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A glial progenitor cell in the cerebral cortex of the adult rat

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Summary

A glial cell subtype, previously classified as a beta astrocyte on the basis of its ultrastructural and radiobiological characteristics, has now been shown to represent the most mitotically active component of the glial population in the grey matter of the cerebral cortex of the young adult rat. The labelling index of 0·83% was evaluated using semithin sections. A role for beta astrocytes as macroglial precursors is supported by the present observations. However, the mechanisms responsible for the intermediate radiosensitivity of these elements remain uncertain.

Introduction

Glial cells in the brain display limited mitotic capabilities (Kaplan & Hinds, 1980; Schultze & Korr, 1981; Sturrock, 1982). The recent demonstration of a population of radiosensitive glial cells in the cerebral cortex of the adult rat (Reyners *et al.*, 1982) prompted us to re-examine the problems of the mitotic activities of the cells in the brain in order to cast additional light on the mechanisms of the glial radiosensitivity and on its consequences. Such a study could also be of some value in elucidating the mechanisms of postnatal gliogenesis in the cerebral cortex. The ontogenetic position of the beta astrocyte among the different glial cell lines is also becoming clearer and the hypothesis that these cells are multipotential glial precursors gains support from the results of the present study.

Materials and methods

Six adult (4-month-old) female Wistar R rats were given repeated i.p. injections of 0.1 ml of [^{3}H]thymidine (0.04 μM ml^{-1}; 1 mCi ml^{-1}) at 24, 16 and 4 h before glutaraldehyde fixative perfusion. This procedure was used in order to obtain a large number of labelled cells. The total injected dose was 1.6 μCi g^{-1} body weight. The right parietal cortex was dissected out of the brain and postfixed in OsO_4 as described previously (Reyners *et al.*, 1982). The tissue was dehydrated with ethanol and embedded in Spurr low viscosity epoxy resin.

Identification of beta astrocytes in semithin sections

In autoradiographs, the precise identification of the glial cells at the optical microscope level was a prerequisite. The criteria established by Ling *et al.* (1973) were used, but only the dark class of oligodendrocytes was considered here, the lighter classes being poorly represented in mature rodents (Ling & Leblond, 1973). Since beta astrocytes had only been described at the electron microscope (EM) level, preliminary tests of identification were carried out using serial pairs of ultrathin and semithin (2 μm) epoxy sections (size: 0.5 × 1.5 mm). The beta astrocytes were first recognized in an ultrathin section using the electron microscope and were carefully located with respect to a number of landmarks (blood vessels). The corresponding 2 μm sections were then scanned under the light microscope and the previously pin-pointed beta astrocytes relocated and re-identified.

With practice, beta astrocytes could be directly recognized in the toluidine blue-stained semithin sections: the very irregular outline of the nucleus, the absence of a 'watery' cytoplasm and a pink metachromatic background staining of the nucleoplasm allowed them to be distinguished easily from the regular astrocytes (protoplasmic or fibrillar astrocytes referred to below as alpha astrocytes). Also, the fact that 50% of beta astrocytes are in satellite position to nerve cells (as opposed to < 10% of alpha astrocytes) was a useful check.

Double-face semithin staining method

In parallel with the above procedures, a special staining method was developed which greatly facilitated the first recognitions of beta astrocytes in semithin sections. Each face of the 2 μm sections was stained separately with toluidine blue (1% toluidine blue in 1% sodium tetraborate solution, 1:6 v/v). The procedure was carried out in a few easy steps. First, the semithin section was floated on a drop of the staining solution on a microscope slide. The slide was warmed on a hot plate (about 80° C) for 2–3 min, then blotted dry with filter paper. It was left on the plate until the section had adhered to the slide. Finally, one drop of

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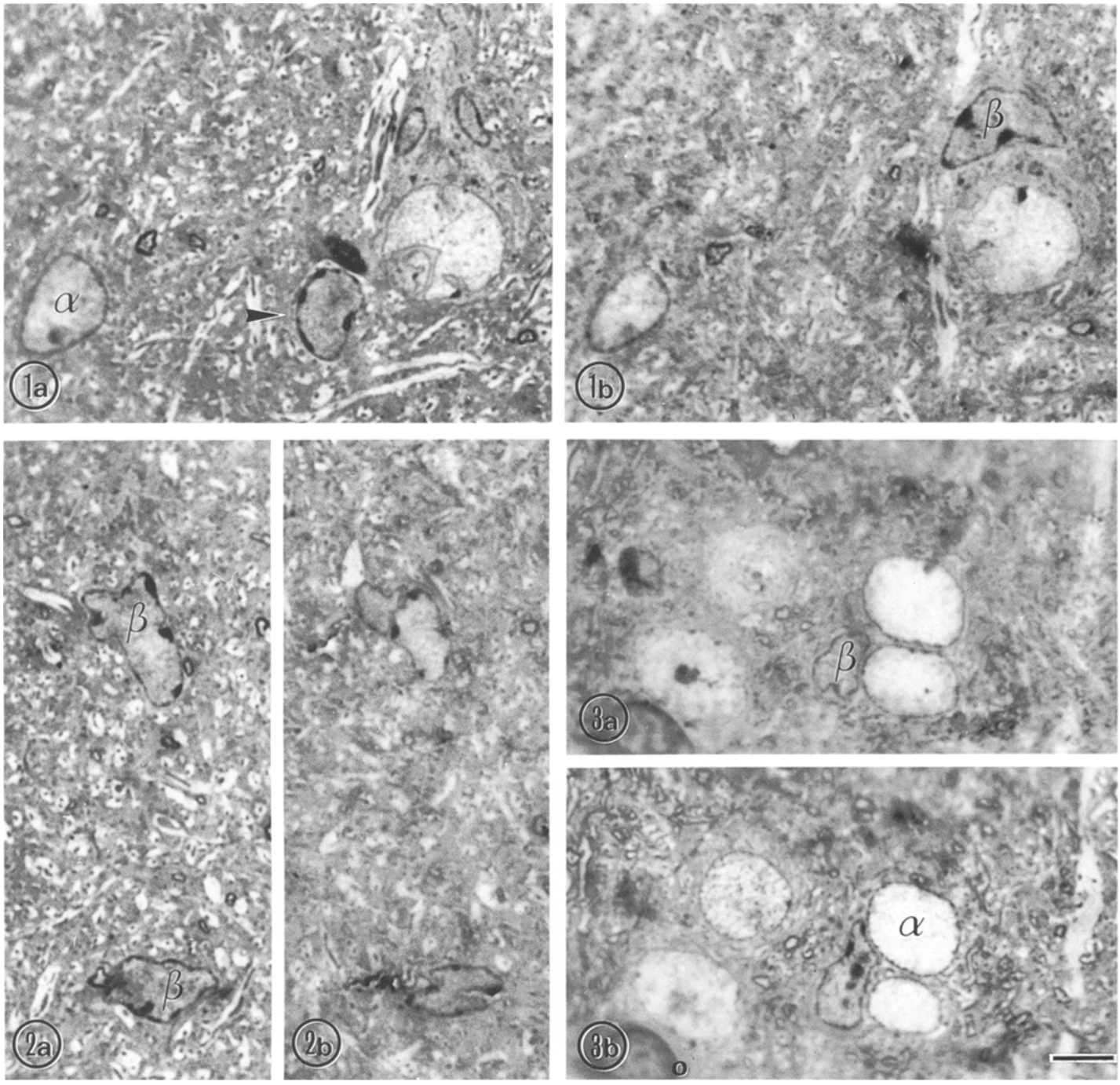
stain was deposited on the upper face of the section again for 2–3 min at 80° C. The penetration of the stain in the Spurr resin is very slow and thus staining remained extremely superficial. Consequently, the images observed with a low depth of focus (oil immersion) objective at the superior and inferior face of the section were different (no image in focus can be found in between), and the degree of the difference between them increased with the irregularity of the structures. Therefore, the very tortuous nuclei of the beta astrocytes were particularly distinguishable: they characteristically display very different nuclear profiles at the upper and lower level of the sections, which contrast with the concentric or slightly eccentric round nuclear outlines of the alpha astrocytes (Figs 1–3). In addition, the



Figs 1–3. Identification of beta astrocytes in semithin sections using the double-face staining method. In each case, micrographs (a) and (b) correspond to the images formed at the upper and at the lower faces of a semithin section, respectively. No image in focus can be obtained between these two levels. Fig. 1b shows the irregular profile of the nucleus of a beta astrocyte (β). The cell is satellite to the dendritic cone of a pyramidal nerve cell. Only two separate fragments of the nucleus remain at the image plane 1a. The arrowhead points to another beta astrocyte nucleus which has entirely disappeared at the level of plane 1b. The regular, ovoid nucleus of an alpha astrocyte (α) is also visible. In Figs 2 and 3, the outlines of the nuclei of different beta astrocytes (β) are shown to vary widely from the upper to the lower image plane level, in contrast to alpha astrocytes (α) and to an oligodendrocyte (o, Fig. 3). Semithin section thickness: 1.8 μm. Scale bar: 5 μm.

method allowed a direct estimation of the section thickness (i.e. the difference between upper and lower focus on the microscope micrometric knob). It is also possible that two different staining methods could be used separately on the two faces of the same section.

Autoradiographic studies

Three serial 2 μm sections were attached to each microscopic slide; only the middle section in the series was coated with diluted (1:1 v/v) Ilford L4 emulsion. After a 2-month exposure, development was carried out using Kodak D19. The coated sections were stained very lightly (Figs 5, 10) in order not to prevent the visibility of the label, particularly when the latter was located above dark staining nuclei as in oligodendrocytes, microglia and endothelial cells. The other sections were stained normally (single-face staining). Labelled cells were first located in the coated section and then identified with the help of the other two sections. The surface of the total observed area of the coated section was determined using a Kontron digitizer.

Semithin section resectioning

The cyanoacrylate method of Johnson (1976) was found to be a most reliable way to remove the semithin sections (even from normal microscope slides) and to recut them as ultrathin sections with minimal damage to the samples. This procedure was used to confirm the identity of a small number of labelled cells (Figs 4–13).

Electron microscopy

The total thickness of the parietal cortex (~ 2 mm) was cut in ultrathin sections to provide glial distribution measurements per unit area of cortex involving all the layers from the pial surface to the upper limit of the corpus callosum. Three 200-mesh grids per rat cortex were screened using a Jeol EM 1200EX. Abercrombie correction for cell density measurements was not used since nuclear areas of beta astrocytes and microglia are highly variable due to the irregularity of their surface.

Results

Normal glial distribution in the parietal cortex

Glial cellular densities, obtained from EM observation of the whole cortex, are given in Table 1. These values are slightly lower than our previous estimations (Reyners *et al.*, 1982) which were carried on at the level of layer V. However, they are much higher than the measurements obtained in ultrathin Araldite sections by Vaughan & Peters (1974), i.e. 134.7 glial cells mm^{-2}. A partial explanation for this discrepancy is that ultrathin sectioning of a large area of Spurr-embedded material induced a compression of the section along the long axis reducing it to 63% of the corresponding length of the semithin section (Figs 7, 12). Corrected data (measured data × 0.63) were thus used in Table 1 for the total glia estimations. The corrected total glial cell density was 251 cells mm^{-2}. From the data of Mori (1971) a density of 600 glial cells mm^{-2} could be calculated, but these observations were made in an oligodendrocyte-rich layer (i.e. layer VI). Total values for glial cells were computed from unit area density counts and from the digitizer measurements of the total area of the 28 semithin sections investigated (i.e. 252.4 mm^2). These total values were required to estimate the labelling index of each glial cell type.

Table 1. Packing densities and total observed number of glial cells in the cerebral cortex of the adult rat. A correction factor of 0.63 has been used to compensate for the tissue compression induced by ultrathin sectioning of a large tissue area.

	Packing density (cells mm^{-2}) (corrected value)	*Total number cells observed*
Alpha astrocyte	178 (112)	28269
Beta astrocyte	53 (33)	8329
Oligodendrocyte	120 (76)	19182
Microglia	47 (30)	7572
All neuroglia	398 (251)	63352

Label distribution on the glial cells

In 28 semithin sections of the parietal cortex, 76 labelled cells were observed. The label was deposited on the glial cells, although one labelled pericyte (but no endothelial cell) was also detected. The distribution of the label on the glial cells was: none on alpha astrocytes, 92% on beta astrocytes (69 cells labelled; Figs 5, 10), 5% on oligodendrocytes (dark nucleus group; four cells labelled) and 3% on microglia (two cells labelled). The estimated total numbers of glial cells in the areas investigated were given above (Table 1). Estimates of the labelling index for each glial cell type were as follows: 0% alpha astrocytes, 0.83% beta astrocytes, 0.021% oligodendrocytes and 0.026% microglia. The total observed number of glial cells was 251 cells mm^{-2} × 252.4 mm^2 surveyed = 63352. So the general label index for all neuroglial cells was 75/ 63352 = 0.0012 (or 0.12%).

Discussion

The present study sought to characterize the frequency of DNA labelling in beta astrocytes. It was hoped that this might help to unravel the fate of these elements and to explain the reason for their particular radiosensitivity (or radioresponsiveness). Numerous studies have already debated the problems of cell replication in the CNS of mature animals. The very high proportion of labelled beta astrocytes observed in the present work (92% of the labelled cells) casts a new light on this problem and provides clues for

future research; it also adds to the knowledge of these cells. However, the present data challenge a number of accepted concepts.

Intermediate radiosensitivity but very low labelling index

The X-ray dose required to eliminate the beta astrocytes (within 24 h) was high, i.e. >15 Gy (Reyners *et al.*, 1982). Below this dose, little or no cell death occurred after single dose exposures. Thus, it was assumed that these cells do not die from the radiobiological mechanism of the 'reproductive (or mitotic) cell death' (which kills the cells after exposure to doses lower than 3 Gy; Okada, 1970) but rather by a process of interphasic cell death.

On the other hand, the present demonstration that beta astrocytes are labelled with [^{3}H]thymidine more than any other cell type of the cerebral cortex could suggest that these cells die during mitosis. However, the very low labelling index (0.83%) of the beta astrocytes has to be taken into account. This percentage also represents the fraction of mitotic beta astrocytes which could directly be killed by the 'reproductive cell death' mechanism. In fact, the labelling index computed here largely over-estimates this mitotic fraction since: (1) thymidine injections were performed over a period of time larger than the synthetic phase of the glia mitotic cycle (9.4 h according to Korr *et al.*, 1975); (2) certain phases of the mitotic cycle have a greater radiosensitivity than others. Consequently, only a minority of beta astrocytes ($\ll$ 0·83%) die from mitotic cell death and the majority of them (all unlabelled beta astrocytes) are thus killed as a consequence of intermitotic cell death after X-irradiation. Although such a dual mechanism of death is unlikely, the major difficulty with such a hypothesis resides in the fact that interphasic cells are generally much more radioresistant than the beta astrocytes, and death usually occurs only after doses much higher than 20 Gy (Okada, 1970). Thus, it seems too early to draw conclusions about the puzzling 'intermediary' radiosensitivity of the beta astrocyte. However, it can be of interest to mention here an additional factor, proposed by Altman *et al.* (1968), suggesting that migratory cells are more radiosensitive than stationary ones: this could be the case for the beta astrocytes.

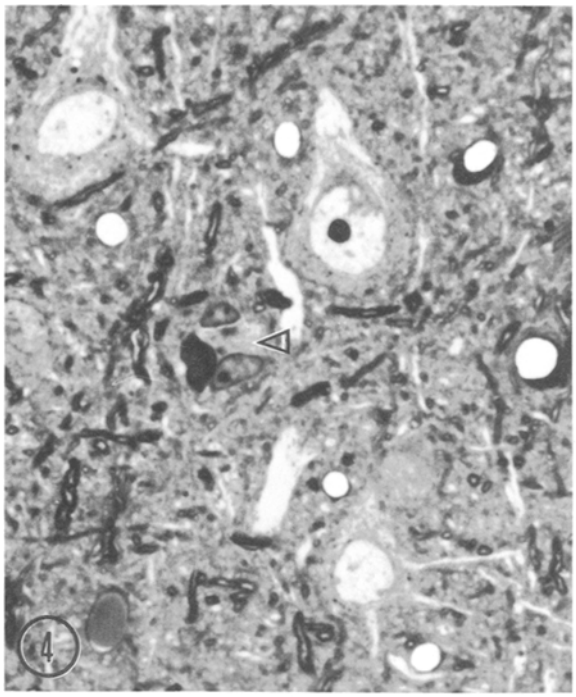
Thymidine incorporation and mitosis in the glial cells of the cerebral cortex

The observation that most of the label was located on the beta astrocytes and nearly absent on the other glial cells is in contradiction with previous findings in the field of gliogenesis in the mature mammalian brain (Kaplan & Hinds, 1980; Schultze & Korr, 1981). However, beta astrocytes represent a recently identified cell category which must have been previously catalogued among the other cell classes, ranging from alpha astrocytes to light and medium-dark oligodendrocytes.

Until now, it has not been possible to identify unequivocally beta astrocytes in paraffin sections; this technique was used for autoradiographic purposes in the studies of Schultze & Korr (1981). Nevertheless, it can be assumed from the data presented above that nearly all of the [^{3}H]thymidine labelled neuroglial cells observed by these authors in the cerebral cortex of adult mice a few hours after injection (Korr *et al.*, 1975) are beta astrocytes, and not a mixed population of astrocytes and oligodendrocytes as proposed. The labelling index they give for the neuroglial cells is 0.2%, this is slightly higher than the value reported here. This could be due to a species difference but also to the fact that white matter and other brain areas were included in the study.

The data presented more recently by Kaplan & Hinds (1980) are more controversial. It is clear in their

Figs 4–13. Identification of [^{3}H]thymidine labelled beta astrocytes by means of ultrathin resectioning of re-embedded semithin sections. Figs 4–6 and 9–11 represent two different sets of serial semithin sections displaying three successive aspects of a labelled cell (arrowhead). Figs 5 and 10 are the middle sections of the groups; they are also the only ones which have been coated with emulsion. Staining of the middle sections was lighter than for the other sections in order to facilitate identification of the label when situated over dark nuclei. The delineated rectangles correspond to the fields of the low magnification electron micrographs; these have been compressed during sectioning (Figs 7 and 12). The small blood vessels have been numbered to facilitate orientation. In Fig. 9, three small arrows point to alpha astrocytes with light and circular nuclei. All inserts represent higher magnification views of the labelled cells. Scale bar: 20 μm. Figs 7 and 12: the cells which were found labelled by light microscope autoradiography (arrowhead) show the irregular nucleus and the thin chromatin rim of the beta astrocyte. n, neuron; o, dark oligodendrocyte. Scale bar: 5 μm. Fig. 8: the beta astrocyte (β) cytoplasm is typically slightly electron dense, dotted with free ribosomes and detached from the surrounding structures. Only one process spreads out from its perikaryon and squeezes between the two other adjacent glial cells (arrow). One of these (o) is a typical oligodendrocyte; the other one (below) was identified as an immature medium-light class oligodendrocyte, rare in adult brains. None of these oligodendrocytes incorporated any label. Scale bar: 1 μm. Fig. 13: this beta astrocyte (β) shows little typical characteristics due to the poorly developed cytoplasm and the absence of cellular processes. Identification is mainly based here on the appearance of the nucleus, the slight tendency to retraction of the cytoplasm and the presence of numerous free ribosomes. Scale bar: 1 μm.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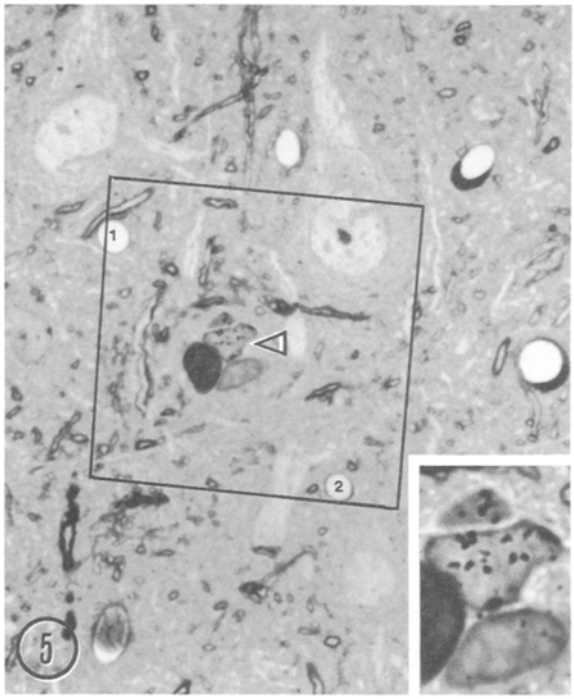
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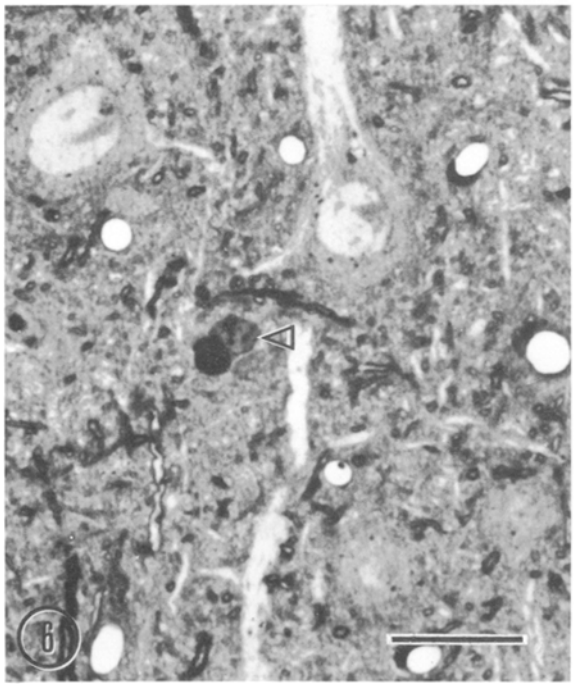
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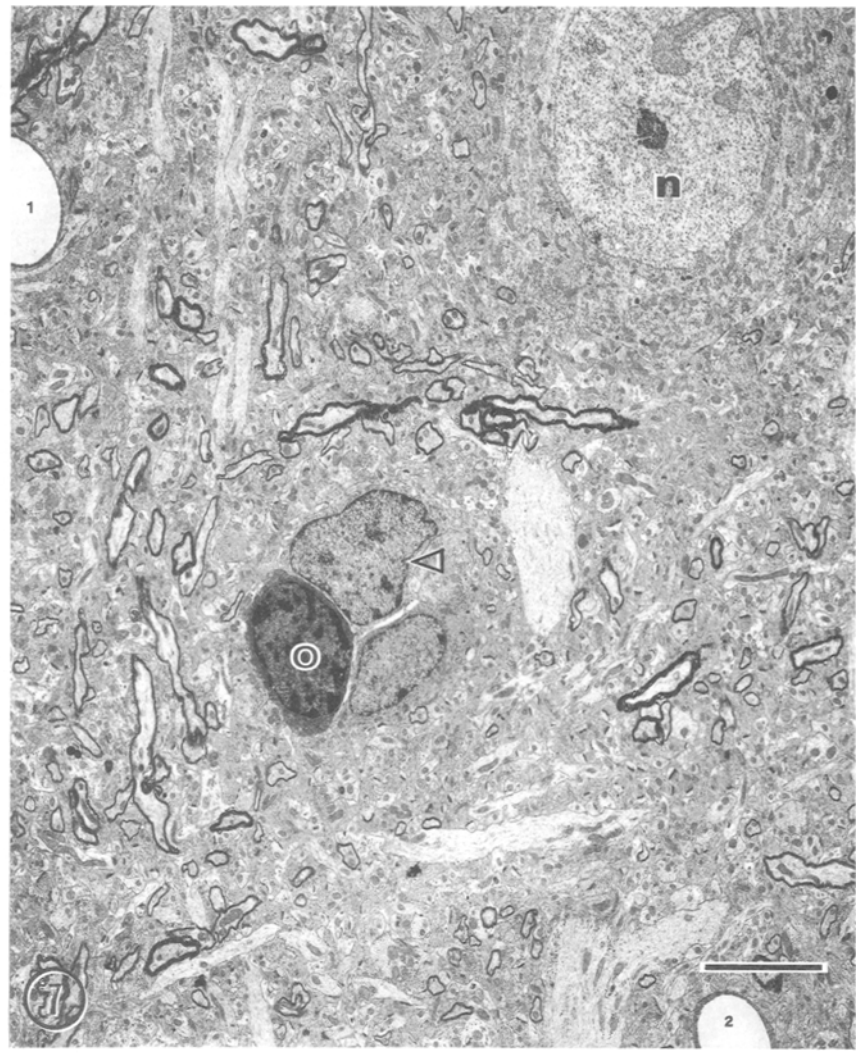
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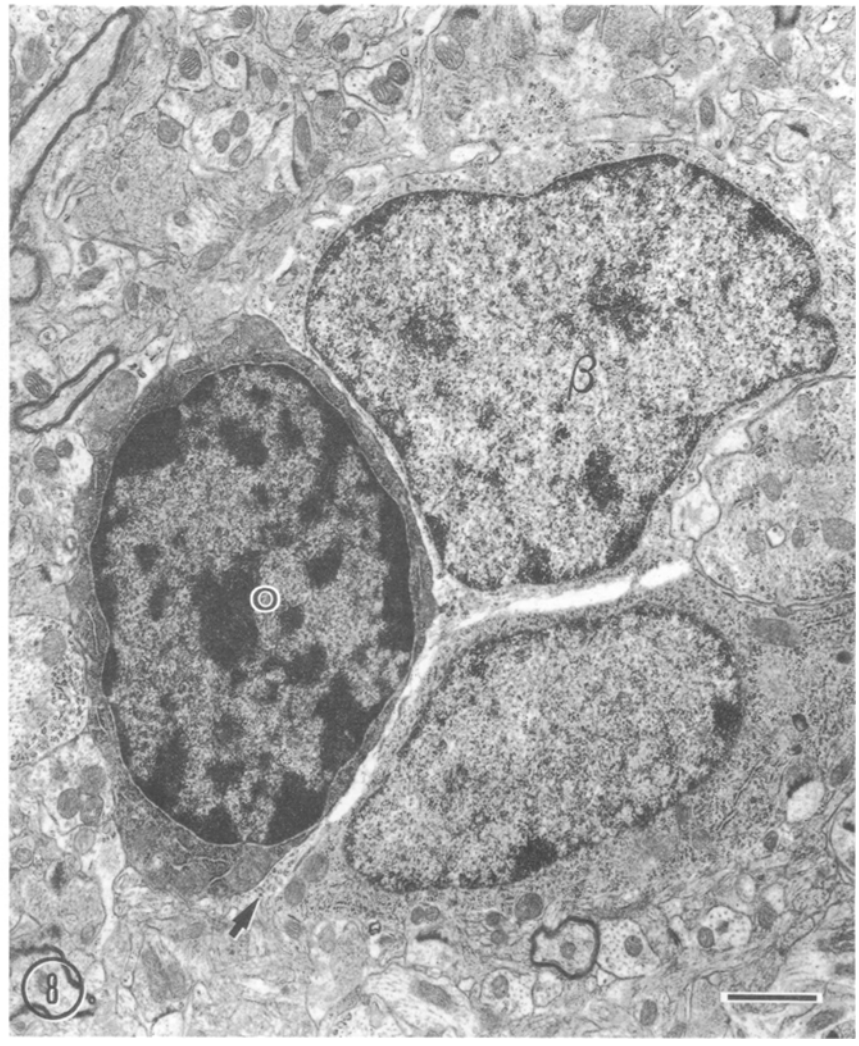
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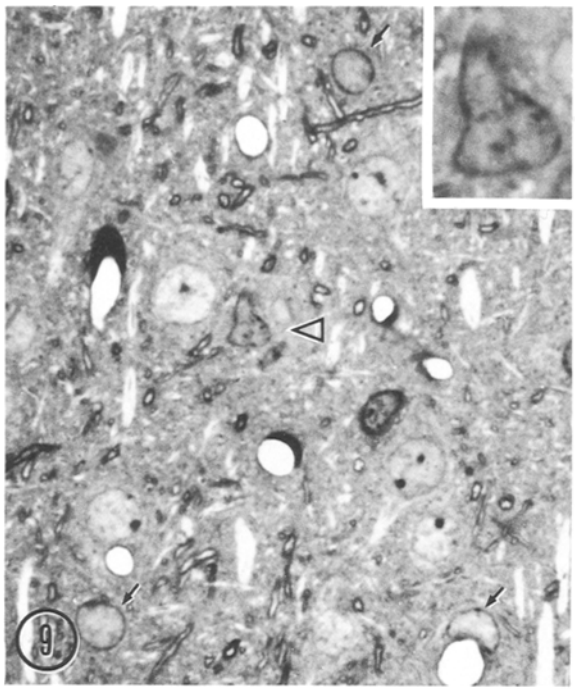

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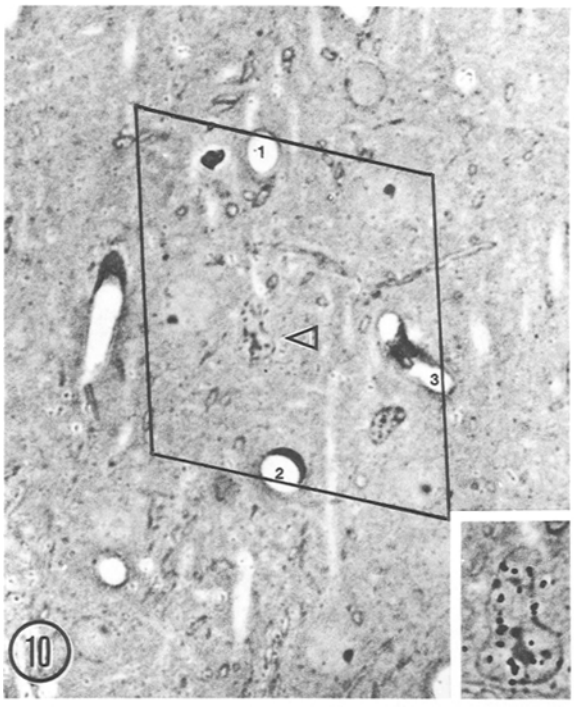

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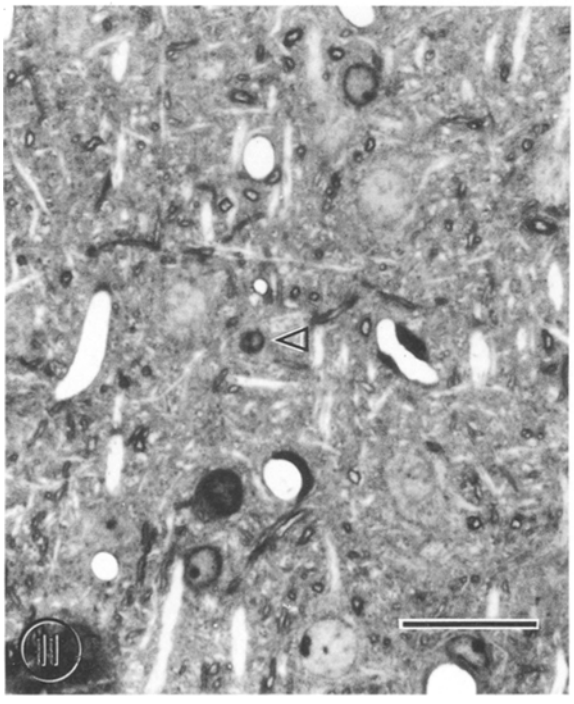

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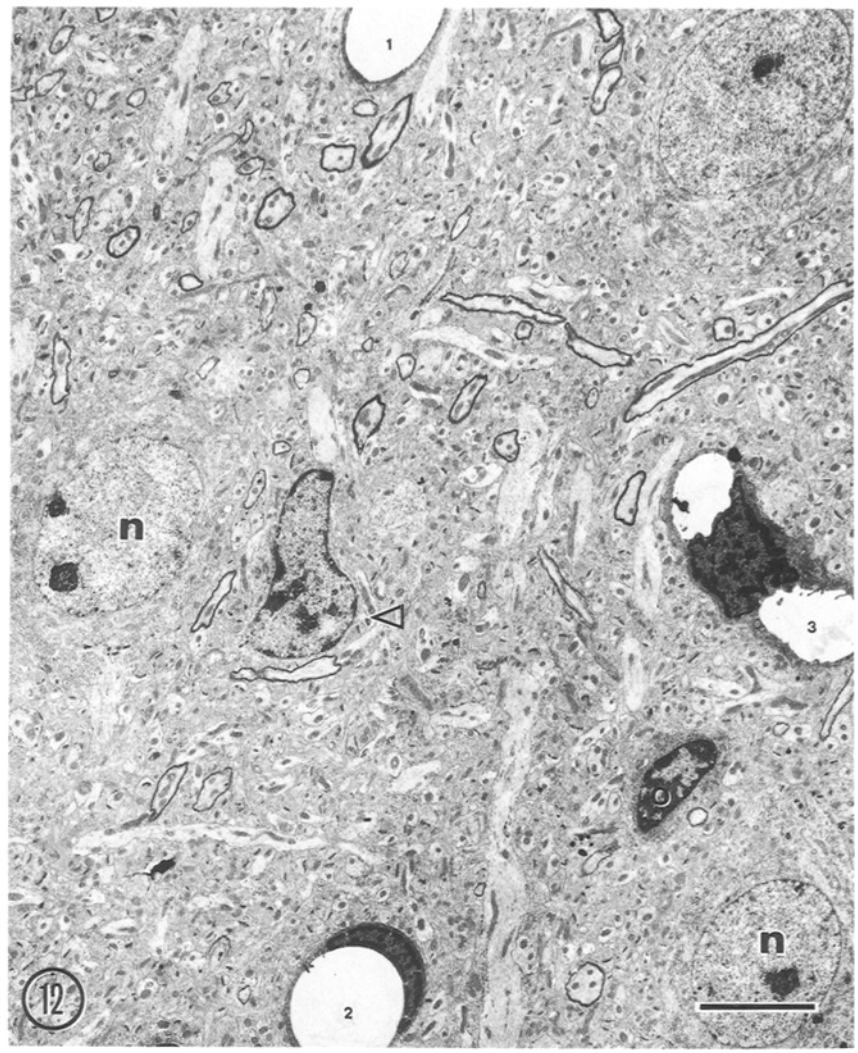

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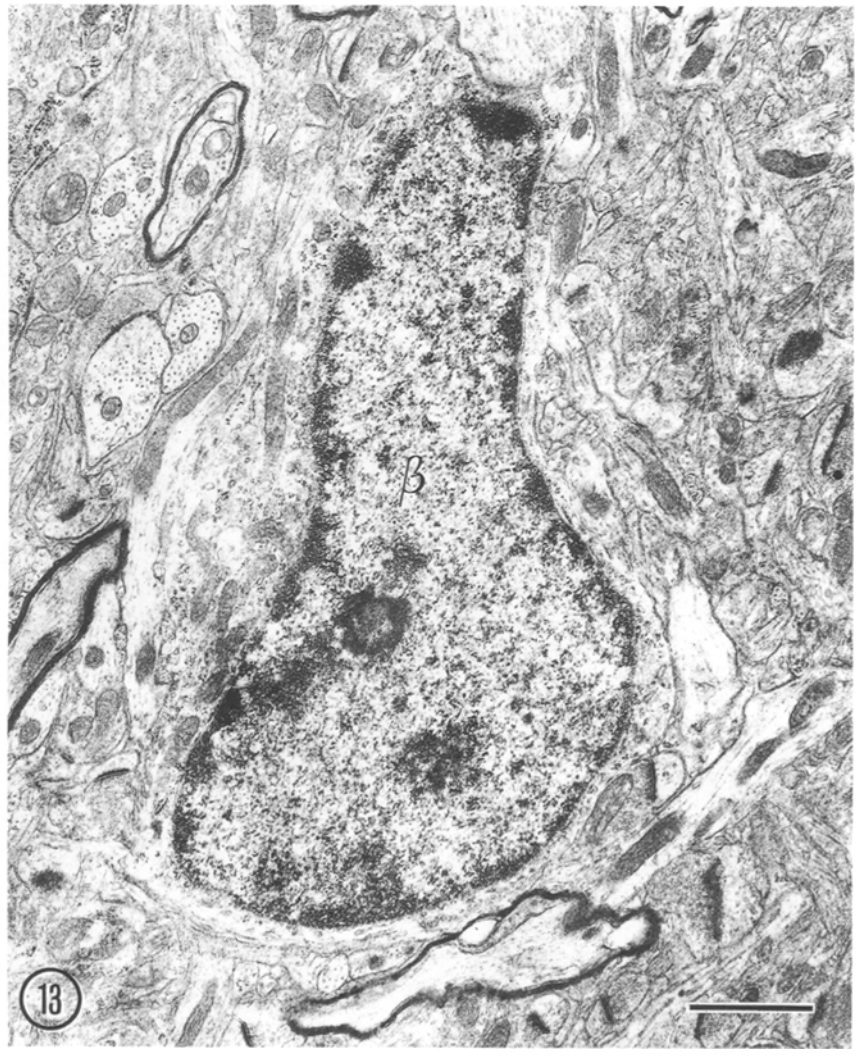

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electron micrographs, derived from re-embedded semithin sections, that some labelled astrocytes were illustrated. At least one of them (their Fig. 1) displays the criteria (gliofilaments) of an alpha astrocyte. We consider that a number of the other labelled cells illustrated represent beta astrocytes (their Fig. 11; the cell in their Fig. 6 is dubious) or light oligodendrocytes (their Figs 2 and 10). With respect to mature oligodendrocytes, the illustrations show clearly that a significant number of them are labelled. However, since there was a delay of 30 days between thymidine injection and animal sacrifice in the report of Kaplan & Hinds (only a few hours in the present work), their labelled cells could certainly be the result of the differentiation of some progenitor cells. This could account for the high number of labelled mature oligodendrocytes as already suggested by Sturrock (1982).

Progressive transformation of beta astrocytes into other glial cell types has already been suggested in our first contribution on this subject (Reyners *et al.*, 1982). They were considered to be 'multipotential cells' with oligodendroglial tendencies; indeed oligodendrocytes are lost as a late consequence of X-irradiation.

As early as 1969, Mori & Leblond noticed that the astrocytes capable of division are smaller and have fewer gliofilament bundles than the non-mitotic ones. In fact, beta astrocytes are smaller than alpha astrocytes and never show gliofilaments; it is often necessary to tilt the section (using a goniometer stage which was not a standard EM component in 1969) in order to see that bundles of filaments that appear to belong to a beta astrocyte are actually part of an adjacent alpha astrocyte.

The beta astrocyte concept and the oligodendrocyte maturation series of Mori & Leblond (1970)

The data presented above showed very low labelling in the oligodendrocytes (dark type). This is in agreement with the series of classical papers by Leblond and co-workers concerning gliogenesis in the white matter of immature rats: the darker class of oligodendrocytes does not incorporate thymidine in contrast to the free subependymal cells and the light and medium classes of oligodendrocytes (Mori & Leblond, 1970). However, more recent reports (Arenella & Herndon, 1984; Ludwin, 1985) have claimed that in certain experimental conditions, mature (dark) oligodendrocytes are also capable of dividing. Although some thymidine incorporation was observed in the dark oligodendrocytes during the present study, the labelling index of these cells is too low (0.021%) to contradict the general theory of Leblond and co-workers. However, even in normal conditions, exceptions to the rule seem to exist. On the other hand, it must be noted that Leblond studied mainly the subependymal region and the corpus callosum of very young rats; only very few data came from the cerebral cortex (Privat, 1975). In addition, Privat clearly stated that free subependymal cells, which represent the earliest progenitors of the macroglial lines, were absent from the cortex at all ages studied. This statement will be commented on further in the next section of this discussion

It is of interest to mention here an old quantitative analysis carried out by Smart & Leblond (1961) long before the development of the oligodendrocyte line theory (Ling *et al.*, 1973). It concerns thymidine incorporation in the white and grey matter of 18 g mice. Most of the label was found first in 'small dark and medium dark nuclei' but passed with time to cells considered as oligodendrocytes and astrocytes. Although Mori & Leblond (1970) later considered these observations (based on paraffin sections) as obsolete, this work was probably pointing to the incorporation of DNA precursor by free subependymal cells in the white matter and by beta astrocytes in the grey matter of semi-mature mammals. Nevertheless, it is also difficult to reconcile this interesting early work with the more recent theory of Mori & Leblond which claims that all oligodendrocytes pass through the stage of light oligodendrocyte during their maturation. Since this stage is considered to be the largest glial cell (Ling *et al.*, 1973; Privat, 1975), it is puzzling that no such large intermediaries were noted in the previous paraffin section study in spite of heavy labelling in the corpus callosum.

Beta astrocytes and the other precursor cells of the brain

Glioblastic cells, also referred to as free subependymal cells or spongioblasts (Ling *et al.*, 1973), have been described in the brain. The ultrastructural characteristics of these cells (Privat, 1975; Sturrock, 1982) are different from these of the beta astrocyte, i.e. nucleus and cytoplasm appear much more electron dense. Endoplasmic reticulum cisternae are well delineated, relatively long and sinuous, much as in the microglial cells; their content also has the same electron density as the surrounding cytoplasm. In addition, the numbers of glioblasts decline in the brain with increasing age (Vaughan & Peters, 1974). Privat (1975) claimed that such cells do not exist in the cerebral cortex throughout the life of the rat.

In contrast, the cells classified as beta astrocytes are a constant population, representing as much as 10% of the total glia in the adult rat brain, even in 30-month-old animals (unpublished data). However, it would appear from the present data that beta astrocytes are functionally equivalent to the free subependymal cells (Ling *et al.*, 1973) which are also the first cellular elements found labelled in the white matter after thymidine injection (Paterson *et al.*, 1973). Some of them transformed into astrocytes, but

the majority were shown to differentiate through light and medium-dark into dark oligodendocytes. From an ultrastructural point of view, the beta astrocytes are very similar to certain cells of the border area of the subependymal region, particularly those cells considered by Privat (1975) to be young astrocytes (his Fig. 14). On the other hand, a few beta astrocytes displaying an unusually large nucleus (>12 μm in length) were observed during the present study, and it was difficult to distinguish these from the light oligodendrocytes. This observation is another argument for considering the beta astrocytes as being functionally equivalent to the free subependymal cells of the corpus callosum. However, it must be pointed out that the light oligodendrocytes are not sharply characterized at the EM level. The considerable differences between the illustrations of light oligodendrocytes provided by Privat (his Figs 5, 7; 1975) illustrate the range of variation still present in this particular group. More recently, Paterson (1983) introduced a new class of immature glial cells in the corpus callosum of the adult mouse. No ultrastructural analysis of these cells has been provided yet, but it can be speculated that they represent one of the missing links between the subependymal precursors and the cortical beta astrocytes.

In view of the new information presented above, the term beta astrocyte could now appear equivocal. It was adopted in our previous contribution (Reyners *et al.*, 1982) due to the clear morphological similarities between beta astrocytes and alpha astrocytes in the cerebral cortex of the adult rat. These cells share a low general electron density and a characteristic chromatin rim around the nucleus. Moreover, the most obvious criterion of the beta astrocytes, the presence of an irregular shaped nucleus, has been observed in gliofilament-rich astrocytes located outside the cerebral cortex (Peters *et al.*, 1976). For such reasons, we believe that both types of cells have often been confounded and classified in the same group (e.g. Mori & Leblond, 1969).

However, in spite of the morphological analogies, the differences are important and numerous. Beta astrocytes never contain gliofilaments, are much more frequently satellites to neuronal cell bodies than alpha astrocytes and never enter into contact with blood vessel basal laminae. The radiosensitivity and the mitotic properties of the alpha and beta astrocytes are entirely different.

In conclusion, 24 h after thymidine injections, no alpha astrocytes and very few oligodendrocytes were labelled. In contrast, labelling of the beta astrocytes was very important. These cells, therefore, appear as the most probable precursors of the oligodendrocytes which contain about 50% of the label at 1 month after thymidine injection as measured by Kaplan & Hinds (1980).

The presence of multipotential glial cells in the brain has recently been mentioned. Raff *et al.* (1984) have demonstrated the existence of a bipotential glial progenitor in the newborn rat. The present demonstration that beta astrocytes represent the major mitotic cells in the brain (excepting the residual glioblasts of the subependymal plate) strongly suggests that in the adult grey matter, a characteristic group of cells is dedicated to the maintenance of the glial populations. This could be particularly true for the oligodendrocytes but also seems possible for the alpha astrocytes. On the other hand, the present work does not provide any satisfactory explanation for the high radiosensitivity of this cell population: the very low labelling index of the beta astrocytes excludes the classical explanation involving the process of the reproductive death of the cells.

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