in contact with the pial surface. It is therefore not surprising that only half of all GFP-positive cells are double labeled by beads applied to the pial surface and this does not imply that half of the GFP-positive cells would not contact the pial surface. Conversely, we know that about 85% of the bead-labeled cells are postmitotic neurons and only 15% are precursor cells as described above. Consistently, in the example depicted in Fig. 4E, the 9% GFP-bead double-labeled cells constitute 23% of the bead-labeled cells (39%). When we analyzed the progeny of these double-labeled precursors isolated from E14 cortex, also a prominent proportion of neuronal clones was detected (37±2%, n=46, Figs 4E,F, 6C,D).

DISCUSSION

Our results show that neither the expression of molecules later specific for astrocytes, such as GLAST, nor the morphology of precursor cells, such as a long radial process, is predictive of a particular progeny. As a result, precursor cells labeled by both these criteria, i.e. radial glial cells, also generate neurons as well as glial cells. Importantly, radial glial cells of the cerebral cortex generate neurons and astrocytes in two distinct lineages, as is typical for cortical precursors in vivo (see, for example, Grove et al., 1993; Reid et al., 1995). It seems therefore unlikely that the sorted precursor cells express a broader potential in vitro than in vivo. These results considerably revise the current view on radial glial cells and have important implications on the nature and origin of precursors involved in neurogenesis of the adult nervous system.

Morphological precursor heterogeneity

A potential link between the morphology of precursor cells in the developing CNS and their progeny has been subject of considerable controversy since the late 19th century (e.g. His, 1889; Schaper, 1897). At early stages, prior to the onset of neurogenesis, all precursor cells have the same morphology

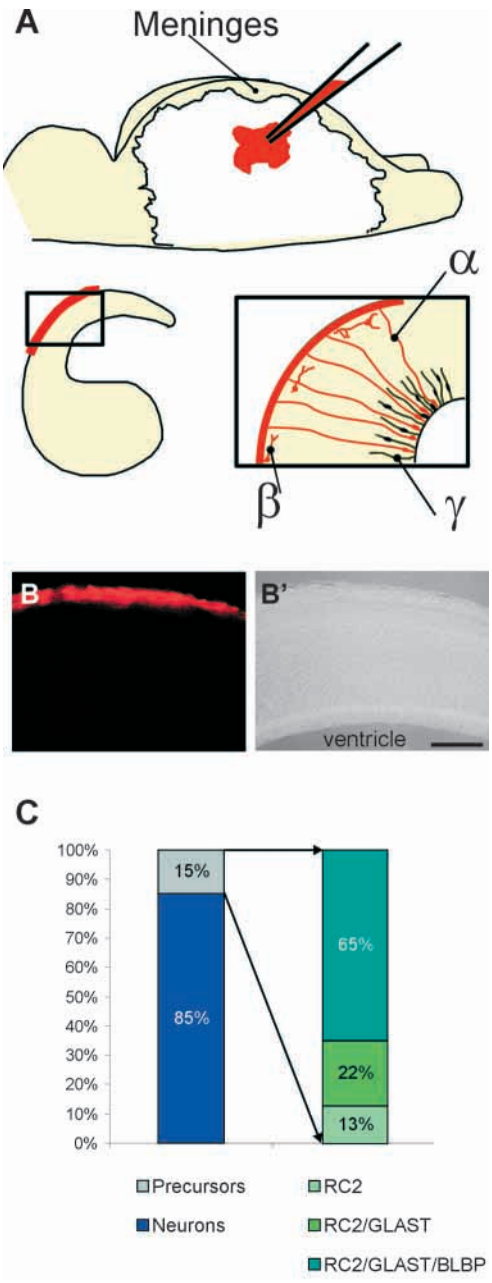

Fig. 5. Fluorescent tracing of cells from the pial surface. (A) The application of the red fluorescent tracers DiI (Voigt, 1989; Götz et al., 1998) or microsphere beads (Katz et al., 1984; Voigt, 1989) on the surface of a cerebral hemisphere after removal of the meninges. A schematic drawing of a section through the cortex indicates the red fluorescent dye on the brain surface (lower left drawing in A) and the back-traced radial glial cells (α), neurons (β) and unlabeled precursor cells with a short process (γ). (B,B') Corresponding micrographs showing beads fluorescence (B) and phase contrast (B') of a 100 μm frontal section of E14 telencephalon 20 minutes after application of the dye on the surface of the cortex. (C) The composition of sorted cells labeled from the pial surface of E14 cortex. β-tubulin-III-immunoreactive cells are depicted in blue and precursor cells in gray. The composition of the precursor cells is depicted in the second bar. Note the similarity in the composition of precursors sorted by their morphology via pial tracing and/or their GFP-content driven by the hGFAP promoter (Fig. 2). Scale bar: 100 μm.

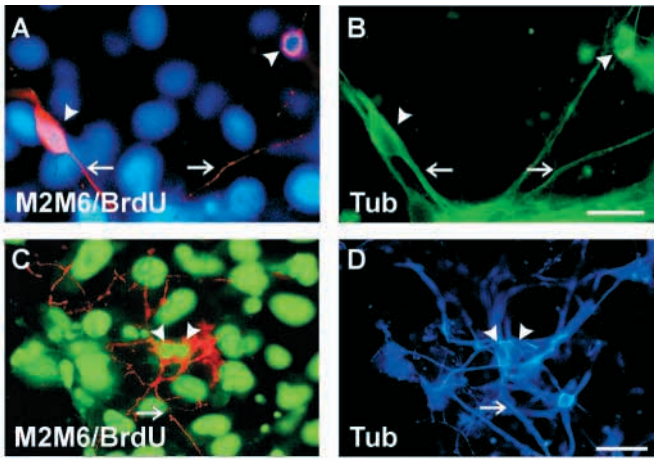

Fig. 6. Examples of the progeny of precursor cells fluorescently labeled from the pial surface and isolated by fluorescence-activated cell sorting. (A-D) Corresponding micrographs of neuronal clones derived from precursor cells of E14 mouse cortex after 7 days in vitro. The clone in A,B is derived from cells labeled by red fluorescent microsphere beads from the pial surface and sorted as depicted in Fig. 4C; the clone in C,D originates from cells double labeled by beads from the pial surface and GFP-content driven by the hGFAP promoter (Zhuo et al., 1997, Fig.1) sorted as in Fig. 4E. Clones of precursor cells that divided in vitro were identified by their M2M6 immunoreactivity (red in A,C; see Fig.3) and BrdU content (blue in A, green in B). β-tubulin-III immunoreactivity (green in B, blue in D) was used to identify the neuronal nature of the progeny. Filled arrowheads indicate triple-labeled cells (M2/M6, BrdU, β-tubulin-III immunoreactive) and arrows depict β-tubulin-III immunoreactive neurites. Note that precursors labeled by both their long radial processes and their glial expression pattern, i.e. radial glial cells, generate pure neuronal clones. Scale bar: 25 μm.

and span the entire thickness of the wall of the neural tube (Sauer, 1935; Stensaas and Stensaas, 1968; Huttner and Brand, 1997). At the onset of neurogenesis, two distinct subsets of precursor cells then differentiate: many precursors have lost their contact with the pial surface of the brain, while some extend processes from the ventricular to the pial surface. The latter are radial glial cells and are distinguished by the loss of tight junctional coupling and their specific antigenic profile from early neuroepithelial cells that also span the entire wall of the neural tube. Neuroepithelial cells are coupled by tight junctions that are lost around the onset of neurogenesis (Huttner and Brand, 1997). This is accompanied by a downregulation of E-cadherin and corresponding upregulation of N-cadherin (Aaku-Saraste et al., 1996). Conversely, the RC2 antigen, GLAST and BLBP, appear around the onset of neurogenesis on cells with long radial processes and are not expressed by earlier neuroepithelial cells (Edwards et al., 1990; Kurtz et al., 1994; Feng et al., 1994; Hartfuss et al., 2000). Similarly, GFAP immunoreactivity in the developing primate cortex appears at early stages of neurogenesis (Levitt and Rakic, 1980; Levitt et al., 1983). Thus, the RC2- and GLAST-immunoreactive cells isolated in our study 3 days after the onset of neurogenesis in the cortex (E11, Bayer and Altman, 1991) clearly differ from neuroepithelial cells, as identified by both their morphological and molecular characteristics.

The morphological tracing of precursors with a long radial process was performed by application of tracers on the surface

of the cerebral hemispheres. The specificity of this previously established labeling (Voigt, 1989; Götz et al., 1998) was reassured by a variety of controls. First, diffusion into the cortex was avoided by using latex microsphere beads that are taken up by endocytosis (Katz et al., 1984). Second, the restriction of the tracer to the place of application was confirmed in sections cut immediately after the labeling and the specificity of labeling was further assured at the cellular level by the observation that only a small population of precursor cells was labeled from the pial surface. Last, we also proved that the tracers were not passed on to unlabeled cells during the dissociation procedure. We are therefore confident that the brief application of tracer on the pial surface of cerebral cortex results in the specific labeling of a subset of precursor cells with processes reaching close to the pial surface.

Indeed, the antigenic profile of this morphologically traced precursor population closely resembled the one selected on the basis of its GFP expression (see also below), further supporting the notion that both techniques label the same precursor population, namely radial glial cells. Almost all precursor cells isolated by labeling from the pial surface express GLAST, and most of them also co-express the RC2 antigen and BLBP. All of these molecules have been described in radial glial cells (Misson et al., 1988; Edwards et al., 1990; Feng et al., 1994; Kurtz et al., 1994; Shibata et al., 1997). Moreover, RC2 and BLBP have been detected in radial glial cells supporting migrating neurons (Misson et al., 1991; Feng et al., 1994; Anton et al., 1997). Despite their radial morphology and radial glial marker expression, half of these precursors generated only neurons and the remainder generated only non-neuronal, mostly astroglial cells. Precursors with long radial processes are therefore not specified towards the glial lineage, but rather contain functionally distinct precursor subsets including a prominent proportion of neuronal precursors. It appears then, that morphological differences between precursor cells, such as a long or short radial process, are not predictive of a particular progeny. We are currently investigating whether precursor cells labeled from the basal or apical surface might differ in other parameters. One might expect, for example, differences in the length of the cell cycle, since dividing cells have to retract their processes during M-phase (Hinds and Ruffet, 1971), which might take longer for precursors with long processes (see also Schmechel and Rakic, 1979).

Molecular precursor heterogeneity

Much of the view of distinct neuronal and glial precursor subsets hinges on the early appearance of GFAP-immunoreactive precursor cells in primate cortex (Levitt and Rakic, 1980; Levitt et al., 1981, 1983). Since retroviral cell lineage experiments in the primate and rodent cortex in vivo confirmed the co-existence of functionally distinct precursor subtypes (Grove et al., 1993; Reid et al., 1995; Kornack and Rakic, 1995), this further supported the suggestion that the precursor subtypes expressing glial markers might be glial precursors. Here we tested this prediction directly by isolating precursor cells that express GFP under the control of the human GFAP promoter (Zhuo et al., 1997). In addition to the transgene regulated by the primate GFAP promoter that is later restricted to astrocytes (Brenner et al., 1994; Zhuo et al., 1997), these precursors also contain GLAST, which is later restricted to astrocytes (Ullensvang et al., 1997). In addition most of these precursors also co-express BLBP, another molecule that is later restricted to astrocytes. Despite all their astroglial characteristics these precursors contain a prominent neuronal lineage. The comparison of the neuronal clones arisen from proliferating precursors to the overall clonal composition even suggests that some GLAST-positive precursor cells differentiate into neurons without further cell division. The detection of a weak GFP signal in some neurons further supports the notion that GFP-expressing precursors generate neurons also in vivo.

During further development, the progeny of these precursor cells changes. While about half of all GFP-positive precursors from E14 cortex generate neurons, hardly any neuronal clones can be detected at E18. At this stage, the progeny of GFP-positive cells consists then almost exclusively of GFAP-positive astrocytes, consistent with previous data on the transformation of radial glia into astrocytes (Voigt, 1989; Culican et al., 1990). This change in progeny is accompanied by a change in the antigenic profile of the sorted precursor cells. While BLBP is increased, the RC2-antigen has decreased during development. Indeed, the analysis of RC2, GLAST and BLBP immunoreactivity in the cortex demonstrates that the antigenic profile of radial glial cells changes during development (Hartfuss et al., 2000). Since there is no quantitative correspondence between the different neuronal and glial lineages of sorted precursor cells and the subtypes identified by their antigenic profile, the change in the antigenic profile might reflect a gradual maturation of radial glial cells that is accompanied by changes in their progeny.

Radial glial cells and neurogenesis

Taken together, precursor cells labeled by the two main characteristics of radial glial cells contain a large proportion of neuronal progenitor cells. This is not unique to the developing cortex, since we also confirmed a neuronal progeny of radial glial cells in other regions of the developing CNS, such as the spinal cord. We therefore suggest that the ubiquitous radial glial cells contribute to the generation of neurons and glial cells throughout the mammalian CNS during development.

Interestingly, radial glial cells in the avian CNS seem to play a similar role. Retrovirally mediated cell-lineage analysis in the developing chick midbrain reported radial glial cells as the earliest component of mixed clones containing neurons and glial cells (Gray and Sanes, 1992). These data, however, could not discriminate whether the radial glial cells are the earliest descendants of a multipotential precursor or radial glial cells themselves are the multipotential precursors. Taken together with our direct evidence for a neurogenic role of mammalian radial glia, it appears as if vertebrate radial glia generate a broad range of descendants, either in different lineages as in the mammalian cerebral cortex or within a single multi-cell type clone, as in the chick midbrain.

Such a broad role as precursors for radial glial cells is further supported by evidence that they also act as neurogenic precursors in the adult CNS. There is a clear link between the persistence of radial precursors and neurogenesis in the adult avian CNS (Alvarez-Buylla et al., 1990). Radial glial cells persist in the forebrain of songbirds in those regions where new neurons are generated (Alvarez-Buylla et al., 1990). Radial glial cells also persist into adulthood in other classes of vertebrates (for a review, see Margotta and Morelli, 1997) and

differ in their intermediate filament composition between amphibians with a high regenerative capacity and reptiles that show less regenerative capacity (Margotta and Morelli, 1997). Thus, even in the adult vertebrate CNS radial glial cells seem to contribute to the generation of neurons such that the presence of radial glial cells is correlated to neurogenesis both in phylogeny and ontogeny.

Radial glial cells and stem cells of the adult CNS

In the forebrain of adult mammals, however, radial glial cells do not persist. There astrocytes and ependymal cells have been implicated as stem cells maintaining a neurogenic potential into adulthood (Doetsch et al., 1999; Johansson et al., 1999). Interestingly, astrocytes and ependymal cells (including tanycytes) arise from radial glial cells (Voigt, 1989; Bruni, 1998). Our results might therefore imply that these cells act as stem cells in the mammalian brain by dedifferentiation into an earlier radial glial-like progenitor state. Indeed, astrocytes can be instructed to resume morphological and/or molecular characteristics of earlier radial glial cells (Hunter and Hatten, 1995; Soriano et al., 1997; Leavitt et al., 1999). For example, transplantation of granule cells into the adult cerebellum induces the re-expression of nestin and the RC2 antigen, two cytoskeletal proteins that are restricted to earlier developmental stages (Sotelo et al., 1994; Soriano et al., 1997). The dedifferentiation of astrocytes into radial glia-like cells is also triggered under in vitro conditions by the addition of neurons or a diffusible protein of about 60kD (Hunter and Hatten, 1995). While these studies suggest that dedifferentiation into radial glial cells serves to support neuronal migration, recent evidence is consistent with their role as a source of de novo generated neurons in the adult cerebral cortex (Leavitt et al., 1999; Magavi et al., 2000). Phototoxic lesions in the adult cortex lead to dedifferentiation of astrocytes into radial glial-like cells re-expressing the RC2 antigen (Leavitt et al., 1999) and at the same time to endogeneous de novo neurogenesis (Magavi et al., 2000). This raises the exciting possibility that dedifferentiation of astrocytes into an earlier radial glial cell type might be accompanied at least under specific conditions by the resumption of the earlier neurogenic potential. A challenge for the future will therefore be to understand the progression of radial glial cells from mainly neuronal to mainly glial lineages and how this process could be reverted.

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