Development 117, 553-561 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Multiple restricted lineages in the embryonic rat cerebral cortex

Elizabeth A. Grove, Brenda P. Williams, Da-Qing Li, Mohammad Hajihosseini, André Friedrich and Jack Price

National Institute for Medical Research,The Ridgeway, Mill Hill, London, NW7 1AA, UK

SUMMARY

We have labelled precursor cells in the embryonic rat cerebral cortex using BAG, a retroviral vector that expresses β-galactosidase. We had previously reported that labelled precursor cells generate clusters of labelled cells that could be classified into four types by their morphological appearance and anatomical distribution (Price and Thurlow, 1988). In this study, we have used immunohistochemistry and intracellular dye labelling to identify the cell types that make up these clusters. We discovered that clusters are almost always composed of a single cell type. In addition to clusters composed entirely of neurones, we found four different types of glial cell clusters. In the grey matter, glial clusters are composed either of protoplasmic astrocytes, or of cells that have an astrocyte morphology, but no glial filaments. In the white matter, clusters are composed of either fibrous astrocytes or oligodendocytes. Our results indicate that each of these different cortical cell types is generated from a separate population of precursor cells.

Key words: cell lineage, retroviral vectors, cerebral cortex, rat

INTRODUCTION

All the neurones and macroglial cells of the cerebral cortex are derived, directly or indirectly, from the precursor cells of the ventricular zone. It is unclear, however, whether the ventricular zone is composed of a single population of precursor cells, each equivalent in their developmental potential; or whether there are many different precursor cell types, each responsible for the generation of a subset of the final cell population.

In a previous study of rat cortex (Price and Thurlow, 1988), we applied the retroviral lineage-labelling technique to this problem using the BAG virus, which encodes the enzyme β-galactosidase (Price et al., 1987). We injected the virus into the cerebral vesicles of rats of 16 days embryonic age (E16) and analysed the resultant clones 14 days postnatally. We were able to distinguish discrete clusters of labelled cells, each of which we interpreted as a clone, that is, the progeny of a single infected precursor cell. The clusters were of four different types. They were made up of either (i) neurones, (ii) astrocytes of the grey matter, (iii) white matter cells or (iv) both white matter cells and neurones (Price and Thurlow, 1988). The white matter cells in this fourth type of cluster were probably oligodendrocytes (or their precursors), since we discovered clones composed of neurones and oligodendrocytes in a subsequent in vitro study. Using a similar approach, we observed the same four types of clusters in the hippocampal formation (Grove et al., 1992).

These findings were similar to those of Luskin et al. (1988), who also described clones composed of a single cell type. Subsequently, Parnavelas et al. (1992) suggested that there are at least two types of neuronal precursor cell, one for pyramidal and another for non-pyramidal neurones.

We interpreted this work to mean that there are separate subpopulations of precursor cells during the period of cortical neurogenesis, and that, with the exception of the N-O precursor, these cells are dedicated to the production of a single neuronal or glial cell type. Clearly, this is a major conclusion if true, because it would settle two important issues. First, it would say that in the cerebral cortex, the most important cell fate decisions are made relatively early in embryonic development. Second, it would remove the fear expressed by Walsh and Cepko (1988, 1992) that clones in the cortex cannot be distinguished from each other because they frequently overlap, since overlap would tend to mix clonal types.

For each type of cluster, however, there were questions left unanswered by our first study. In the case of the grey matter astrocyte clusters, there was simply a question of cell identification. These cells had been identified by their morphology and by the classical astrocyte property of having end feet contacting either blood vessels or the pial surface. Not all cells had obvious end feet, however, and astrocyte morphology is not a very precise criterion. Consequently, we could not be sure that all members of these clusters were astrocytes.

For both the neuronal and white matter glial clusters there were more profound questions. In some neurones, the β-galactosidase reaction product was found throughout the cell body and dendrites, making these cells easy to identify morphologically. Cortices analysed on P14, however, had few such clusters. Most putative neurones were stained only

around their nucleus and in one or two spots in the cytoplasm. At the time of our first study, we had no independent criterion to show that all such cells were neurones.

The data on white matter glia were incomplete for a different reason. Our original analysis at P14 was too early for differentiation to have occurred in most of the cells of the white matter (Parnavelas et al., 1983). Although myelination in the external capsule begins soon after birth in the rat (Wood and Bunge, 1984), most of the white matter clusters that we observed were made up of morphologically undifferentiated cells. We called these cells 'horizontal cells' because many of them were orientated along the axon tract. We assumed that they were glial precursor cells, but we did not know whether they would give rise to astrocytes, oligodendrocytes, or both.

In this study, we have again used retroviral labelling together with a variety of strategies to identify the different cell types in the cortex. We show that the dispersed clusters of labelled cells that appear in infected cortices are, with the exceptions we have already noted, composed of a single cell type. This is true not only in the grey matter, as we had previously supposed, but also in the white matter of the external capsule.

MATERIALS AND METHODS

Retroviral production and injection

Ecotropic BAG virus was produced, titred and concentrated as previously described (Price et al., 1987). Titres of virus between 10^5 and 10^7 per ml were used for injection depending on the experiment. Pregnant rats, on day 16 of gestation (E16: plug day being day zero), were anaesthetized with a mixture of Hypnorm (Jansen, Oxford, UK) and Hypnovel (Roche, Welwyn Garden City, UK) administered intraperitoneally. Following laparotomy, roughly 0.5 μl of virus, including 10 μg/ml of polybrene (Sigma) and a trace of Indian ink, was injected through the uterine wall into the left cerebral vesicle of each embryo using a hand-held Hamilton syringe and a 30 G needle. The wound was closed by suture, the rat was allowed to recover and the injected pups were born normally at term.

Preparation of brain tissue

Either 14 days (P14), or 28-29 days (P28/29) after birth, the injected animals were killed by perfusion under terminal Hypnorm/Hypnovel anaesthesia with 2% paraformaldehyde + 0.2% glutaraldehyde in 0.1 M Pipes buffer. The brains of these animals were left overnight in the same fixative, before sectioning on a vibratome. Sections were cut at either 150-200 μm (for all experiments involving either X-Gal staining alone, or X-Gal staining plus Lucifer yellow injections), or 70 μm (for experiments involving immunohistochemistry). X-Gal staining to detect β-galactosidase was as previously described (Price et al., 1987).

The brains of P3 β-galactosidase transgenic mice (Beddington et al., 1989) were perfused and fixed as above, then frozen on dry ice and cut at 20 μm on a cryostat. These sections were then X-Gal stained.

Cell injections

Fixed, X-Gal-stained sections were immobilised in small Petri dishes under an epifluorescence microscope. Individual X-Gal$^+$, nuclear-stained cells were identified by bright-field microscopy and impaled using a micromanipulator with a glass microelectrode (tip diameter less than 1 μm), filled with a 4% solution of Lucifer Yellow (Sigma) in water. The cell was filled by passing a negative DC current of 5 nA for 10-15 minutes.

Cell culture

Cultures were prepared from the cerebral cortex of E16 rat embryos as previously described (Williams et al., 1991). Briefly, cells were dissociated and plated onto monolayers of cortical astrocytes at a density of 2×10^5 per 13 mm coverslip. The cortical astrocytes were prepared as described by Noble and Murray (1984). Cultures were grown in Dulbecco's modified Eagle's medium, supplemented as described by Bottenstein and Sato (1979). Fresh medium was added every 2-3 days. One day after plating, cultures were infected with a titre of BAG that we had previously shown would give no more than ten marked clones per coverslip.

Immunohistochemistry

After staining for X-Gal, sections were treated with 1% Triton X-100 for 60 minutes, then incubated overnight in primary antiserum diluted in phosphate-buffered saline (PBS). The antisera used were mouse anti-GFAP (Boehringer-Mannheim, diluted 1:50) or mouse anti-MAP2 tissue culture supernatant, diluted 1:50 (antibody AP14, Binder et al., 1986). After extensive washing, the sections were incubated in an HRP-conjugated goat anti-mouse immunoglobulin serum (diluted 1:100, Bionuclear Service Ltd.). After further washing, sections were reacted in diaminobenzidine (5 mg/ml) plus 0.01% hydrogen peroxide in 50 mM Tris/HCl, pH 7.4.

Cultures were fixed after 10 days in vitro with 4% paraformaldehyde for 15 minutes at room temperature. After brief treatment with 1% Triton X-100, they were stained overnight at 4°C with a rabbit anti-β-galactosidase serum diluted 1:1000 (Williams et al., 1991), followed by fluorescein-coupled goat anti-rabbit immunoglobulin (diluted 1:100, Bionuclear Services Ltd.). Cultures were then fixed in acidified alcohol for 15 minutes at −20°C, then incubated for 30 minutes at room temperature with anti-MAP2 diluted 1:50. Finally, the cultures were incubated with biotinylated goat anti-mouse IgG1 (diluted 1:100, Bionuclear services Ltd.), followed by streptavidin coupled to 7-amino-4-methylcoumarin-3-acetic acid (diluted 1:100, Molecular probes Inc.). Cultures were mounted in glycerol, PBS solution (Citifluor). As controls, primary rabbit or mouse antibodies were replaced with preimmune serum or normal ascites fluid, respectively. No staining was observed in these controls. No staining was observed with the anti-β-galactosidase serum in cultures where no virus was administered.

RESULTS

Types of cluster

In one series of embryos, we injected the BAG retrovirus on E16 and analysed the resultant clones on P14. This repeated our previous experiment (Price and Thurlow, 1988), but here we used a lower titre of virus in order to obtain a lower clonal density (average 9±4 clusters per brain) and hence reduce the chance of clones being superimposed. As before, we discovered discrete clusters of cells, which were preliminarily classified using the anatomical and morphological criteria employed previously (Price and Thurlow, 1988; Moore and Price, 1992). Putative neurones were usually X-Gal stained exclusively in or around the nucleus, and/or in a small number of spots in their cytoplasm (see below). Neuronal clusters were usually composed of between one and four cells, occasionally more.

Table 1. Distribution of types of clusters found in BAG labelled cerebral cortices

Type of cluster	Percentage occurrence
Neurones	62
Grey matter astrocytes	9
White matter cells	13
Neurones + white matter cells	6
Three type mixtures	4
Unidentified	7

The data are from 6 brains, infected at low density (9±4 clones per brain). We did not include brains in which clusters were concentrated in one quadrant of the cortex. In these cases, it seemed likely that virus had been injected into the cortex itself rather than the ventricle, with the result that infections were clumped.

Clusters of putative astrocytes were larger, and composed of cells with an astrocytic morphology and, in many cases, blood vessel end feet. Other clusters were confined to the white matter of the external capsule or corpus callosum.

A series of 6 brains were analysed in this group (Table 1). As previously, most of the clusters were of a single type - neurones, grey matter astrocytes, or white matter cells - with two exceptions. Even with this low clonal density we saw, in a small number of cases, the same juxtaposition of labelled neurone and white matter cells that we had seen previously (Price and Thurlow, 1988). Rarely, clusters included all three of the identifiable cell types (Table 1). No other mixes were observed. In addition, a small number of clusters were composed of cells that could not be identified.

The identification of neurones

Neurones had been tentatively identified as those cells with the characteristic nuclear X-Gal staining. We did a number of experiments with further series of BAG infected brains to verify this identification. First, we injected Lucifer Yellow into 15 X-Gal$^+$ putative neurones from three infected brains, fixed at P14. This revealed the entire morphology of the cells, and in each case the nuclear-labelled cells were unambiguously neuronal (Fig. 1). The spots of cytoplasmic staining that we had noted were usually at the base of dendrites, or at dendritic branch points (Fig. 1).

Three other features of the distinctive X-Gal staining pattern became apparent. First, it was transient. We injected a series of E16 embryos that we allowed to survive until P28/29. Nuclear-labelled cells were rare in these brains. Instead there were many morphologically identifiable neurones (Fig. 2).

Second, the nuclear labelling pattern was not the result of the *lac-Z* gene being carried by a retrovirus, or expressed by the viral promoter. Sections were taken from the cerebral cortices of transgenic mice that carry the *lac-Z* gene driven off the rat β-actin promoter (Beddington et al., 1989). When stained with X-gal, these sections contained many cells labelled around the nucleus and in small spots in the cytoplasm, just like those we had found in BAG-infected rat brains (Fig. 3).

The third feature was that neurones in culture also had the nuclear staining pattern. Clones were labelled with BAG in cultures from E16 cortex as we have previously described (Williams et al., 1991). In many of the BAG-labelled neurones, the X-Gal reaction product was restricted to the nucleus (data not shown). Interestingly, they showed the same pattern when the β-galactosidase was detected by indirect immunofluorescence with an anti-β-galactosidase serum (Fig. 7). Hence, the restricted X-Gal-staining pattern reflects not only β-galactosidase activity, but also the actual distribution of the protein.

We also used immunohistochemistry to identify BAG-labelled neurones. MAP2 specifically labels dendrites in the cerebral cortex (Escobar et al., 1986). We used anti-MAP2 antibody (Binder et al., 1986) visualised by immunoperoxidase to ask if nuclear-labelled cells had MAP2$^+$ dendrites. We stained a series of sections from five brains fixed at P14 in which there were 12 nuclear-stained cells. The antibody stained many but not all of the dendritic profiles in the sections. Similarly, it stained seven of the twelve X-Gal$^+$ cells (Fig. 4). The remainder were either MAP2$^-$, or the staining was inconclusive. Another series of sections from P14 rats were stained with an antibody against the astrocyte marker, GFAP. We looked at 107 nuclear stained cells; none were GFAP$^+$ (data not shown). Similarly in sections from P28/29 animals, we looked at 46 cells with a neuronal morphology. All were GFAP$^-$ (Fig. 2). So, many of the nuclear-labelled cells were MAP2$^+$, but none stained with GFAP.

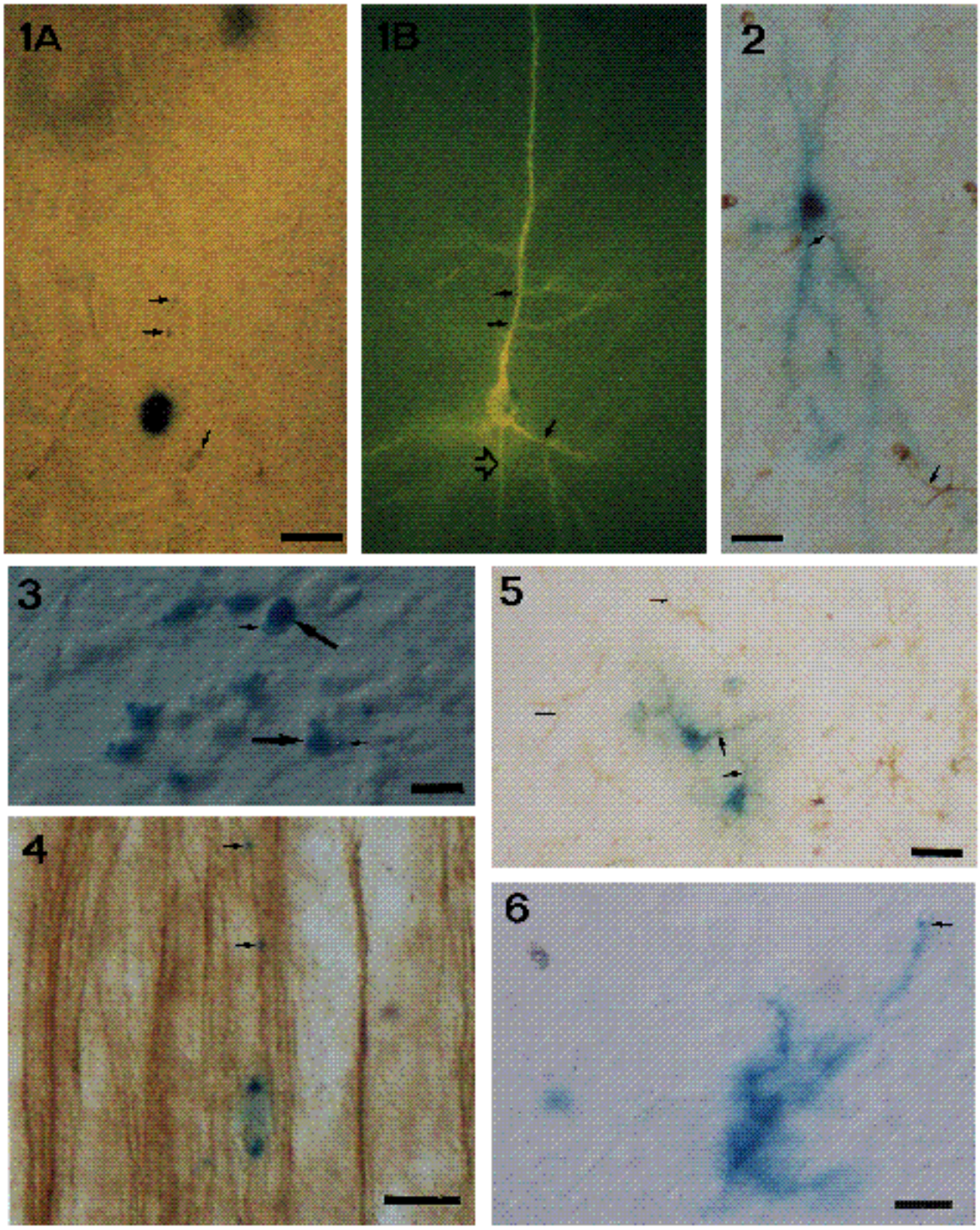
The identification of grey matter astrocytes

Several astrocyte clusters were identified by the criteria described above in sections taken from brains analysed at both P14 and P28. These sections were stained with anti-GFAP. We looked at 24 clusters, a total of 143 cells. Because of problems with tissue processing, six cells could not be defined as GFAP positive or negative, and one entire clone was questionable. This clone was composed of seven grey matter cells, two of which were GFAP$^+$. The other five looked like astrocytes, but were not clearly GFAP stained. All the remaining cells of the 24 clusters, however, were GFAP$^+$ (Fig. 5). As in the previous study (Price and Thurlow, 1988), these clusters were essentially confined to the grey matter. There was one exception, a pair of white matter astrocytes in an otherwise exclusively grey matter cluster (Fig. 6). These two cells were similar to the fibrous astrocytes found in white matter clusters, except that one had a blood vessel end foot, something rare in exclusively white matter astrocyte clusters (Fig. 9).

In addition to many typical astrocyte clusters, we found two clusters of cells that were astrocytic in morphology, yet were different in a number of other respects. They were both large clusters of over a hundred cells that had finely branched processes but no identifiable blood vessel end feet. At least some cells in clusters of GFAP$^+$ astrocytes usually have such end feet. Both clusters were found in older (P28) animals. We stained the sections containing these two clusters with anti-GFAP, and found that all the cells in the clusters were GFAP$^-$ (Figs 10, 11).

The identification of white matter clusters

In this work and in our previous study, clusters of white matter cells at P14 were composed predominantly of morphologically undifferentiated cells that we thought were probably glial precursors (Price and Thurlow, 1988). To test



Figs 1-6

this hypothesis, we allowed injected animals to survive until P28/29 before sectioning the brains, X-Gal staining the sections, and counterstaining with anti-GFAP. We looked at a series of 39 white matter clusters from six animals.

The clusters were of three sorts. Nine (22%) were indistinguishable from the undifferentiated cells that we had seen at P14 (data not shown, see Price and Thurlow, 1988). In the majority of the clusters (25: 61%), however, all the cells

Fig. 1. A nuclear labelled cell, filled with Lucifer Yellow. (A) Bright-field microscopy reveals the X-Gal reaction product to be limited to the cell body and to three points of staining some distance from the cell body (arrows). B shows the same cell filled with Lucifer Yellow. The cell has the distinctive morphology of a pyramidal neurone, including a basally directed axon (open arrow). The spots of staining are revealed as dendritic branch points (small arrows). Scale bar, 20 μm.
Fig. 2. A pyramidal neurone from an animal analysed on P28. The dendrites of this cell are clearly X-Gal stained (in comparison with Fig. 1A). The section was counterstained with anti-GFAP, and many $GFAP^+$ astrocyte processes can be seen to be stained (arrows). The $X\text{-}Gal^+$ cell is $GFAP^-$. Scale bar, 20 μm.
Fig. 3. X-Gal-stained cells in the cortex of a P3 β-galactosidase transgenic mouse. The cells show nuclear staining (large arrows) and staining of spots of cytoplasm (small arrows), similar to the pattern observed in BAG-infected tissue. Since expression of this vector begins to be turned off in embryonic life (Beddington et al., 1989), not all cells are stained. Scale bar, 20 μm.
Fig. 4. An X-Gal-stained neurone, counterstained with anti-MAP2. The dendrites containing the spots of staining (arrows). are $MAP2^+$. Scale bar, 20 μm.
Fig. 5. Part of a cluster of X-Gal-stained grey matter astrocytes, counterstained with anti-GFAP. Several $GFAP^+$ astrocytes can be seen (finer arrows), including the two blue cells (larger arrows). Scale bar, 20 μm.
Fig. 6. Two white matter cells, part of a cluster otherwise restricted to the grey matter. Note the blood vessel end foot (arrow). Scale bar, 20 μm.

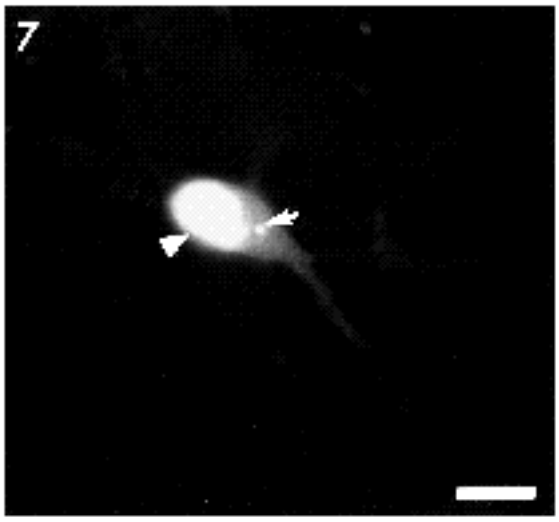


Fig. 7. A cortical neurone, infected with BAG in culture, stained with anti-β-galactosidase serum. The staining is restricted to the nucleus (arrowhead) and a cytoplasmic spot (arrow). Scale bar, 10 μm.

had the morphological features of oligodendrocytes (Figs 12, 13). Their processes ran largely parallel with the axonal tract, and often had club-shaped endings (Wood and Bunge, 1984). Their cell bodies were often found in rows, resembling intrafascicular oligodendrocytes. The oligodendrocyte clusters varied considerably in size, from as few as two cells per cluster to as many as 25 cells. The oligodendrocytes were $GFAP^-$, in contrast to the strongly $GFAP^+$ processes of the fibrous astrocytes (see below). Astrocyte processes also tended to run roughly parallel with each other, but at an angle to the processes of the oligodendrocytes.

A dominant feature of all the oligodendrocyte clusters was that they were very tightly grouped, so that individual cell bodies were rarely outside the halo of processes formed by other members of the cluster (Fig. 12).

The third type of white matter cluster (7 examples: 17%) was made up entirely of $GFAP^+$ astrocytes (Figs 8, 9). These clusters were also variable in size ranging from 1 to 17 cells. In most cases they looked exactly like the other surrounding fibrous astrocytes. White matter astrocyte clusters were more dispersed than oligodendrocyte clusters, nearest neighbours commonly being between 50 and 300 μm apart (Fig. 8). A common feature of all white matter clusters in P29 brains was that they often spread over into the deeper layers of grey matter. This was more prominent than it had been at P14.

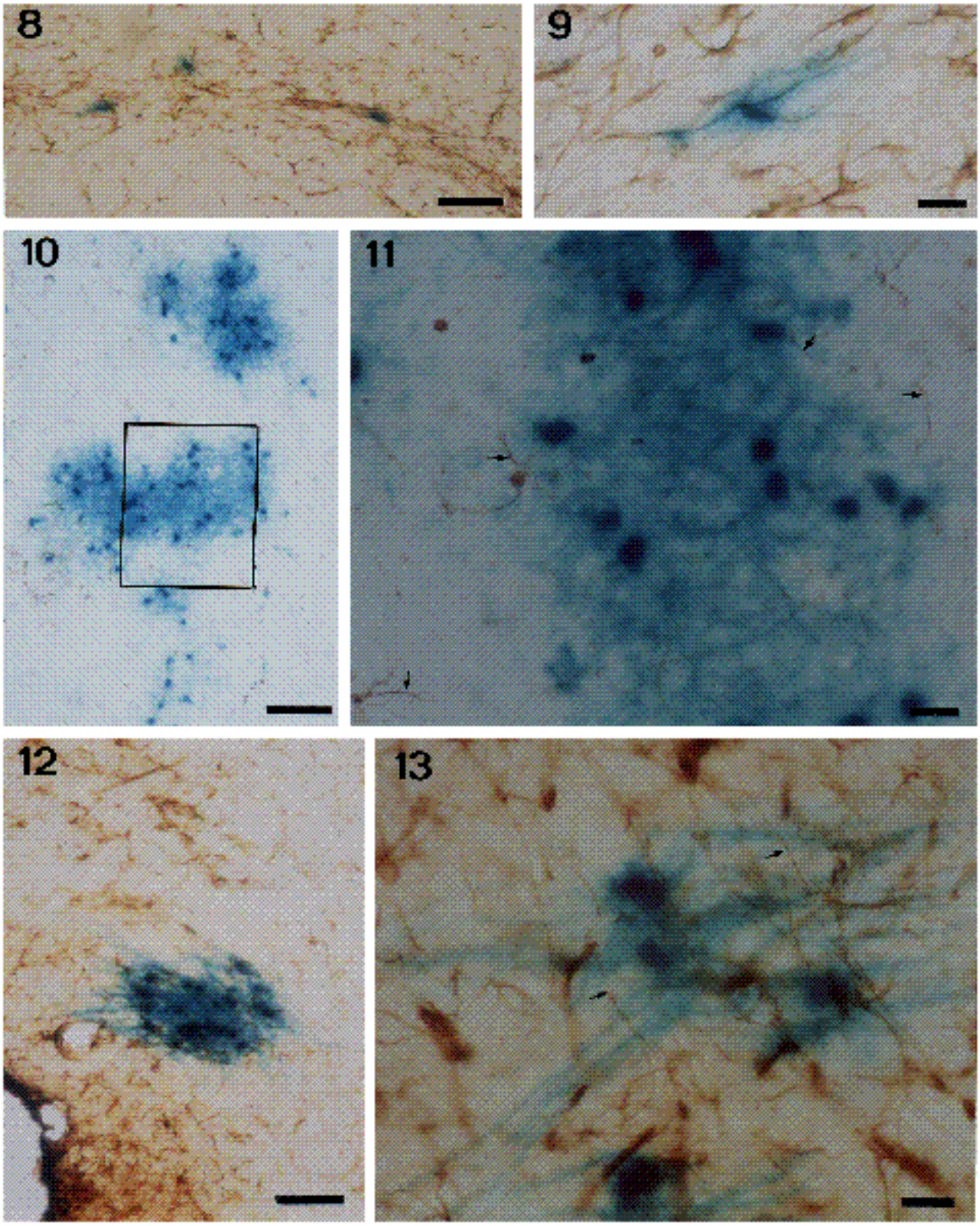
We were particularly interested in whether any white matter clusters included both oligodendrocytes and astrocytes. In no case were astrocytes found within the tight clusters of oligodendrocytes, neither were clusters of oligodendrocytes found within the larger area covered by the clusters of astrocytes. Only in brains in which there were more than 20 white matter clusters (data not included above), were there a few cases in which clusters of astrocytes were found 0.5-1 mm from clusters of oligodendrocytes.

DISCUSSION

The BAG retrovirus, when introduced into the cerebral vesicles of E16 embryos, labels discrete clusters of cortical cells, as detected at P14. These clusters are more dispersed than those found in the retina, using similar techniques (Turner and Cepko, 1987; Turner et al., 1990), but somewhat similar to those found in the chick optic tectum after longer survival times, where some types of neurone spread several hundred micrometers in the tangential plane (Gray and Sanes, 1991; Martinez et al., 1992). There are two important questions to be asked of these clusters of labelled cells. First, of what cell types are they composed? Second, what do they tell us about the precursor cells from which they are derived, particularly in the absence of independent evidence that each cluster is a complete clone?

In this study, we have shown that the majority of clusters were composed of a single cell type. The exceptions were the small number of clusters composed of neurones and white matter cells, and the rare clusters composed of all three of the major neural cell types. Neuronal clusters could be identified in P14 animals by a distinctive X-Gal staining pattern, which in neurones highlighted the nucleus, and the base and branch points of dendrites. We have shown that cells with this staining pattern had a neuronal morphology (as revealed by filling the cells with Lucifer Yellow) and were $GFAP^-$. The nuclear staining of neurones, though transient, could have important practical consequences for those studies in which nuclear and non-nuclear localised β-galactosidase are used as independent lineage labels (Galileo et al., 1990).

Many (but not all) nuclear-stained cells had $MAP2^+$ dendrites. Since not all cortical neurones were $MAP2^+$, it is



Figs 8-13

not surprising that this was also true of some X-Gal-labelled neurones. Consequently, the MAP2 staining helped confirm that the nuclear labelled cells were neurones although it is not a definitive neuronal marker.

Putative grey matter astrocyte clusters were of two types. The majority were made up of $GFAP^+$ cells, some of which had blood vessel end feet. A small proportion, however, were $GFAP^-$, and had highly branched processes, but no

Fig. 8. Low-power micrograph of part of a cluster of white matter astrocytes. Three blue cells can be seen, counterstained with GFAP. Scale bar, 100 μm.
Fig. 9. High-power micrograph of a X-Gal$^+$ white matter astrocyte. The blue cell is also stained with the anti-GFAP antibody. Scale bar, 20 μm.
Fig. 10. Low-power micrograph of a part of a cluster of putative grey matter astrocytes that are GFAP$^-$. This cluster was composed of over 100 cells. Scale bar, 100 μm.
Fig. 11. High-power micrograph of the boxed area of Fig. 10. Several GFAP$^+$ grey matter astrocytes can be seen (arrows), but none of the blue cells are GFAP$^+$. Note also the morphological differences between these blue cells and the GFAP$^+$ astrocytes of either the grey matter (Fig. 5) or white matter (Fig. 9). Scale bar, 20 μm.
Fig. 12. Low-power micrograph of a cluster of white matter oligodendrocytes. Scale bar, 100 μm.
Fig. 13. High-power micrograph of part of an oligodendrocyte cluster, counterstained with anti-GFAP. Note that the blue cells are GFAP$^-$. Their processes run roughly parallel with one another, and in the direction of the axons of the external capsule. The processes of the fibrous astrocytes, some of which are indicated by arrows, are GFAP$^+$ and also tend to run parallel, but in a different direction from those of the oligodendrocytes. Scale bar, 20 μm.

detectable blood vessel end feet. The identity of these GFAP$^-$ cells remains uncertain, since they resembled neither neurones nor oligodendrocytes. They could be the cells previously described as β-astrocytes (Reyners et al., 1982), which are now thought to be adult precursor cells (Reyners et al., 1986). They could be microglia. We would expect to see some clones of microglia (even though this cell type is not thought to be derived from the neuroepithelium) because there are microglial precursors in the ventricular zone (Alliot et al., 1991). Cell type-specific markers are needed to resolve this issue.

The identification of white matter clusters is difficult at P14 because, although the clusters were relatively undispersed and easily recognised, the cells were undifferentiated. By P28/29, however, the cells in the majority of white matter clusters had become either GFAP$^-$ oligodendrocytes, or GFAP$^+$ fibrous astrocytes. A minority remained undifferentiated. We presume that these clusters would have differentiated into either astrocytes or oligodendrocytes if left longer. An important point is that clusters were never composed of both mature and undifferentiated glial cells, indicating that the differentiation of sister cells may be synchronous. Similar synchrony of differentiation is shown by oligodendrocytes in vitro, where it is thought to reflect an intrinsic timing control mechanism (Temple and Raff, 1986).

In summary, the dispersed clusters of cells were composed of a single cell type, with a small number of exceptions. What does the homogeneity of clusters tell us about the precursor cells that generate them? The first conclusion is that each cluster of labelled cells is unlikely to be the progeny of more than one infected precursor cell since overlaps would tend to generate mixed clusters. Most of the data presented here were from animals with relatively few clusters per brain, so a priori overlaps seemed unlikely.

The possibility of superimposition, however low, makes rare mixed clusters difficult to interpret. We have argued that the neurone/white matter clones are genuine, because they occur at a frequency of between 6% (this study) and 9% (Price and Thurlow, 1988), and because we have identified clones of neurones and oligodendrocytes (N-O clones) in cultures from embryonic cortex (Williams et al., 1991). We consider it likely that these are two descriptions of the same phenomenon. The clusters composed of neurones, astrocytes, and white matter cells were less frequent, have not been identified in cortical cultures (Williams et al., 1991), and so are more questionable.

The dispersion of clones

If clusters are composed of sister cells, can we conclude that each cluster is a complete clone, that is, the entire progeny of the ventricular zone cell that was infected? We had previously concluded that this was correct (Price and Thurlow, 1988). Within a cluster, cells were seldom separated by more than a few hundred micrometers, yet neighbouring clusters were generally separated by many millimetres. For clusters to be related, ventricular zone cells, or their immediate progeny, would have to disperse considerably, yet generate discrete clusters with no stragglers between them. This seemed unlikely, although there is now evidence that some cortical cells do achieve a relatively modest degree of tangential dispersion both in the intermediate zone (O'Rourke et al., 1992) and in the ventricular zone (Fishell et al., 1992). Walsh and Cepko (1992), however, have recently proposed that tangential dispersion of cortical clones, at least in the grey matter, is considerably greater than previously thought. Using a retroviral library together with PCR analysis, they concluded that related clusters in the grey matter were frequently separated by as much as 1.5 mm, and occasionally by as much as centimetre. The widely dispersed clones appeared to be predominantly neuronal, although cell identification was uncertain in many cases. Interpretation of these data is complicated by the possibility that some apparently split clones could in fact be multiple infections by one or more members of the library (Kirkwood et al., 1992). Nonetheless, we must consider the possibility that clusters of different types could be related, and that a single infected ventricular zone cell could give rise to more than one cell type.

A strong argument against this hypothesis comes from experiments in culture. If cells from E16 cortex are dissociated, labelled with BAG, and cultured at various cell densities and in various media, then the resultant clones are composed of a single cell type, just as in vivo (Williams et al., 1991, and unpublished observations). The only exceptions are the N-O clones. Yet naturally in these cultures, there is no histogenesis and clones remain tightly clustered. If precursor cells have a broader potential in vivo than we have supposed, why do the in vivo and culture experiments concur so well? The culture data suggest, therefore, that if clones split, they split only into clusters of the same type, a conclusion that seems for the most part to be consistent with the findings of Walsh and Cepko (1992).

We have shown that clusters in the white matter were composed of either astrocytes or oligodendrocytes, suggesting that the immediate precursors of each cell type are different. This finding is surprising in two regards. First, precursor cells from the optic nerve, called O-2A cells, can generate both oligodendrocytes and astrocytes in tissue cul-

ture (Raff, 1989). Thus we might have expected to see clusters composed of both cell types in vivo. The absence of such clusters could mean that the O-2A cell never generates astrocytes in vivo. Alternatively, both astrocyte and oligodendrocyte clusters could be derived from O-2A cells if the fate of each O-2A cell were determined before clonal expansion.

The second surprise was that the related oligodendrocytes were in such tight clusters, whereas the white matter astrocytes were spread over several hundred micrometers. We interpret this to mean that related astrocytes disperse as they divide and mature, but that the immediate precursor cell of each oligodendrocyte cluster - probably the 'proligodendrocyte' (Gard and Pfeiffer, 1990) or 'oligodendroblast' (Skoff et al., 1976) - divides extensively without migration. Several studies both in vivo and in culture, however, have suggested that the oligodendrocyte precursor is migratory (Small et al., 1987; Lachappelle et al., 1990; Gansmuller et al., 1991). The migratory phase, therefore, probably takes place before the clonal expansion that generates the clusters that we see.

In conclusion then, virally labelled clusters in both the white and grey matter were mostly composed of a single cell type. Since overlap of clones would tend to give mixed clusters, we can conclude that all the members of a cluster are sister cells. Further, the homogeneity of clusters means that cortical precursor cells are fated well before their terminal mitosis to generate a single cell type. An outstanding question is, at what point does this fate restriction occur? The data presented here, together with our earlier tissue culture results, suggest that it is the ventricular zone cells that become dedicated. Whether or not all of the dedicated precursor populations are ventricular zone cells, our data suggest there are five restricted populations that generate: (1) $GFAP^+$ grey matter astrocytes, (2) $GFAP^-$ astrocyte-like cells, (3) fibrous astrocytes, (4) oligodendrocytes or (5) neurones.

Our findings are consistent with Luskin et al. (1988) who described clones composed of single cell types in the mouse cortex, albeit that the cell types were defined only by morphology. Our data are also consistent with those studies that have demonstrated restricted glial lineages in cultures from postnatal rat cortex (Vaysse and Goldman, 1990; Goldman and Vaysse, 1991; Miller and Szigeti, 1991). In addition, Parnavelas et al (1992) have suggested that there are two distinct neuronal precursors, one for pyramidal and one for non-pyramidal neurones. This would increase the number of restricted precursors to at least six, and the number becomes seven if we include the N-O precursor. We have not yet identified the origin of grey matter oligodendrocytes, although they may arise from the same precursors as the white matter oligodendrocytes since in older animals the oligodendrocyte clusters extend into the grey matter.

The diversity of precursor types in the cortex sets it apart from other CNS regions that have been studied, where precursor cells seem to be predominantly multipotent (Turner and Cepko, 1987; Gray et al., 1988; Holt et al., 1988; Wetts and Fraser, 1988; Galileo et al., 1990; Leber et al., 1990; Turner et al., 1990; Gray and Sanes, 1991). Presumably, multipotent precursor cells also predominate in the cerebral cortex at earlier stages of development. We now need to identify these multipotent cells and discover when and how they give rise to the dedicated precursor cells we have described.

We would like to thank Andrew Matus for giving us antibodies and Rosa Beddington for supplying us with β-galactosidase transgenic mice. We acknowledge the financial support of the Medical Research Council, the Multiple Sclerosis Society of Great Britain and Northern Ireland, the Commission of the European Communities, the British Council, and the International Spinal Cord Research Trust.

REFERENCES

Alliot, F., Lecain, E., Grima, B. and Pessac, B. (1991). Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain. *Proc. Natl. Acad. Sci. USA* **88**, 1541-5.

Beddington, R. S. P., Morgenstern, J., Land, H. and Hogen, A. (1989). An in situ transgenic enzyme marker for the midgestation mouse embryo and the visualisation of inner cell mass clones during early organogenesis. *Development* **106**, 37-46.

Binder, L. I., Frankfurter, A. and Rebhun, L. I. (1986). Differential localization of MAP-2 and tau in mammalian neurons in situ. *Ann. NY Acad. Sci.* **466**, 145-166.

Bottenstein, J. E. and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76**, 514-517.

Escobar, M. I., Pimenta, H., Caviness, V. S., Jacobson, M., Crandall, J. E. and Kosik, K. S. (1986). Architecture of apical dendrites in the murine neocortex: dual apical dendritic systems. *Neurosci.* **17**, 975-989.

Fishell, G., Mason, C. A. and Hatten, M. E. (1992). Lateral dispersion of premigratory, neural progenitors within the ventricular zone of cerebral cortex. *Soc. Neurosci. Abstr.* **18**, 926.

Galileo, D. S., Gray, G. E., Owens, G. C., Majors, J. and Sanes, J. R. (1990). Neurons and glia arise from a common progenitor in chicken optic tectum: demonstration with two retroviruses and cell type-specific antibodies. *Proc. Natl. Acad. Sci. USA* **87**, 458-462.

Gansmuller, A., Clerin, E., Kruger, F., Gumpel, M. and Lachappel, F. (1991). Tracing transplanted oligodendrocytes during migration and maturation in the shiverer mouse brain. *Glia* **4**, 580-590.

Gard, A. L. and Pfeiffer, S. E. (1990). Two proliferative stages of the oligodendrocyte lineage ($A2B5^+O4^-$ and $O4^+GalC^-$). under different mitogenic control. *Neuron* **5**, 615-625.

Goldman, J. E. and Vaysse, P. J.-J. (1991). Tracing glial cell lineages in the mammalian forebrain. *Glia* **4**, 149-156.

Gray, G. E. and Sanes, J. R. (1991). Migratory paths and phenotypic choices of clonally related cells in the avian optic tectum. *Neuron* **6**, 211-225.

Gray, G. E., Glover, J. C., Majors, J. and Sanes, J. R. (1988). Radial arrangement of clonally related cells in the chicken optic tectum: lineage analysis with a recombinant retrovirus. *Proc. Natl. Acad. Sci. USA* **85**, 7356-7360.

Grove, E. A., Kirkwood, T. B. L. and Price, J. (1992). Neuronal precursor cells in the rat hippocampal formation contribute to more than one cytoarchitectonic area. *Neuron* **8**, 217-229.

Holt, C. E., Bertsch, T. W., Ellis, H. and Harris, W. A. (1988). Cellular determination in the xenopus retina is independent of lineage and birth date. *Neuron* **1**, 15-26.

Kirkwood, T. B. L., Price, J. and Grove, E. A. (1992). The dispersion of neuronal clones across the cerebral cortex. *Science* **258**, 317.

Lachappelle, F., Lapie, P., Gansmuller, A., Villarroya, H., Baumann, N. and Gumpel, M. (1990). What have we learnt about the jimpy phenotype expression by intracerebral transplantations? *Ann. NY Acad. Sci.* **605**, 332-345.

Leber, S. M., Breedlove, S. M. and Sanes, J. R. (1990). Lineage, arrangement and death of clonally related motoneurons in chick spinal cord. *J. Neurosci.* **10**, 2451-2462.

Luskin, M. B., Pearlman, A. L. and Sanes, J. R. (1988). Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. *Neuron* **1**, 635-647.

Martinez, S., Puelles, L. and Alvarado-Mallart, R. M. (1992). Tangential

neuronal migration in the avian tectum: cell type identification and mapping of regional differences with quail/chick homotopic transplants. *Dev. Brain Res.* **66**, 153-163.

Miller, R. H. and Szigeti, V. (1991). Clonal analysis of astrocyte diversity in neonatal rat spinal cord cultures. *Development*, **113**, 353-362.

Moore, R. and Price, J. (1992). the distribution of clones of neurons in the rat somatosensory cortex. *J. Neurocytol.* **21**, 737-743.

Noble, M. and Murray, K. (1984). Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell. *EMBO J.* **3**, 2243-2247.

O'Rouke, N. A., Dailey, M. E., Smith, S. J. and McConnell, S. K. (1992). Diverse migratory pathways in the developing cerebral cortex. *Science* **258**, 299-302.

Parnevelas, J. G., Luder, R., Pollard, S. G., Sullivan, K. and Lieberman, A. R. (1983). A qualitative and quantitative ultrastructural study of glial cells in the developing visual cortex of the rat. *Phil. Trans. R. Soc. Lond. B.* **301**, 55-84.

Parnevelas, J. G., Barfield, J. A., Franke, E. and Luskin, M. B. (1992). Separate progenitor cells give rise to pyramidal and non-pyramidal neurons in the rat telencephalon. *Cerebral Cortex.* **1**, 463-468.

Price, J. and Thurlow, L. (1988). Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. *Development* **104**, 473-482.

Price, J., Turner, D. and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* **84**, 156-160.

Raff, M. C. (1989). Glial cell diversification in the rat optic nerve. *Science.* **243**, 1450-1455.

Reyners, H., Gianfelici De Reyners, E. and Maisin, J.-R. (1982). The beta astrocyte: a newly recognised radiosensitive glial cell type in the cerebral cortex. *J. Neurocytol.* **11**, 967-983.

Reyners, H., Gianfelici De Reyners, E., Regniers, L. and Maisin, J.-R. (1986). A glial progenitor cell in the cerebral cortex of the adult rat. *J. Neurocytol.* **15**, 53-61.

Skoff, R. P., Price, D. L. and Stocks, A. (1976). Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. I. Cell proliferation. *J. Comp. Neurol.* **169**, 291-312.

Small, R. K., Riddle, P. and Noble, M. (1987). Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve. *Nature* **328**, 155-157.

Temple, S. and Raff, M. C. (1986). Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. *Cell* **44**, 773-779.

Turner, D. and Cepko, C. (1987). Cell lineage in the rat retina: a common progenitor for neurons and glia persists late in development. *Nature* **328**, 131-136.

Turner, D. L., Snyder, E. Y. and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron* **4**, 833-845.

Vaysse, P. J. J. and Goldman, J. E. (1990). A clonal analysis of glial lineages in neonatal forebrain development in vitro. *Neuron* **5**, 227-235.

Walsh, C. and Cepko, C. L. (1988). Clonally related cortical cells show several migration patterns. *Science* **241**, 1342-1345.

Walsh, C. and Cepko, C. L. (1992). Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science* **255**, 434-440.

Wetts, R. and Fraser, S. E. (1988). Multipotential precursors can give rise to all major cell types of the frog retina. *Science* **239**, 1142-1145.

Williams, B. P., Read, J. and Price, J. (1991). The generation of neurons and oligodendrocytes in the cerebral cortex from a common precursor cell. *Neuron* **7**, 685-693.

Wood, P. and Bunge, R. P. (1984). The biology of the oligodendrocyte. In *Oligodendroglia. Advances in Neurochemistry, Vol. 5.* (ed. W. T. Norton), pp. 1-46. New York: Plenum.

(Accepted 16 November 1992)