COEXISTENCE OF NEURONAL AND GLIAL PRECURSOR CELLS IN THE CEREBRAL VENTRICULAR ZONE OF THE FETAL MONKEY: AN ULTRASTRUCTURAL IMMUNOPEROXIDASE ANALYSIS[1]

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Abstract

The cytological composition of the proliferative zones in the fetal monkey occipital lobe was examined at the light and electron microscopic levels by immunoperoxidase localization of glial fibrillary acid protein (GFA), a protein that is present in astrocytes and radial glial cells but not neurons. During the peak of neurogenesis at embryonic day 80, two distinct classes of proliferative cells, GFA-positive and GFA-negative, are intermixed in the ventricular and subventricular zones. Both cell types are readily recognized in different phases of the mitotic cycle along the ventricular surface. The results indicate that, contrary to prevailing views, (1) glial and neuronal cell lines coexist within the fetal proliferative zones and (2) the onset of glial phenotypic expression occurs prior to the last cell division.

All neurons, with few exceptions, are generated near the surface of the embryonic cerebral ventricles at sites far from their ultimate positions in the adult mammalian brain (see Sidman and Rakic, 1973; Rakic, 1975a for reviews). Glial cell proliferation is thought to occur at sites distant to the ventricles, although the glioblast must originate from the neuroepithelium at some time (Jacobson, 1978). The uncertainty concerning the developmental stage at which glial and neuronal cell lines diverge in the proliferative zone has been a persistent problem in neuroembryology for almost a century. From his classic observations on the embryonic ventricular zone,[2] His (1889) suggested that the proliferative zone is composed of two cell lines, one that gives rise to neurons (*Keimzellen* or "germinal cells") and the other that produces glia or glioblasts ("spongioblasts"). Examination of similar histological material induced Schaper (1897a, b) to propose that the ventricular zone is comprised of a single, homogeneous, mitotically active cell population which produces "indifferent" cells that migrate into the intermediate (mantle) zone to give rise to both neurons and

glial cells. This hypothesis was supported by Sauer (1935), who showed that His's two cell classes simply represent different phases of the mitotic cycle of the same cell type.

More recently, autoradiographic data obtained from studies using pulse and/or cumulative [3H]thymidine (3H-TdR) labeling of ventricular zone cells have also been interpreted as supporting the view that the ventricular zone contains a homogeneous population of dividing cells (Fujita, 1963; Sidman et al., 1959). These results, taken in conjunction with studies using 3H-TdR to determine the time of cell origin, have indicated to many investigators that the glial cells in a given structure are usually generated only after neuronal production ceases (e.g., Angevine, 1970; Berry and Rogers, 1965; Fujita, 1965; Hicks and D'Amato, 1968; see Jacobson, 1978 for review) and have led to the widely held view that the ventricular zone may be comprised of only a single precursor cell population. According to Fujita (1963, 1966), this lone precursor (or "matrix") cell first gives rise to neurons; only later, after the completion of neurogenesis, is it supposed to switch to glial production and then finally transform into a mature ependymal cell. Fujita's formulation is representative of the prevailing view (e.g., see Boulder Committee, 1970; Jacobson, 1978).

Contrary to this notion, detailed quantitative analysis of the cell proliferation cycle in the telencephalon raises the possibility that the ventricular zone in young rodents consists of at least two cell populations with different generation times (Gracheva, 1969; Waechter and Jaensch, 1972). Furthermore, studies using Golgi and electron microscopic methods have indicated that glial cells, particularly radial glial cells and Bergmann glial cells, exist during late stages of neurogenesis (Rakic,

[2] When describing the principal embryonic zones of the central nervous system, the nomenclature suggested by the Boulder Committee (1970) will be used. Under this terminology, two proliferative zones, the *ventricular* and *subventricular* zones, correspond to the germinal matrix zones of His (1889).

1971, 1972; Schmechel and Rakic, 1979). More recently, immunocytochemical staining for the specific glial fibrillary acid protein (GFA) (Bignami et al., 1972; Eng, 1980; Eng and Bigbee, 1978; Eng et al., 1971; Ludwin et al., 1976) provided more direct evidence of the presence of radial glial cells during early periods of neurogenesis in the human cerebrum (Antanitus et al., 1976; Choi and Lapham, 1978) and in all major subdivisions of the fetal monkey central nervous system (Levitt and Rakic, 1980). This method showed that the number of radial glial cells increases during periods of peak neuronal proliferation and subsides only after neuronal migration ceases (Levitt and Rakic, 1980). Thus, it has become increasingly evident that neuronal and glial proliferative precursor cells must coexist at relatively early stages of brain development. However, light microscopic studies cannot determine conclusively whether both neurons and glial cells arise from a single mitotically active cell population, as suggested by Schaper (1897a, b), or whether there exist two distinct classes of proliferative cells in the ventricular and subventricular zones, as advocated by His (1889). In addition, previous electron microscopic studies failed to display ultrastructural features that would permit classification of the dividing cells into multiple classes (Fujita and Fujita, 1963, 1964; Stensaas and Stensaas, 1968).

The present study is the first use of electron microscopy in conjunction with a sensitive immunohistochemical technique (Sternberger, 1979) in the fetal brain. The combination of these techniques for the localization of GFA has allowed, for the first time, the direct determination of whether two classes of proliferative cells exist in the ventricular zone during the peak of neurogenesis in the cerebral wall.

Materials and Methods

Rhesus monkeys (*Macaca mulatta*) of 80 and 123 embryonic (E) days were used for this ultrastructural immunohistochemical study. These ages represent, respectively, the period of peak neurogenesis and the period by which all neurons have been generated in the occipital lobe (Rakic, 1974). In addition, sections from two fetal monkeys of approximately the same ages were prepared for light microscopic immunohistochemistry (Levitt and Rakic, 1980). The gestational period of the rhesus monkey is about 165 days. Animals were bred and pregnancies were dated as previously described (Rakic, 1973).

Anti-GFA immunoperoxidase staining of the occipital lobe was performed for light and electron microscopic localization of glial cells. The embryos at each age were perfused transcardially with a 1.25% formaldehyde, 1.0% glutaraldehyde phosphate-buffered fixative (pH 7.4). The brains were then immersed in the fixative for 24 hr at 4°C prior to dissection of the occipital lobes. One lobe was processed for polyester wax embedding (Sidman et al., 1961) and subsequent antibody staining using the peroxidase-antiperoxidase (PAP) technique as previously described (Levitt and Rakic, 1980). The other occipital lobe was used for GFA localization at the ultrastructural level. Coronal 20- to 30-µm-thick sections of this lobe were cut using a vibratome (Oxford Instruments); then, the sections were rinsed overnight at 4°C in a phosphate-buffered saline solution (PBS) prior to exposure to the

antibody. During the first incubation, sections were bathed for 7 hr at room temperature in a 1:150 dilution of rabbit antibody directed against GFA (Eng and Rubinstein, 1978; the antibody was supplied by Dr. L. F. Eng, Veterans Administration Hospital, Palo Alto, CA), followed by a 5-hr incubation at 4°C. The antibody was prepared in PBS containing normal swine serum, diluted 1:20. (In order to preserve ultrastructural quality, our methodology did not include detergent in the incubation steps.) The tissue next was rinsed five times with cold PBS over a 1-hr period and then washed overnight in PBS at 4°C. Control sections were processed as noted above, substituting normal rabbit serum for the primary antibody. The following day, the sections were allowed to warm to room temperature, rinsed twice with PBS, and then incubated for 5 hr at room temperature in a 1:50 dilution of horseradish peroxidase (HRP)-linked swine anti-rabbit immunoglobulin G (Dako), followed by an additional 5 hr at 4°C. Next, the tissue was rinsed as described for the first incubation. Finally, the HRP was visualized with the 3,3'-diaminobenzidine reaction. The vibratome sections were mounted in a glycerol:PBS solution (3:1), examined and photographed light microscopically, and then processed for electron microscopic analysis.

For electron microscopy, the tissue was trimmed into 3- to 4-mm-wide segments which spanned the entire width of the occipital cerebral wall, from the ventricular to the pial surfaces. Following a 30-min rinse in 0.1 M phosphate buffer, the tissue was osmicated (2% OsO_4, 60 min), briefly stained *en bloc* with uranyl acetate (15 to 30 min), dehydrated in a graded series of alcohols and propylene oxide, and finally embedded in a 1:1 mixture of Epon 812 and Araldite 8005. The sections were flat mounted on plastic coverslips which, in turn, were glued to plastic blocks. Then, the tissue blocks were trimmed into pyramids containing the proliferative and intermediate zones. Thick plastic sections (0.5 to 1.0 µm) were collected, counterstained lightly with toluidine blue, and examined to determine the distribution and number of mitotic figures lining the ventricular surface. Next, silver thin sections were collected on Formvar-coated grids and examined with a JEOL 100A electron microscope. These thin sections were not counterstained with either uranyl acetate or lead citrate in order to improve the contrast between the HRP reaction product and the surrounding unlabeled tissue.

GFA-positive cells situated in the proliferative zones were identified in over 300 micrographs printed at magnifications ranging from × 2,000 to × 75,000. Each mitotic figure identified in the thick plastic sections was also localized ultrastructurally for final determination of the presence or absence of GFA staining. The sensitivity and specificity of the GFA antibody and the immunohistochemical methods utilized here for the localization of glial cells are well documented (Eng and Kosec, 1974; Levitt and Rakic, 1980; Ludwin et al., 1976). There is little doubt concerning the specificity of GFA as a major constituent of astroglial filaments (Bignami et al., 1972; Bignami and Dahl, 1974; Eng, 1980; Eng and Bigbee, 1978; Eng and Kosec, 1974). In the results presented below, the ultrastructural localization of the immuno-
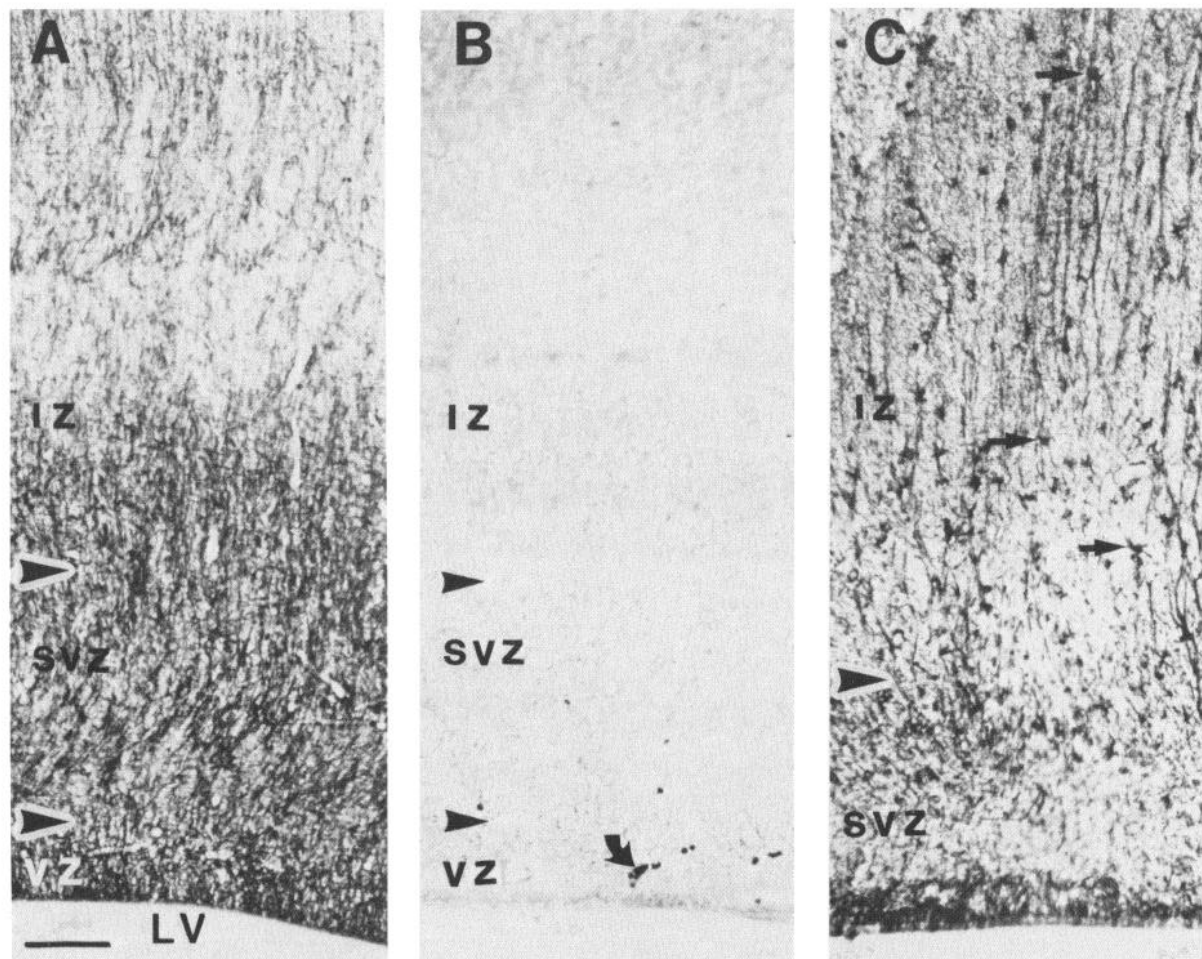
Figure 1.[3] Immunoperoxidase localization of GFA-positive glial cells in uncounterstained 25-μm vibratome sections of the occipital lobe. *Horizontal arrowheads* indicate the approximate boundaries between the primary embryonic zones. Lateral cerebral ventricle is at the bottom of the figures. *Marker bar* = 150 μm. *A,* Cerebral wall of occipital lobe of 80-day-old monkey fetus (E80) shows dense array of positively stained radial glial cells and their elongated fibers. The glial shafts extend from the cell somata, situated within the ventricular and subventricular zones, through the intermediate zone and enter the developing cortical plate, which lies above the area encompassed by the photograph. *B,* An adjacent section, used as a control, was processed identically to that illustrated in *A* except that normal rabbit serum was substituted for the GFA antibody. No glial cells or their fibers are stained in this preparation. The small dark spots (*curved arrow*) in the ventricular zone are red blood cells whose hemoglobin has endogenous peroxidase-like activity. *C,* As in younger specimens, the cerebral wall in a 123-day-old monkey fetus contains densely staining radial glial fibers. In addition, there are numerous transitional profiles representing radial glial cells transforming into mature astrocytes (*arrows*), which are situated predominantly in the intermediate zone. Differentiated astrocytes are conspicuously absent at E80 (*A*). Also, note the decrease in the number of radial fibers, particularly in the proliferative zones, compared to the E80 specimen (*A*).

peroxidase reaction product within the embryonic glial cells correlates closely with previous electron microscopic characterizations in mature astrocytes (Maunoury et al., 1979; Schachner et al., 1977).

Results

The proliferative zones of the occipital lobe in the rhesus monkey fetuses at both E80 and E123 contain a large number of cells stained positively for GFA intermixed with cells containing no reaction product (Fig. 1).

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[3] The abbreviations used on the figures are: C, chromatin; Cy, cytoplasm; IZ, intermediate zone; LV, lateral cerebral ventricle; N, nucleus; SVZ, subventricular zone; VZ, ventricular zone.

As reported previously (Levitt and Rakic, 1980), the only morphologically differentiated cells which contain the fine brown HRP reaction product are glial in nature. There is no immunoperoxidase staining in the control material (Fig. 1*B*) or in any neuronal elements in either the E80 or E123 specimens.

Light microscopic examination. At E80, the cerebral occipital wall contains denser GFA staining than at E123 or any other stage previously examined (Levitt and Rakic, 1980). The reaction product at E80 is located mostly in radial glial cells, the perikarya of which are situated within the ventricular and subventricular zones (Fig. 1). The GFA-positive radial glial processes course in a palisade-like fashion through the intermediate zone and developing cortical plate to terminate in conical end-

feet along the pial surface. As is evident in the 25-μm vibratome sections, large numbers of the radial glial processes run in dense fascicles from the proliferative zones into the intermediate zone, where the fascicles usually split into single processes prior to entering the developing cortical plate. Fibrous and protoplasmic astrocytes are not evident in the occipital lobe at this embryonic age.

The number of positively stained radial glial fibers seems to decrease by E123, although some areas of the occipital lobe still contain an abundance of densely stained radial processes (Fig. 1C). Many of the GFA-positive perikarya of radial glial cells are located now in the subventricular zone, and a large number of their elongated processes still traverse the expanded width of the cerebral wall to terminate at the pial surface. In addition to the radial glial cells, positively stained, morphologically differentiated astrocytes are situated in the intermediate zone and cortex at E123 (Fig. 1C). However, the ventricular zone in most regions of the occipital lobe at this stage has been depleted of proliferative germinal cells and contains only maturing ependymal cells and some radial glial cell somata. As expected, the specialized ependymal cells do not stain positively for GFA (Eng, 1980). Neuronal proliferation in the monkey occipital lobe has ceased by E123 (Rakic, 1974, 1975b), so that all

dividing cells remaining in the subventricular zone at this age are presumably glial. Indeed, most cells in this zone are positively stained with the immunoperoxidase method at E123 (Fig. 1).

The light microscopic observations of the ventricular and subventricular zones at E80 indicate the presence of two cell populations. GFA-positive cell somata lie at varying distances from the ventricular surface. Intermingled among these is a population of cells that does not stain positively and thus is presumed to contain the neuronal precursor population. A crucial question is whether or not the two cell classes are determined developmentally before or after final cell division.

During the peak of neurogenesis at E80, there is considerable cell proliferation at the ventricular surface, which contains many mitotic figures. At the light microscopic level, a number of these dividing cells appear to have a thin cytoplasmic rim containing the HRP reaction product, while in the others, the cytoplasm seems to be unlabeled (Fig. 2). This finding raises the possibility that two distinct mitotically active cell populations exist within the ventricular zone. Thus, two distinct precursor cell types (glial and neuronal) may proliferate side by side. Light microscopic examination of the immunoperoxidase labeling does not provide sufficient resolution to answer this question because it could be argued that
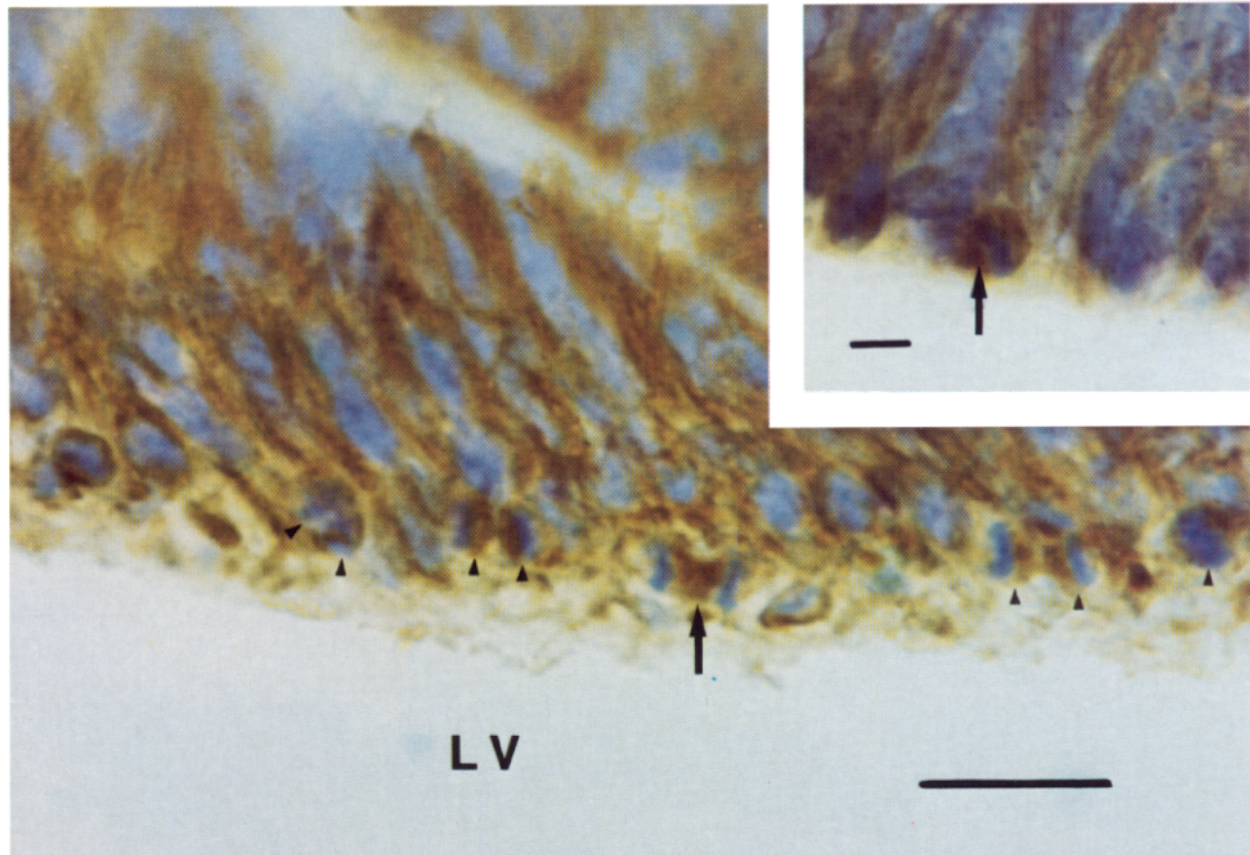

Figure 2. Vericolor photomicrograph of cresyl violet counterstained 8-μm-thick polyester wax embedded section. Numerous mitotic figures (*arrowheads*) are situated along the ventricular surface of the occipital lobe in an 80-day-old monkey fetus. The *arrowheads* on the *far left* point to GFA-negative mitotic cells which lie under a GFA-positive process. Two adjacent daughter cells are positively stained (*arrow*). At higher magnification (*inset*), a dividing cell in metaphase (*arrow*) contains the brown HRP reaction product. Although it appears here that the positive HRP staining is in the cytoplasm, it is not possible at the light microscopic level to exclude the possibility that the product lies within a glial process which surrounds the dividing cell. *Marker bar = 20 μm. Inset marker bar = 5 μm.*
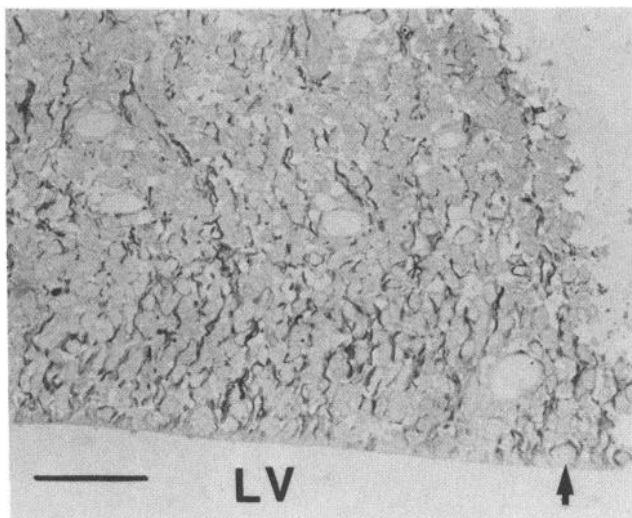
Figure 3. Numerous radial glial fibers are densely stained for GFA in a 0.5-μm-thick plastic section through the cerebral wall of the E80 specimen. The positively labeled processes penetrate the ventricular and subventricular zones in a wavy, but approximately parallel fashion. The *arrow* indicates an uncounterstained cell that is outlined by a dense cytoplasmic rim of HRP reaction product. Counterstaining with toluidine blue shows that this cell is in the anaphase stage of its mitotic cycle (see also Fig. 8). *Marker bar = 50 μm.*

the apparently positively stained mitotic cells at the ventricular surface only represent basal processes of postmitotic glial cells wrapping around the somata of the dividing neuronal precursors (Fig. 2). Thus, analysis at the ultrastructural level is essential in order to determine the location of the HRP reaction product within ventricular zone cells.

Electron microscopic examination. A thorough inspection of the ventricular surface in the 0.5-μm-thick plastic sections permits a somewhat more detailed characterization of the cells stained positively for GFA (Fig. 3). The cytoplasmic rim of each mitotically active cell is either clearly stained or completely unstained by the antibody-HRP complex (Figs. 7 and 8 *insets*). The light counterstaining of the 0.5-μm-thick sections with toluidine blue enables clear visualization of the mitotic figure in each cell in addition to the HRP reaction product. Of the 15 mitotic cells examined in the thick sections of the E80 specimen, approximately 60% were labeled by the GFA antibody. Following their identification in the thick sections, these mitotic cells were examined in thin sections under the electron microscope.

Ultrastructural examination of the E80 specimen at low power reveals a number of positively stained cell bodies and their processes throughout the ventricular zones (Fig. 4). Although the staining is occasionally pale (probably due to either detergent omission or less GFA), each cell can easily be classified as either GFA-positive or GFA-negative. The cell perikarya are situated at varying distances from the ventricular surface, many in the DNA-synthesizing region at the outer edge of the ventricular zone. Distal processes containing the HRP product can usually be followed for only a short distance in a single ultrathin section. Basal processes arising from both

Figure 4. Low power electron micrograph of the ventricular and subventricular zones of the monkey occipital lobe at E80. Labeled cells (*arrowhead*) are distinguished readily from GFA-negative cells (*arrow*). This preparation reveals the heterogeneity of cells in the proliferative zones. The GFA-positive basal processes of the glial cells are intermingled with unstained cells of the ventricular and subventricular zones. In this and all subsequent electron micrographs, lead citrate and uranyl acetate counterstaining of the ultrathin sections has been omitted (see "Materials and Methods"). Thus, contrast of the electron micrographs is low compared to that in routinely counterstained sections. Counterstaining was omitted to render more visible the HRP reaction product located in GFA-positive cells. *Marker bar = 10 μm.*

labeled and unlabeled cells can often be traced directly to their termination in the form of an end-foot along the ventricular surface. Commonly, GFA-positive and -negative endings alternate at this surface (Fig. 5). Such a regular pattern of labeled and unlabeled cells present at this embryonic age corroborates previous light microscopic observations that indicated that GFA-positive processes run between columns of unlabeled ventricular zone

cells (Levitt and Rakic, 1980; see also Fig. 2). The labeled and unlabeled processes are in intimate contact with each other at the ventricular surface; desmosomes are prevalent, joining stained with unstained fibers (Fig. 5). Both types of endings contain an abundance of clear, large vacuoles, mitochondria, and cilia projecting into the ventricular cavity.

Electron microscopic examination reveals that, in addition to the GFA-positive cells located at varying depths throughout the ventricular zone at E80, a number of the mitotic cells situated along the ventricular surface contain HRP reaction product in their cytoplasm. Again, there is a clear distinction between the cytoplasm of stained and unstained cells in different phases of mitosis (Figs. 6 to 8). The mitotic cells lack long proximal processes, consistent with the idea that precursor cells round up while undergoing cell division (Stensaas and Stensaas, 1968; Seymour and Berry, 1975). Both labeled and unlabeled mitotic cells contain the typical organelles observed

in proliferative cells of the central nervous system (Fujita and Fujita, 1963, 1964; Stensaas and Stensaas, 1968). Previous investigators noted that it is not possible to distinguish whether there is more than one class of mitotically active cells on the basis of the usual ultrastructural criteria. Thus, there is no obvious ultrastructural difference between the labeled and unlabeled cell types apart from the presence or absence of the antibody staining reaction product (Figs. 6 to 8). Within the cytoplasm of stained cells, the label was distributed evenly as a ring around the entire circumference of the mitotic figure (Figs. 6 and 8). Therefore, after division, both daughter cells presumably contain an approximately equal amount of the GFA. This is supported by the observation that obvious daughter cells are both stained (cf., Fig. 2).

The typical, rather globular HRP reaction product is located mainly on fibrillary structures in the labeled radial processes, on the cytoplasmic surface of the plasma
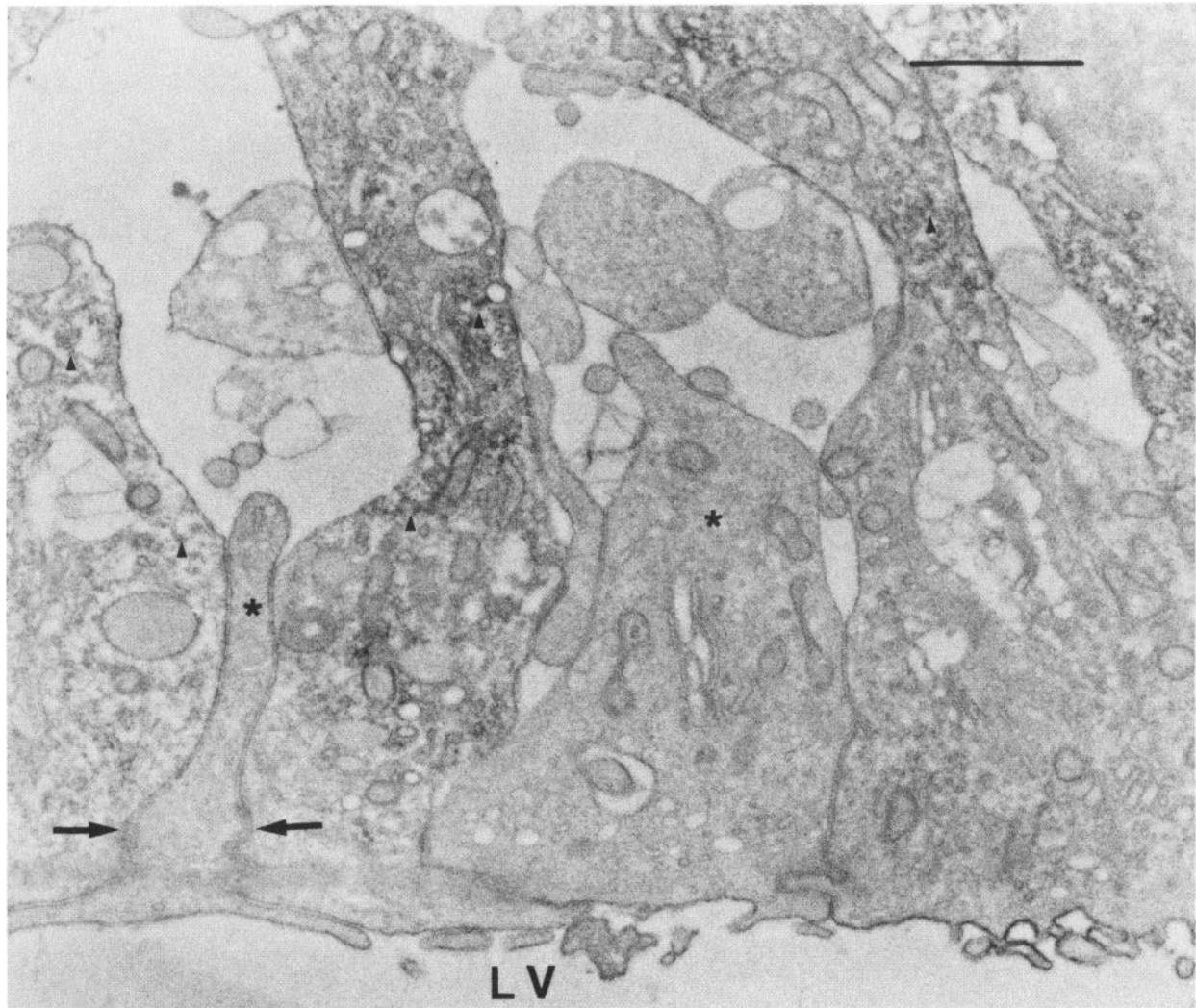

Figure 5. Basal processes in the ventricular zone of the occipital lobe of an E80 specimen. There is a roughly alternating pattern of GFA-negative (*) and GFA-positive processes (*arrowheads*) that terminate along the ventricular surface. Desmosomal junctions (*arrows*) are formed between both types of processes. All the basal processes appear to contain an identical complement of organelles, including mitochondria and large, clear vacuoles. Typical projecting cilia are commonly visible along the surface of the lateral ventricle in both labeled and unlabeled cells. *Marker bar* = 1 μm.
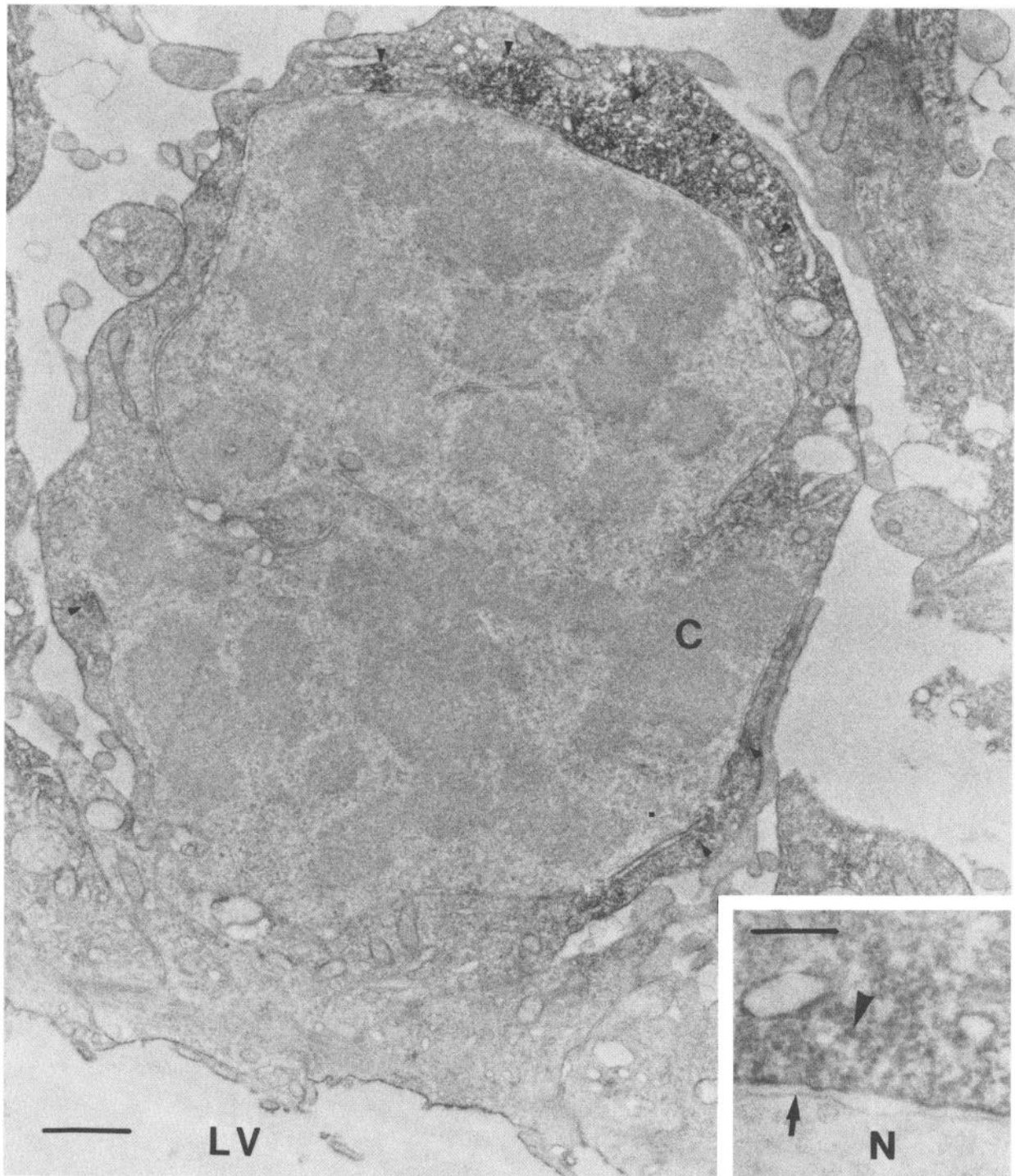
Figure 6. A dividing cell in late prophase situated at the ventricular surface of the occipital lobe in the E80 monkey. Even in this uncounterstained section, the clumped chromatin is evident in the nucleus, which during this mitotic phase still remains separated from the cytoplasm by a nuclear envelope (*inset, arrow*). The *arrowheads* denote the HRP reaction product, indicative of GFA-positive staining. The dense, globular reaction product (*arrowhead*) is seen more clearly at higher power in the *inset*. *Marker bar = 1 μm. Inset marker bar = 0.2 μm.*

membrane, outside mitochondrial membranes, and diffusely throughout the cytoplasm (Figs. 5, 6, and 8). The inner compartments of the Golgi complex, endoplasmic reticulum, mitochondria, and the cell nucleus are totally devoid of reaction product (Figs. 5, 6, and 8). This pattern of labeling is typical of the ultrastructural localization of GFA (Eng and Bigbee, 1978; Maunoury et al., 1979;

Schachner et al., 1977). The present study was not designed to precisely localize GFA within cytoplasmic organelles or to determine the site of its synthesis. The localization of the antigen-antibody binding site depends upon many factors, including fixation, antibody access to different tissue compartments, and staining parameters. Thus, it is difficult with our methodology to define pre-
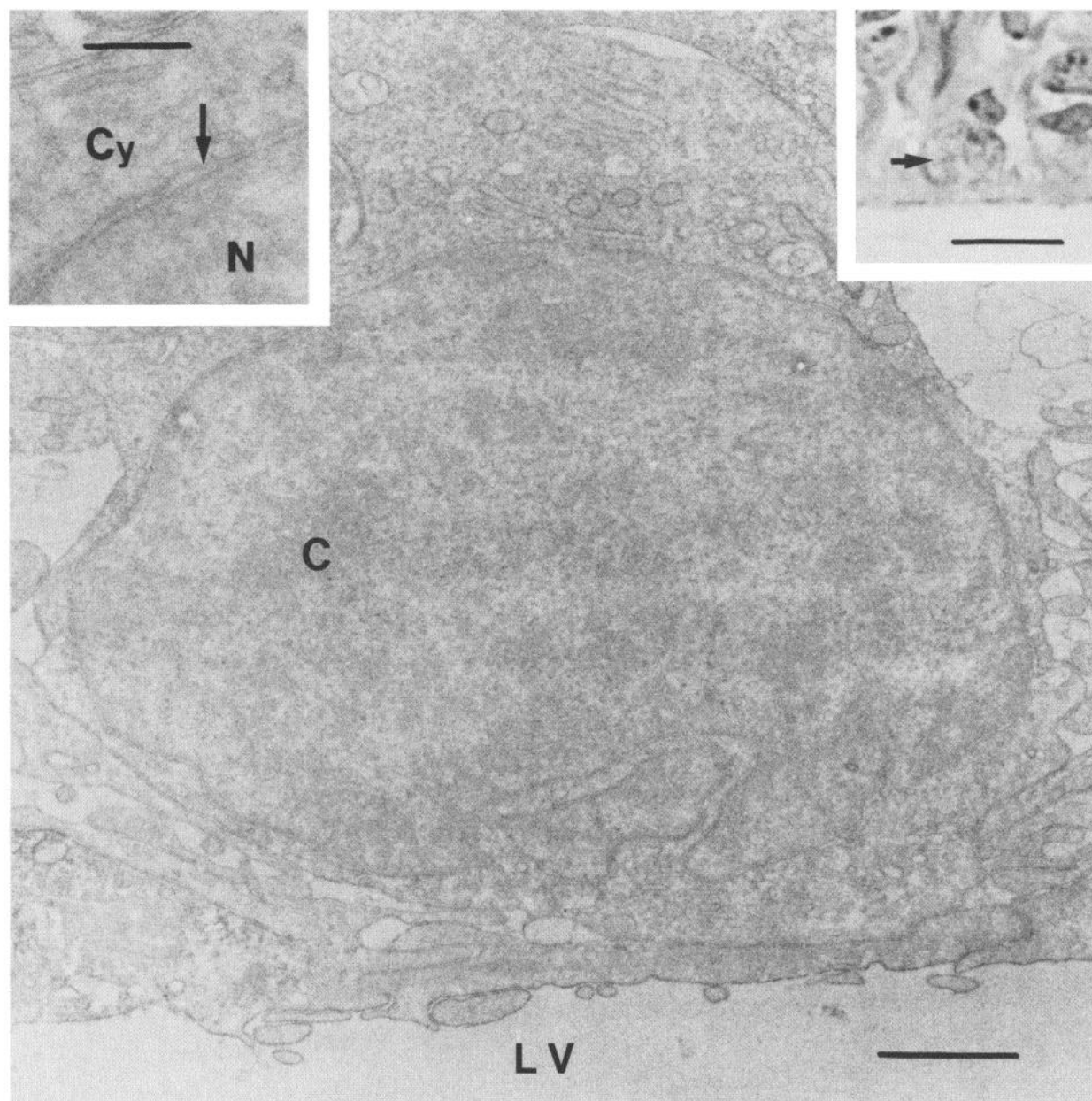
Figure 7. A GFA-negative cell in early prophase is situated in the ventricular zone of the occipital lobe in the E80 specimen. The lightly clumped chromatin in the cell nucleus is characteristic of this mitotic phase; this feature is visible more readily in the 0.5-μm-thick plastic section (*arrow, right inset*) of the same cell. Note the lack of any HRP reaction product in the cytoplasm in this mitotic cell at both the light and ultrastructural levels. The absence of any GFA staining in the cytoplasm is more evident at higher power (*left inset*). The nuclear envelope (*arrow*), normally present in prophase, separates the nucleus from the cytoplasm. Note that the nucleus and cytoplasm show identical contrast due to lack of the HRP reaction product and the omission of the lead citrate and uranyl acetate counterstaining. *Marker bar = 1 μm. Right inset marker bar = 10 μm. Left inset marker bar = 0.2 μm.*

cisely the normal intracellular sites of GFA. However, for our purposes, the simple identification of the positively and negatively staining cells is sufficient.

Discussion

The present immunocytochemical study provides new insights into two major issues in developmental neurobiology. First, we have demonstrated the heterogeneity of the proliferative zones during the peak period of neurogenesis in the rhesus monkey occipital lobe by showing

the coexistence of mitotically active neuronal and glial precursor cells. Secondly, we have shown that the two cell lines begin to diverge prior to completion of final cell division. These results stand in contrast to the widely held views that neurogenesis precedes gliogenesis and that the ventricular zone is composed of a single cell type during neurogenesis (see Boulder Committee, 1970; Jacobson, 1978 for review).

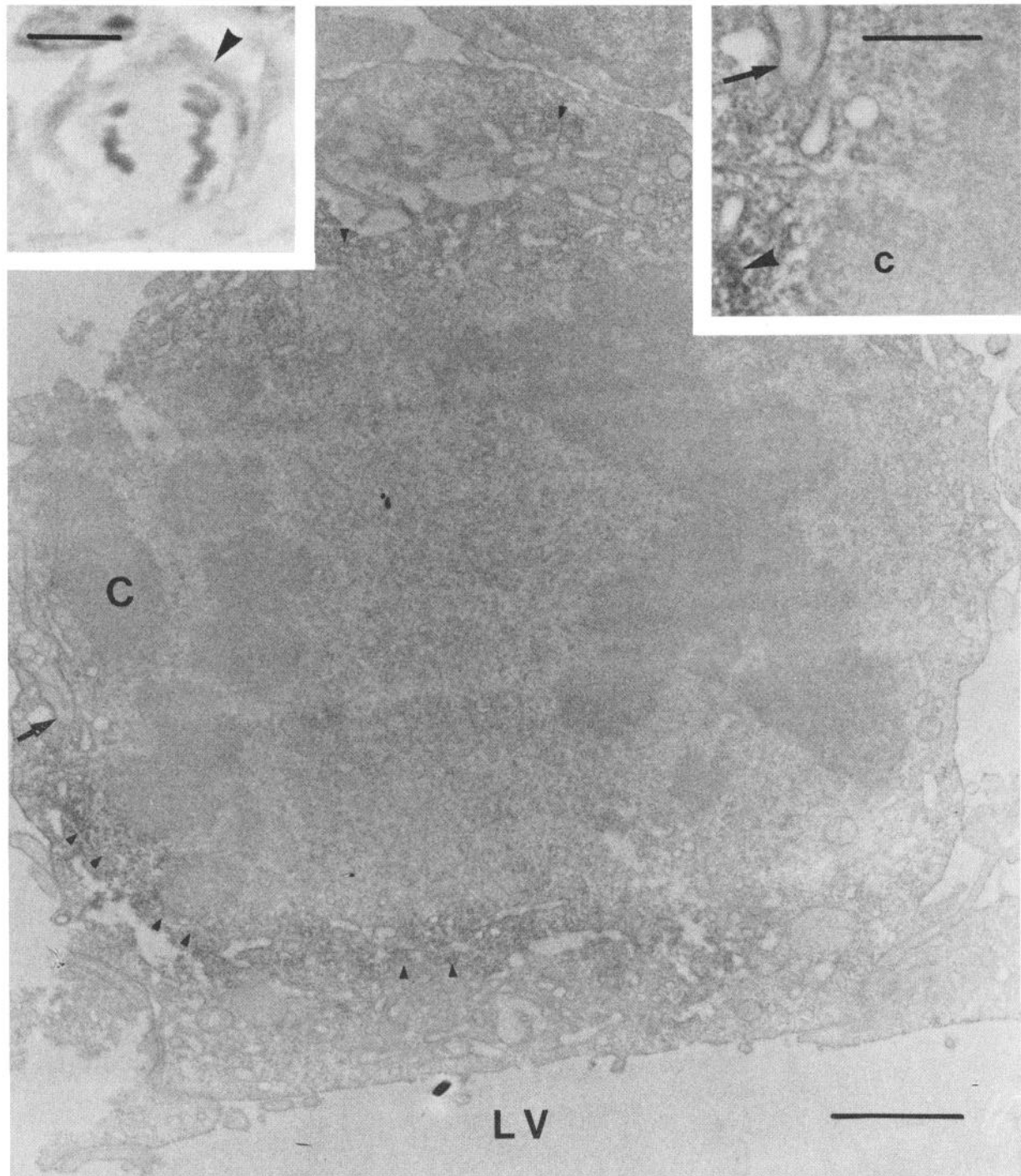*GFA as a specific marker of developing glial cells.* The GFA antigen was selected as a marker for glial cell

Figure 8. A GFA-positive dividing cell situated in the occipital lobe in the E80 monkey fetus; this same cell is illustrated in the 0.5-μm section in Figure 3. Distinct chromatids (*C*), characteristic of anaphase, are evident in both the light (*left inset*) and ultrastructural views. A densely labeled cytoplasmic rim (*arrowhead*) surrounding the nucleus is prominent in the 0.5-μm-thick plastic section (*left inset*). The HRP reaction product is evident also throughout the cytoplasm (*arrowheads*) at the ultrastructural level. An unstained mitochondrion (*arrow*), and the typical HRP reaction product (*arrowhead*), characteristic of anti-GFA immunoperoxidase staining, are visible more clearly at higher magnification (*right inset*). The nuclear envelope has disintegrated by this phase of the mitotic cycle. In spite of this, the GFA reaction product remains at the periphery of the perikaryon and does not intermix with the nuclear material. *Marker bar = 1 μm. Left inset marker bar = 5 μm. Right inset marker bar = 0.4 μm.*

differentiation during brain development only after careful consideration. This structural protein is well documented as a specific component of astroglial filaments that are present in protoplasmic and fibrillary astrocytes as well as in radial and Bergmann glial cells (see Eng, 1980 for review). The GFA protein is found neither in non-nervous tissues nor in either developing or mature oligodendrocytes and neurons. Furthermore, it should be emphasized that those proliferating cell populations of the central nervous system that can be identified readily

as consisting of purely neuronal precursors, such as the external granule cells of the cerebellum or the granule cells of the hippocampal dentate gyrus, do not stain positively for GFA (Bignami and Dahl, 1973; Levitt and Rakic, 1980). Taken together, all available data support the contention that GFA is a specific, stable gene product of glial cells of astrocytic lineage and that it is not normally expressed in any other cell classes.

Although several other macromolecules such as non-neuronal enolase (NNE; EC 4.2.1.11) and S-100 protein have been utilized as glial cell markers in the adult brain (Cicero et al., 1970; Ludwin et al., 1976; Schmechel et al., 1978), the case for their usefulness as differentiation antigens during development is less convincing. For example, NNE or its hybrid form has been detected in mature cerebellar stellate and basket neurons, in non-glial brain tissues such as choroid plexus and ependymal cells, and even in non-brain tissue such as liver (Schmechel et al., 1980). Thus, this protein cannot be considered as a specific gene product of glial cells, since several other cell types, including developing neurons, express NNE (Schmechel et al., 1980). The S-100 protein is a highly soluble macromolecule that presents considerable difficulties in its use as a glial differentiation marker in the developing brain. This protein has been found in both neurons and glial cells using a variety of fixation and staining methods, and controversy still exists as to whether its localization is truly specific to glial cells (cf., Varon and Somjen, 1979). The protein is localized only to glial cells in brains that are extremely well fixed. However, S-100 protein is found in both glial and neuronal elements when the tissue is fixed only moderately or inadequately (Eng and Bigbee, 1978; Hartman et al., 1977). The presence of S-100 protein in neurons may be due to intercellular transfer of this protein, and such a problem would be especially acute in tightly packed regions such as the embryonic ventricular zone, which in any event is difficult to fix well. These problems concerning the utilization of S-100 protein raise questions about its suitability for use (as in DeVitry et al., 1980) in determining cell lineages in developing brain. Therefore, given the available data, it seems that GFA best meets rigorous criteria for use as a specific glial differentiation marker.

Heterogeneity of the cerebral proliferative zones. As noted in the introduction, His (1889) proposed that the germinal epithelium of the cerebral wall consists of two precursor cell classes, one neuronal and the other glial (Fig. 9A). Furthermore, he thought that glial cells are organized in the form of a multinuclear syncytium. Subsequently, an opposing view was elaborated by Schaper (1897a, b), who considered the ventricular zone to be composed of a single precursor cell type giving rise to indifferent cells (Fig. 9B). The idea of a homogeneous ventricular zone gained support from Sauer's (1935) demonstration that ventricular zone cells with different shapes and nuclear locations simply represent various phases of the cell mitotic cycle, from detailed ultrastructural examinations in chick embryos (Fujita, 1966; Fujita and Fujita, 1963, 1964), and from studies by Fujita (1963) and Sidman et al. (1959) that showed that all cells in the proliferative zone could be labeled by ^{3}H-TdR. Furthermore, investigations of the time of cell origin with this

autoradiographic technique were interpreted by many investigators to show that, in general, neurons of the vertebrate central nervous system are generated prior to glial cells (see the introduction). For example, Fujita (1963, 1966) proposed that the germinal cells first give rise to neurons and that later, after cessation of neuronal production, these proliferative cells undergo some undefined changes to become glial precursor cells (Fig. 9C).

There are at least two problems in the interpretation of ^{3}H-TdR autoradiographic labeling after either short or long survival times. First, labeling of all ventricular cells with cumulative administration of ^{3}H-TdR does not necessarily imply a homogeneous cell population. Rather, it indicates only that all cells in this zone are synthesizing DNA during this period. Secondly, embryonic glial cells may have prolonged periods of cell division, resulting in the dilution of the ^{3}H-TdR label to an undetectable level in the mature brain. Thus, the ^{3}H-TdR technique is not adequate to determine the onset of differentiation of glial cells during the development of a given structure.

The first indication that neuronal and glial cell lines coexist in the embryonic brain was provided by descriptions of radial glial cells. This class of glial cells was described in the spinal cord by the Golgi impregnation method at the end of the last century (Golgi, 1885; Ramón y Cajal, 1890; Retzius, 1894). Because radial glial cells are present and increase in number along with neurons prior to and during the peak of corticogenesis (Levitt and Rakic, 1980; Rakic, 1972, 1974, 1975b; Schmechel and Rakic, 1979), the precursors for these cell types theoretically must be present concomitantly (Fig. 9D). However, prior to this report, the actual location and coexistence of the two precursor cell types had never been established.

The use of an immunological probe in the present study reveals for the first time the heterogeneous character of the ventricular zone by demonstrating the presence of two distinct mitotically active cell types (GFA-positive and GFA-negative) (Figs. 6 to 8). Therefore, although His's (1889) description of a cellular syncytium proved to be wrong, his concept that two classes of proliferative cells coexist in the ventricular zone, one giving rise to neurons and the other to glia, gains support by the present immunocytochemical study (Fig. 9D).

Onset of glial phenotypic expression as indicated by the presence of GFA. Our results demonstrate the presence of at least two mitotically active precursor cell types in the embryonic ventricular zone. The localization of a differentiation antigen, GFA, in cells that are actively dividing and synthesizing DNA also indicates that proliferative cells are committed to their specific lineage prior to final mitosis. Thus, the onset of expression of a specific cell phenotype is not rigidly linked to *final* mitosis. This concept has been documented for the processes of erythropoiesis and chondrogenesis (see Caplan and Ordahl, 1978; Marks et al., 1978; Rutter et al., 1973 for reviews). Likewise, in the developing sympathetic nervous system, mitotically active cells that are destined to become sympathetic neurons contain the specific enzymes which are involved in the synthesis of the neurotransmitter norepinephrine (Cohen, 1974; Rothman et al., 1978).

It should be emphasized that the present results do not resolve the questions of whether and at what stage a
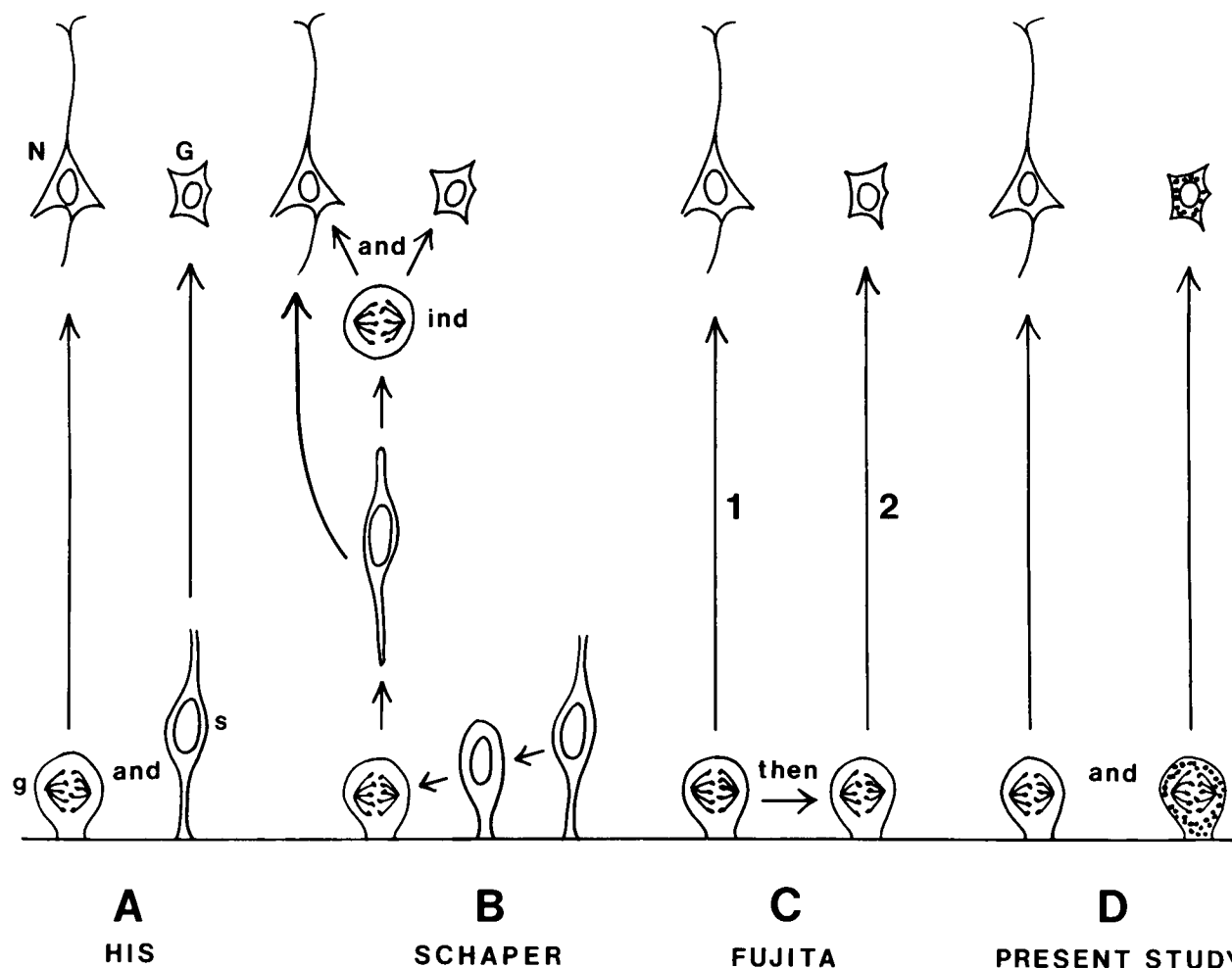
Figure 9. A schematic diagram summarizing in a simplified manner previous theories of the origin of neuronal and glial cell lines (*A, B,* and *C*) in comparison to the scheme suggested by the results of the present study (*D*). *A,* His (1889) distinguished two types of cells in the germinal matrix (now called ventricular) zone lining the surface of the embryonic ventricles (represented by the *horizontal line*). The first type is the rounded "germinal cell" (*g*), which contains a mitotic nucleus in proximity to the ventricular surface. The second is the columnar "spongioblast" (*s*), with a nucleus that can lie at various distances from the ventricle. His believed that the "germinal cells" give rise to neurons (*N*), whereas the "spongioblasts," which he held to form a syncytium, give rise to glia (*G*). Thus, His proposed that separate and different neuronal and glial precursor lines coexist in the ventricular zone. *B,* Schaper (1897a, b) considered that His's two cell types actually represented different phases of a single type of cell; this was confirmed later by Sauer's (1935) study. Furthermore, Schaper suggested that the mitotically active cells of the ventricular zone are indifferent cells (i.e., not yet determined as either glial or neuronal). He supposed these cells to give origin to migrating cells which move into the overlying intermediate (mantle) zone, where they yield either neurons directly or both neurons and glia after further divisions as indifferent (*IND*) cells. Schaper's theory, therefore, supported the homogeneity of the ventricular zone. *C,* Tritiated thymidine autoradiographic evidence led to Fujita's (1963) scheme, which also emphasizes the homogeneity of the ventricular zone. According to Fujita, the dividing cell population in the ventricular zone first gives rise to neurons (*1*); then, after neurogenesis ceases, this same dividing population changes proliferative programs and begins to produce glial cells (*2*). Thus, Fujita suggested that at any given time, the proliferative population is homogeneous and composed of cells which first give rise to neurons and only later to glia. *D,* In the present study, we have demonstrated that both GFA-positive (*stippled*) and GFA-negative mitotic cells coexist in the ventricular zone at the time of peak neurogenesis during midgestation in the monkey occipital lobe. Therefore, the labeled cells already possess a glial differentiation marker and accordingly may be a committed glial precursor cell line. The data point to the coexistence of distinct neuronal and glial precursors in the ventricular zone, implying that the proliferative cell population is heterogeneous in its composition.

single, indifferent germinal cell gives rise to both glial and neuronal precursor types. Neither do we address the issue of whether all or only some astrocytes pass through the stage of radial glial cell phenotype. Finally, we do not know whether the unlabeled cells consist of multiple cell classes, including some "indifferent" cells. Therefore, although our data demonstrate clearly that at E80, there already exist distinct neuronal and glial precursors in the ventricular zone of the monkey occipital lobe, the details of the lineage relationships between these cells remain unclear. However, as noted above, the fact that cellular populations that are known to contain proliferating neuronal percursors, such as those which produce the hippocampal and cerebellar granule cells, do not express detectable levels of GFA indicates that transient expression of this marker by neuronal precursor cell lines is unlikely. Given the early coexistence (by E47) of neurons and differentiated radial glial cells in the cerebral wall of

monkey embryos (Levitt and Rakic, 1980), we favor the hypothesis that neuronal and glial lines maintain separate identities from the time that neurogenesis is initiated in a given structure (Fig. 9D). However, we cannot rule out the possibility that some glial precursor cells arise from dividing cells which had at some point generated neurons. Even at E80, we are certain only that many of the unlabeled cells in the ventricular zone represent a neuronal precursor line which eventually forms layer III of the visual cortex (Rakic, 1974). Theoretically, a fraction of the unlabeled cells could be either indifferent cells yielding both neuronal and glial precursors or cells which have ceased producing neurons and subsequently changed over to glial production without having yet expressed GFA. These two possibilities seem to be unlikely; to test them, one would need glial markers that are present in the proliferative cell population at ages before the first neurons are generated.

The present study demonstrates that the precursor cell population of the ventricular zone contains at least two cell types. In the future, the use of immunocytochemistry may provide a means for subdividing the germinal population into even more than two classes and for a precise spatial separation of the proliferative zone into areas that produce neurons destined for specific cytoarchitectonic fields. The possibility of detecting cell classes by immunocytochemical techniques will also provide new criteria for defining the onset of cell differentiation that cannot be attained by conventional morphological changes alone.

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