

## Autoradiographic Investigation of Cell Proliferation in the Brains of Rats and Cats

JOSEPH ALTMAN<sup>1</sup>

*Psychophysiological Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts*

**ABSTRACT** Cell proliferation in the brains of rats and cats was investigated autoradiographically. Two young adult rats were injected intraperitoneally with 2 mc of thymidine- $H^3$  and killed after a two weeks' exchange period. Two adult cats were injected intraventricularly with 0.5 mc of thymidine- $H^3$  and killed one week later. Labeling of cell nuclei in the brain, presumed to reflect DNA turnover and cellular proliferation, was investigated. In the rats, some neuroglia cells were found labeled in all parts of the brain, suggesting a low rate of glial proliferation. In addition, circumscribed small regions with numerous labeled neuroglia and microglia cells were seen in several brain regions, suggesting the occurrence of local glial proliferative reactions in these presumably normal brains. A few apparently labeled neurons were seen in the neocortex, and a proliferative region of granule cells was identified in the dentate gyrus of the hippocampus. In the cats labeling of glia cells was highest in the midline region, near the point of injection of the radiochemical, with a gradient of decreasing number of labeled cells both laterally and in the anteroposterior direction of the neuraxis. Neurons with apparently labeled nuclei were observed in the midline cortex bilaterally in both animals. These results indicate that glia cells can multiply in the brains of young adult rats and adult cats and they support the possibility that new neurons may be formed in forebrain structures, both in rodents and carnivores.

In a previous autoradiographic study (Altman, '62a), evidence was obtained of thymidine- $H^3$  uptake by glia cells in the visual pathway of young adult rats in which intracranial administration of this specific precursor of chromosomal DNA was combined with electrolytic destruction of the lateral geniculate body. In addition to the numerous labeled glia cells in the visual pathways, which presumably underwent degenerative and regenerative proliferation, we also observed some labeled glia cells and a few neurons (Altman, '62b) in brain regions which were not directly affected by the lesions and are not known to have direct structural connections with the damaged areas. This latter result suggested that cell multiplication (primarily of glia cells, but possibly also of neurons) may be a "normal" postnatal phenomenon in the brains of mammals.

In this pilot study an attempt was made to throw light on two questions raised by the latter finding. Firstly, will glia cells (and possibly neurons) show uptake of labeled thymidine in young adult rats when their brains are not damaged or other-

wise interfered with experimentally; that is, is cell multiplication in the brain a "normal" phenomenon? Secondly, is cell proliferation in the central nervous system restricted to "lower" mammals, such as rodents, or does it also occur in such "higher" forms as carnivores?

### MATERIALS AND METHODS

**Rats.** Two young adult, Long-Evans hooded rats (four months of age and weighing 250 gm) were injected intraperitoneally with 2 mc of thymidine-methyl- $H^3$  (specific activity 6.59 C/mM, 0.074 mg of the chemical dissolved in 2 ml of sterile water). The labeled thymidine was administered in two 1 mc-doses, with one week interval between the injections. At the end of the two week exchange period, the rats were killed by cardiac perfusion with 10% neutral formalin.

**Cats.** Two adult cats (exact age unknown, weight about 2,500 gm) were used. The animals were anesthetized with Nembutal and, under aseptic conditions, the

<sup>1</sup>This study was supported by the U. S. Atomic Energy Commission. Assistance in various phases of this work was provided by Elizabeth Altman, William J. Anderson, and Gopal Das.

radiochemical was injected stereotaxically into the third ventricle. To accomplish this, a 27-gauge needle was lowered into the third ventricle and firmly fixed there, and with a 30-gauge needle 0.2–0.3 ml of cerebrospinal fluid was withdrawn through the larger needle stock. The withdrawn fluid was replaced gradually (over a period of 45 minutes) with 0.2 ml of isotonic saline containing 500  $\mu\text{C}$  of thymidine-methyl- $\text{H}^3$  (specific activity 6.59 C/mM, amount of chemical 0.018 mg). In both animals a small amount of fluid was seen extruding around the needle during injection and after withdrawal of the needle. The cats were killed after one week survival by cardiac perfusion with 10% neutral formalin.

All the brains were further fixed for one week in neutral formalin, embedded in paraffin, and then coronal sections were cut at 7  $\mu$ . Following deparaffinization, the unstained sections, and some that were prestained with gallocyanin chromalum, were coated with melted Kodak NTB-3 nuclear emulsion. After exposure in sealed lightproof boxes for four months at 5°C, the slides were developed in D-19 at 20°C for six minutes and fixed in the usual manner. The unstained, emulsion-coated sections were stained with cresyl violet after developing.

## RESULTS

### *Cell proliferation in the brains of rats*

*Proliferation of glia cells and ependymal cells.* Some labeled neuroglia cells were found in all parts of the brain (figs. 1–3). These included interfascicular glia cells, seen in such fibrous structures as the white matter of the medulla, optic tract, fimbrium of hippocampus, fornix, anterior commissure, cortical radiation, and corpus callosum. Interfascicular and perivascular neuroglia cells in gray matter were observed in the medulla, cerebellum, midbrain, hypothalamus, thalamus, hippocampus, septum, amygdala, caudate nucleus, and neocortex. Labeled perineuronal glia cells were identified with certainty in some structures with large neurons, such as the motor nuclei of the medulla and midbrain, in the rhinencephalon, and neocortex. Many of these la-

beled glia cells were found singly or as pairs, surrounded by numerous unlabeled cells. As these intensely or mildly labeled neuroglia cells were seen in all parts of the brain, and were present in areas with no signs of brain trauma, they appeared to represent a normal process of glial proliferation.

In addition to the few scattered glia cells with thymidine uptake in all parts of the brain, circumscribed foci with large number of glia cells were observed in several regions (fig. 4). Such foci were seen in the two presumably normal brains in the medulla, thalamus, subthalamus, caudate nucleus, putamen, pyriform area, amygdala, and in the neocortex. Some of these foci could be identified without the labeling by obvious increases in the number of glia cells, and also by the presence of histiocytes (fig. 7). In other areas that contained many labeled neuroglia cells, the small increase in the total number of cells would not have permitted identification of the reaction without autoradiography. Blood vessels surrounded by many labeled cells were generally observed adjacent to the focus of glial reactions.

In the anterior horn of the *fourth* ventricle, on both sides and in both animals, numerous mildly and a few intensely labeled ependymal cells were observed. These labeled cells were seen in the lateral and medial walls of the ventricles, but were particularly numerous in the roof of the anterior horn (fig. 5) from which a narrow band of labeled cells could be traced dorsolaterally (fig. 6). The number of cells in the dorsal roof of the lateral ventricle is considerable and as many as one-third of these were mildly labeled. As the band passes from the ventricle in dorsolateral direction and beneath the corpus callosum, the number of cells gradually decreases, though it remains recognizable as a distinct band of darkly stained cell nuclei. A good proportion of these cells were mildly labeled. (This aggregate of cells is in close proximity to, and may be part of, the subcallosal fasciculus.)

In the available sections no labeled ependymal cells were seen in the walls of the third ventricle and only an occasional labeled cell in the fourth ventricle. Whether the labeled cells of the

lateral ventricles are related to the cells of the chorioid plexus could not be established.

*Apparent uptake of thymidine by neurons.* In addition to the occasional labeled glia cells, a few neurons were found with overlying reduced silver grains in the neocortex in both animals (figs. 8-12). The labeling of some of these neurons could be attributed to the labeling of perineuronal satellites in intimate contact with underlying neurons. In others, perineuronal glia cells could not be seen beneath the labeled nucleus and in these (e.g., fig. 9) the labeling could be attributed to uptake of thymidine by nuclei of neurons themselves. In some sections as many as four or five such neurons were recognized in the cortex, in others a few or none were seen. Significantly, no such apparently labeled neurons were seen in the medulla, brain stem, diencephalon, or basal ganglia. (In some regions of the cerebellum labeled cells were seen in the granular layer, but it could not be determined whether these were labeled glia or granule cells.)

In all autoradiographic sections of the dentate gyrus of the hippocampus several mildly labeled neurons, up to a dozen in some sections, were seen bilaterally in both animals (figs. 13-17). In all instances these labeled cells could be identified as granule cells (spherical or oval cells with minimal cytoplasm); labeled pyramidal cells were not seen. In general, the labeled granule cells were observed in the superficial part of the granular layer adjacent to the molecular or plexiform layer, though an occasional labeled granule cell was also seen in the deeper parts of the granular layer. The labeled hippocampal neurons were particularly abundant inside the corners of the dentate gyrus. None of these labeled cells, or other cells of the dentate gyrus, showed signs of mitotic activity. A few labeled cells resembling neuroglia were also seen in the molecular layer in these regions, but there was no obvious sign of pathological gliosis.

#### *Cell proliferation in the brains of cats*

*Proliferation of glia cells and ependymal cells.* The majority of labeled glia cells

was concentrated in both cats in midline brain structures bilaterally near the entrance of the needle used for injection of the radiochemical. In one animal (RCT 151), the largest concentration of labeled glia cells was seen in the midline region of thalamus (habenular, mediodorsal, parafascicular and centromedian nuclei) and hypothalamus, with relatively fewer labeled glia cells in the medial parts of the lateral gyrus of the cerebral hemispheres. In the other animal (RCT 152), the largest concentration of labeled glia cells was seen more dorsally in the medial cortex, with fewer labeled glia cells in the medial nuclei of thalamus and hypothalamus. In both animals labeled glia cells were seen in fornix and corpus callosum (fig. 18), with highest concentration near the midline and decreasing number of labeled cells laterally. The number of labeled cells was approximately equal in homologous structures bilaterally. However, in cat RCT 152, in which the largest concentration of labeled cells was seen in the medial cortical areas, the labeled cells were less numerous on the side of entrance of the needle and near the needle track. In this region histiocytes were present, many of which were labeled. On the opposite ("normal") side a large number of oligodendrocytes were labeled and the labeling of perineuronal satellite cells was quite common. The number of labeled cells decreased abruptly laterally and in the most lateral parts of the neocortex labeled cells were not seen in either animal. A gradient of cell labeling, furthermore, was observed also in the anteroposterior direction, the number of labeled cells falling off anteriorly and posteriorly from the region of the needle track. A few labeled glia cells were seen as far caudally as the white matter and granular layer of the cerebellum, and near the fourth ventricle in the medulla. No labeled cells (except one in one animal) were encountered in the spinal cord.

These findings suggested a dependence of cell labeling on sluggish diffusion of the intraventricularly administered radiochemical from the region of injection both by ventricular and subarachnoid routes. The evidence also indicates a dependence of cell labeling on differential

rates of regional reactivity, for only few labeled cells were present in some midline regions and large clusters in others. For instance, in the ependymal wall of the third ventricle labeled cells were very scarce in one animal and altogether absent in the other. Conversely, several structures far removed from the midline contained relatively large number of labeled glia cells. Thus, many labeled glia cells were seen bilaterally in the caudate nucleus in one animal, and far ventrolaterally in the amygdala in the other.

*Apparent uptake of thymidine by neurons.* In both animals several neurons were found in the neocortex with overlying silver grains (figs. 20-36). In many instances the origin of the reduced grains could be attributed to perineuronal satellites that were in intimate contact with the neurons. In other cases no such satellites could be detected, though their possible presence could not be ruled out. The inherent ambiguity regarding the origin of the reduced grains in these neurons is illustrated in figure 32. It shows how an imaginary horizontal section of 7  $\mu$  thickness (parallel lines) through the lower neuron with a satellite might have produced an image not dissimilar from that of the upper, apparently labeled neuron. Among the apparently labeled neurons several were observed with pairs of darkly staining nuclei, one or both of which were labeled (figs. 33, 34, 36). No apparently labeled neurons were seen in the thalamus or other brain structures.

#### DISCUSSION

Thymidine is a specific precursor of DNA, and it is generally accepted (Hughes, '59) that DNA is metabolically inert, excepting during chromosomal replication prior to cell division. The autoradiographic demonstration of uptake of labeled thymidine by cell nuclei is therefore considered an indication that the labeled cells were either preparing for mitosis when the thymidine became available or that they are products of cells so labeled (Hughes, et al., '59; Leblond, et al., '59; Taylor, et al., '57). Using this technique, several investigators (Messier and Leblond, '60; Schultze and Oehlert, '60) observed the labeling of occasional glia cells

in the central nervous system. It was later reported (Altman, '62a; Koenig, et al., '62) that, as a consequence of brain trauma, glia cells become labeled in large numbers at sites of experimentally provoked gliosis. This latter finding added support to the interpretation that the labeling reflects cell proliferation.

The aim of this pilot study was to investigate whether thymidine uptake will occur in the brains of normal young adult rats and adult cats in the absence of experimental brain damage. In the first part of the experiment two apparently normal rats from a healthy colony were used and, in order to avoid possible chemical damage to their brains, the radiochemical was injected intraperitoneally rather than intracranially. If the brains studied represent normal material, as was intended, the evidence of labeling occasional glia cells over the entire brain would indicate normal cellular turnover. The occurrence of several small foci of gliosis would then suggest that local inflammatory processes, and glial reaction to them, are prevalent in "normal" rat brains. The other possibility is that inadvertently we were investigating pathological material, and that some or all of the demonstrated cellular multiplication is attributable to brain trauma of unknown origins.

Further investigation is required to determine the nature and significance of the high rate of ependymal cell multiplication observed in the roof of the lateral ventricles, on both sides and in both animals. The proliferating ependymal cells, it would seem, migrate beneath the corpus callosum and move around the wall of the caudate nucleus. The destination of these proliferating cells and their possible further differentiation have not been established.

The method of intraventricular injection, employed with the cats for reasons of economy, was found to be ill-suited for the study of global uptake of thymidine by cells of the brain. Thymidine uptake was largely, though not exclusively, restricted to midline structures bilaterally. These included areas bordering the third ventricle (midline thalamic nuclei, hypothalamus) as well as others (medial cortex) far removed from the ventricular

system, which were presumably reached by subarachnoid diffusion from the region of the needle track. The high rate of glia cell labeling in the medial cortex on the side opposite the needle track, where no signs of brain trauma could be detected, may reflect the normal turnover rate of glia cells. The significance of the considerable perineuronal satellite proliferation (somewhat suppressed on the damaged side) remains to be elucidated. However, the possibility of a mirror focus effect (Morrell, '61) from the contralateral region of damage cannot be excluded.

The apparent labeling of neurons in the brains of rat and cat is of great interest, since it is generally assumed that in mammals, and other higher vertebrates, neurons do not multiply postnatally. This finding raises many questions which cannot be resolved at present and call for further experiments. As was pointed out, the very fact of uptake of thymidine by neurons is dubious in many instances. Thus, the labeling of a large number of neuron nuclei in the cortex of cats may have been due in many (if not most) instances to uptake by perineuronal satellites situated over the nuclei of neurons. This ambiguity could be resolved only by preparing sections of 1-2  $\mu$  thickness for autoradiography. On the other hand, several neurons were found in the rat cortex with light labeling, in which the possibility of perineuronal satellites being the source of the label could be ruled out with some confidence. Similarly, the frequent labeling of granule cells in the hippocampus is of great interest, as this region is devoid of perineuronal satellites.

The strongest argument against the assumption of postnatal neuronal multiplication in higher vertebrates is the absence of mitotic figures in neurons. But is it not possible that primitive precursor cells (such as ependymal cells) may multiply and become differentiated into neurons? Such a hypothetical process of neurogene-

sis cannot be investigated except with a labeling technique, such as employed in this investigation. On the other hand, even successful demonstration of true labeling of neuron nuclei leaves some doubt whether the uptake of thymidine might not reflect some process of DNA turnover that does not lead to cell multiplication. Experiments are in progress in our laboratory designed to deal with some of the questions raised.

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PLATE 1

EXPLANATION OF FIGURES

Labeled glia cells in the rat brain. (In these and all the following photomicrographs focusing is on the reduced emulsion grains; underlying stained sections are slightly out of focus.)

- 1-2 Intensely labeled glia cells in fimbrium of hippocampus. Prestained with gallocyanin chromalum,  $\times 900$ .
- 3 Lightly labeled glia cells in cortical radiation. Gallocyanin chromalum,  $\times 900$ .
- 4 Focus of gliosis in amygdala. Gallocyanin chromalum,  $\times 384$ .

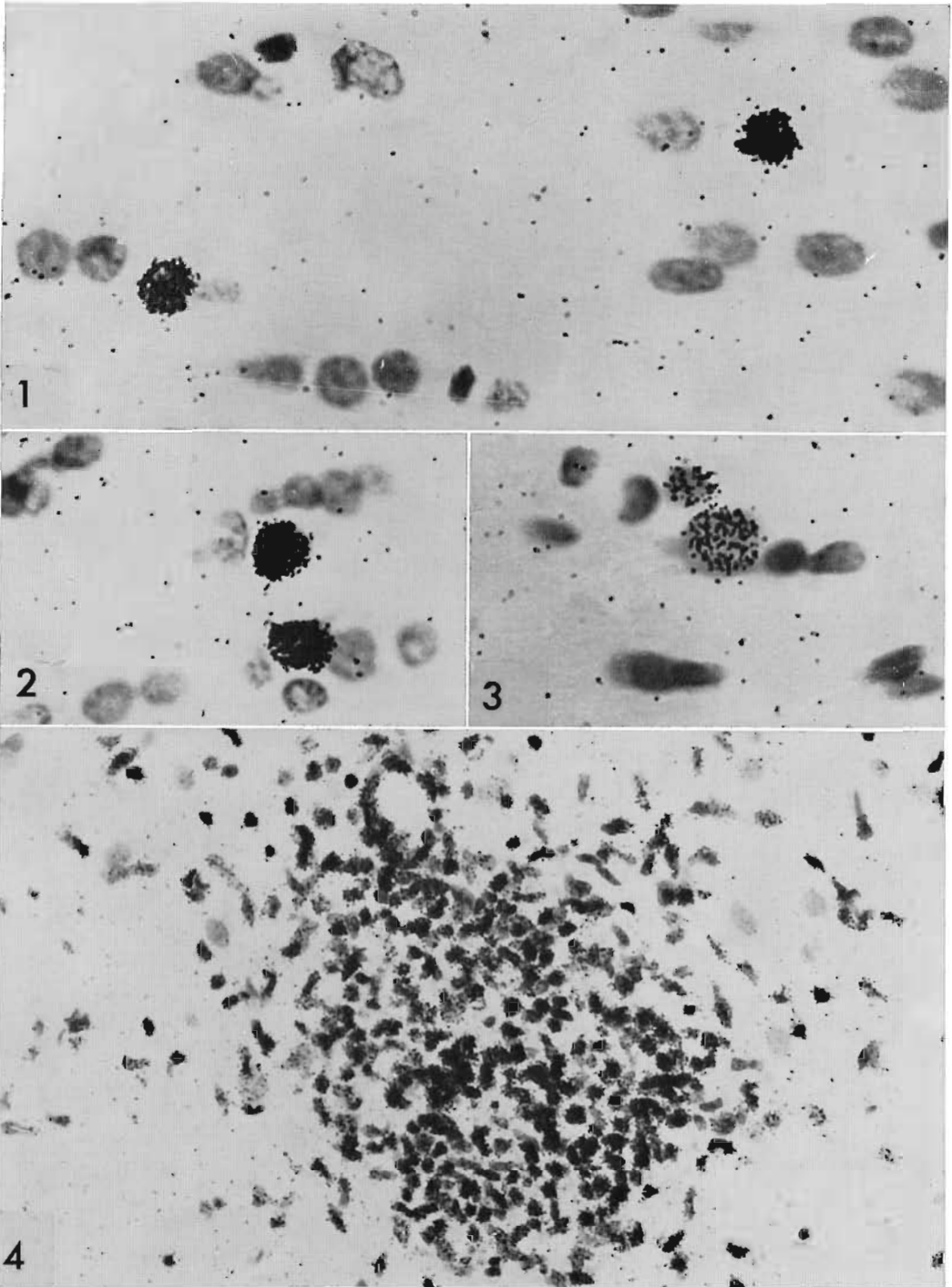


PLATE 2

EXPLANATION OF FIGURES

Labeled glia cells and ependymal cells in the rat brain.

- 5 Lightly labeled subependymal (and glial?) cells in the roof of the anterior horn of the lateral ventricle. Galloxyanin chromalum,  $\times 490$ .
- 6 Subcallosal band of cells over caudate nucleus, apparently migrating from the roof of the lateral ventricle. Galloxyanin chromalum,  $\times 960$ .
- 7 Focus of relatively mild gliosis in medulla with histiocytes. Cresyl violet (stained after developing),  $\times 960$ .



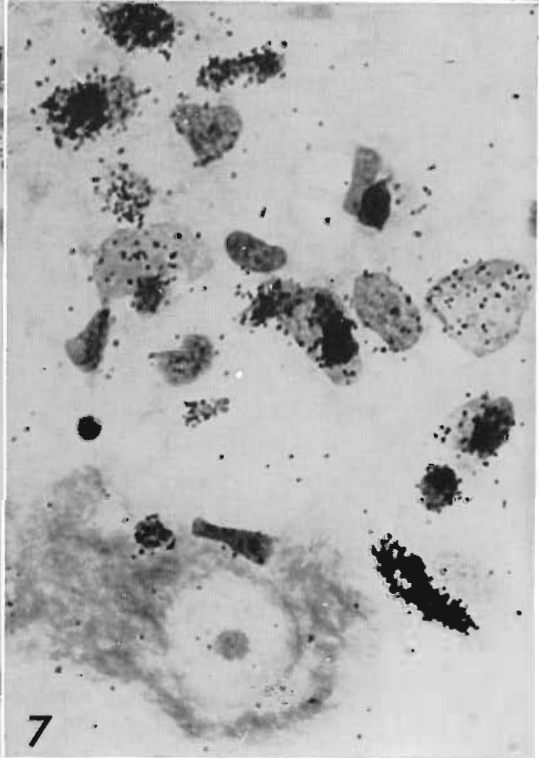
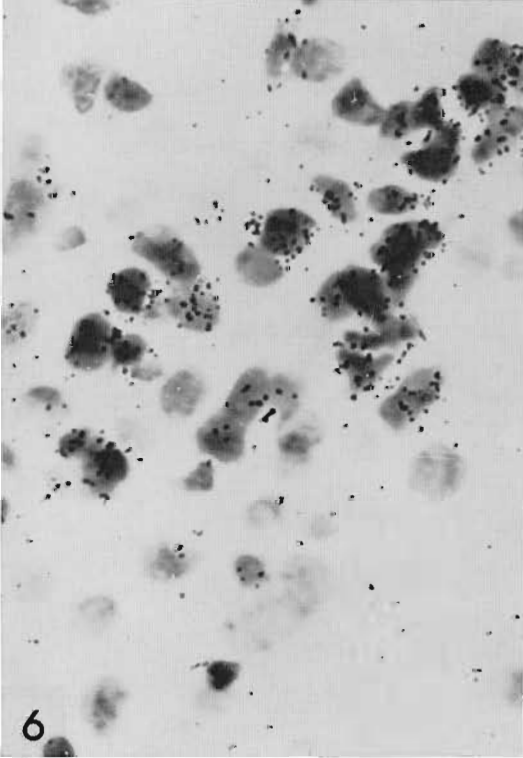
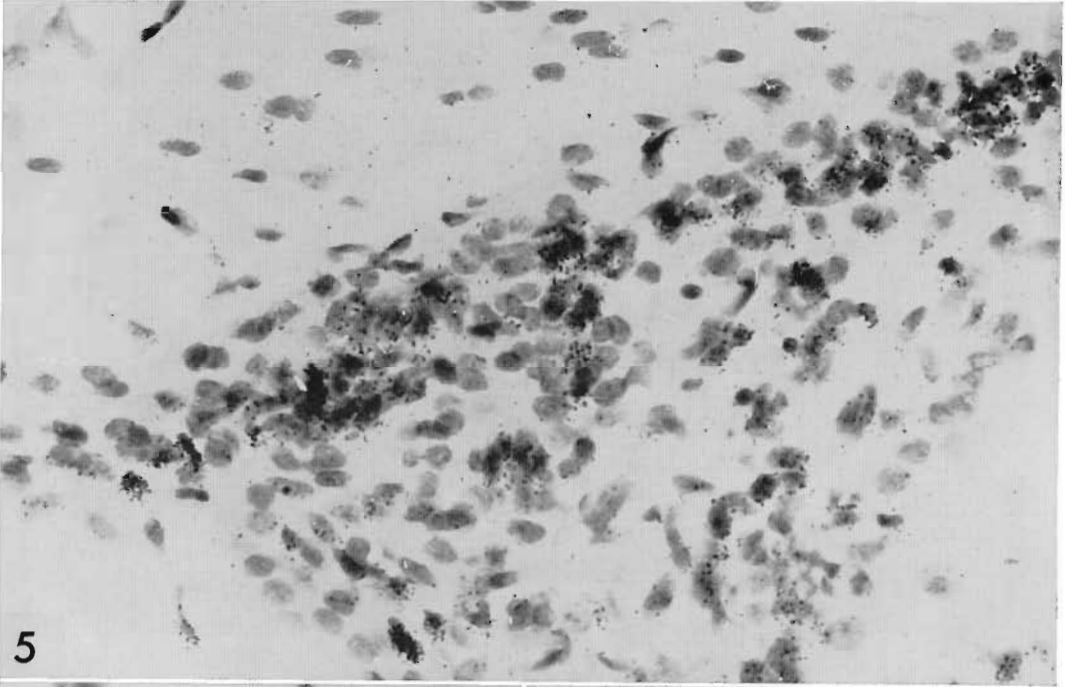


PLATE 3  
EXPLANATION OF FIGURES

Apparently labeled neurons and neuron-like cells in cortex of rat.

- 8 Intensely labeled pyramidal cell. Cresyl violet,  $\times$  1,890.
- 9-10 Lightly labeled neurons. Gallocyanin chromalum,  $\times$  1,536.
- 11 Binucleate neuron-like cell with both nuclei labeled. Gallocyanin chromalum,  $\times$  1,536.
- 12 Binucleate neuron-like cell with one nucleus labeled. Gallocyanin chromalum, oil immersion,  $\times$  3,072.

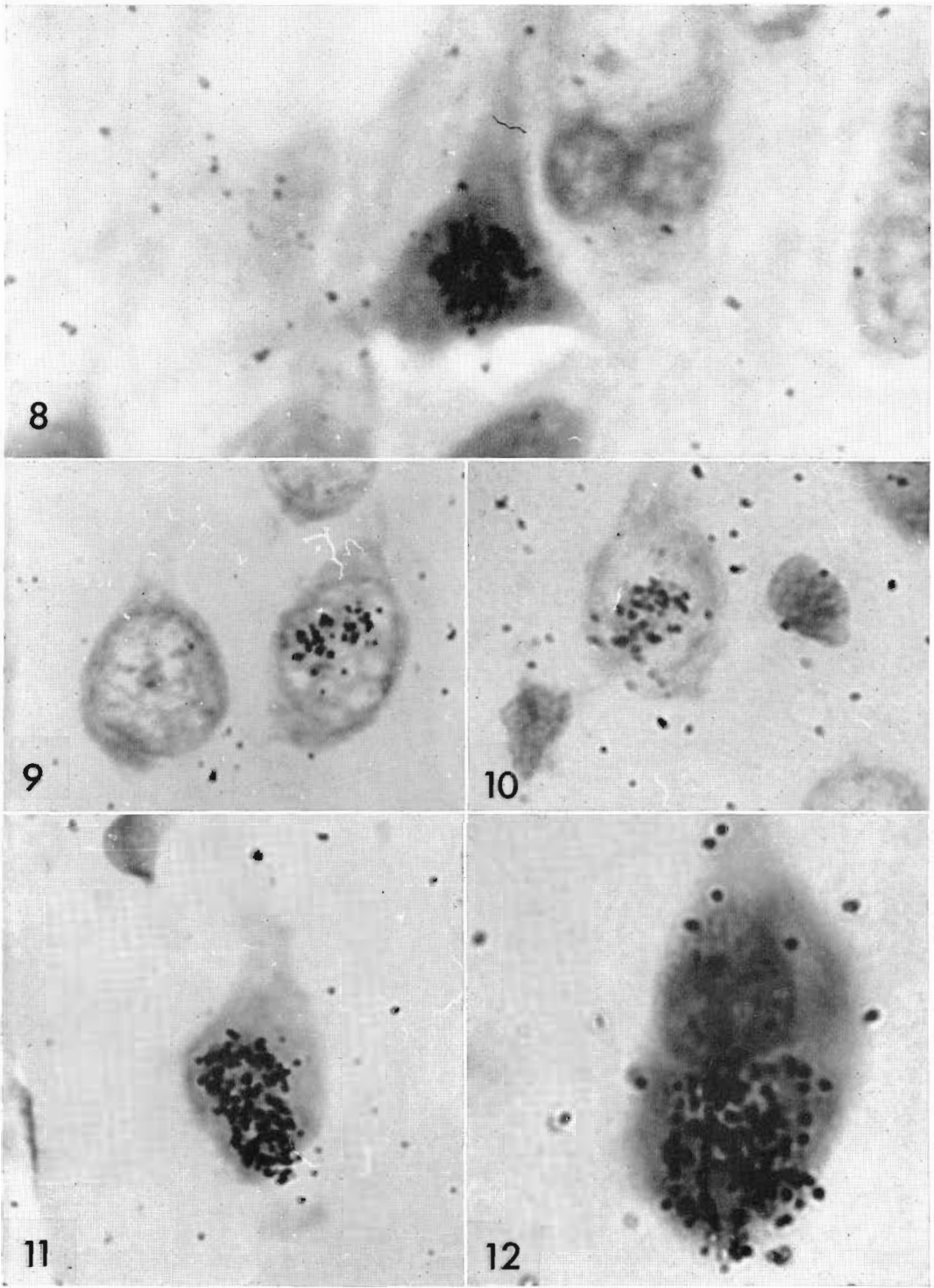


PLATE 4

EXPLANATION OF FIGURES

13-17 Labeled granule cells in dentate gyrus in hippocampus of rat.  
Gallocyanin chromalum,  $\times 1,536$ .

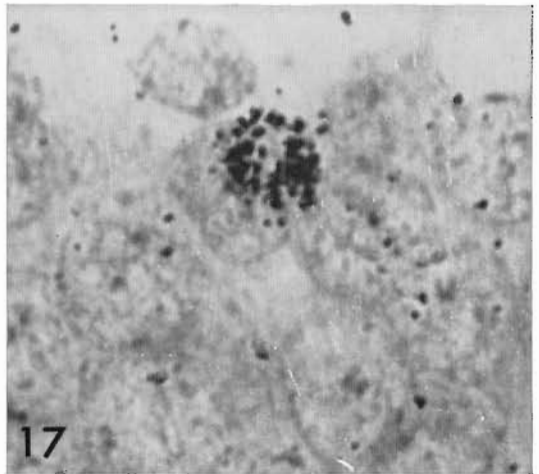
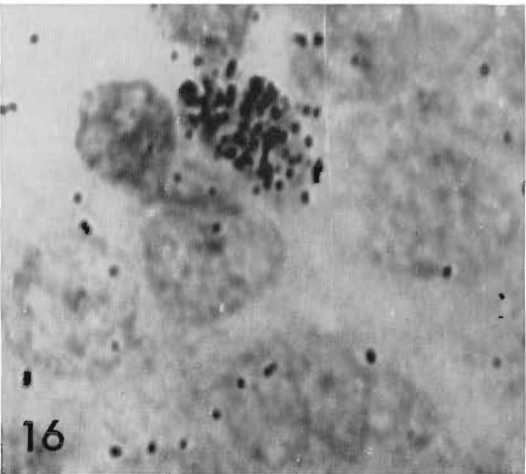
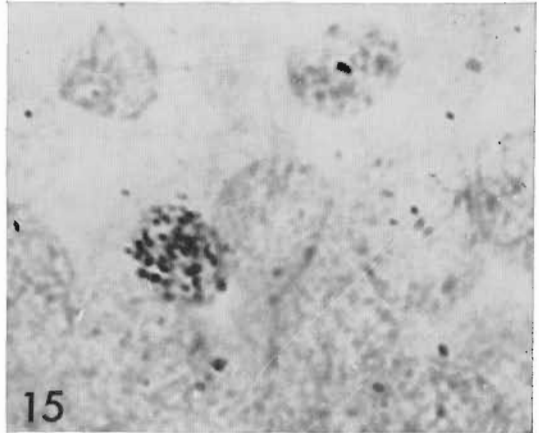
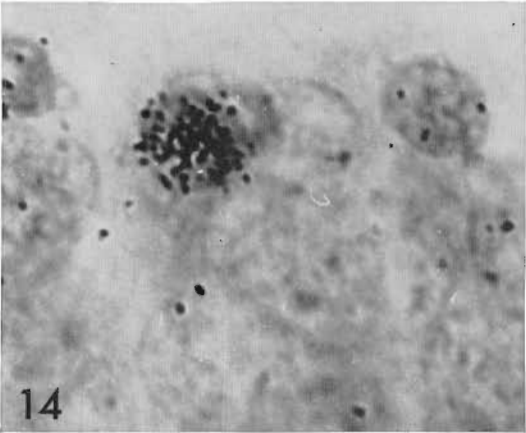
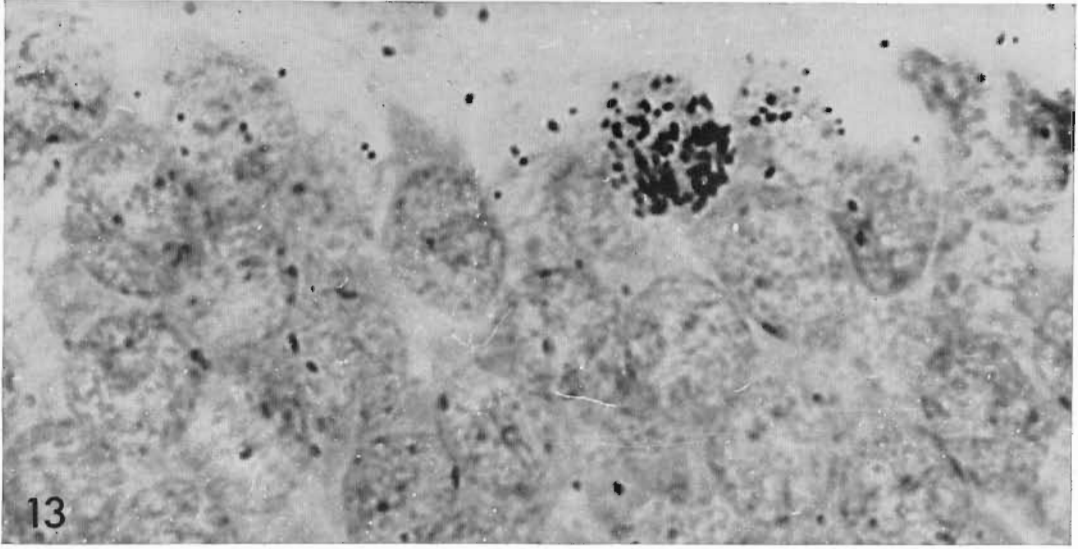


PLATE 5

EXPLANATION OF FIGURES

Labeled glia cells in the brain of cat.

- 18 Labeled interfascicular glia cells in corpus callosum. Cresyl violet,  $\times 492$ .
- 19 Labeled perineuronal glia cells in dorsomedial nucleus of thalamus. Cresyl violet,  $\times 1,229$ .

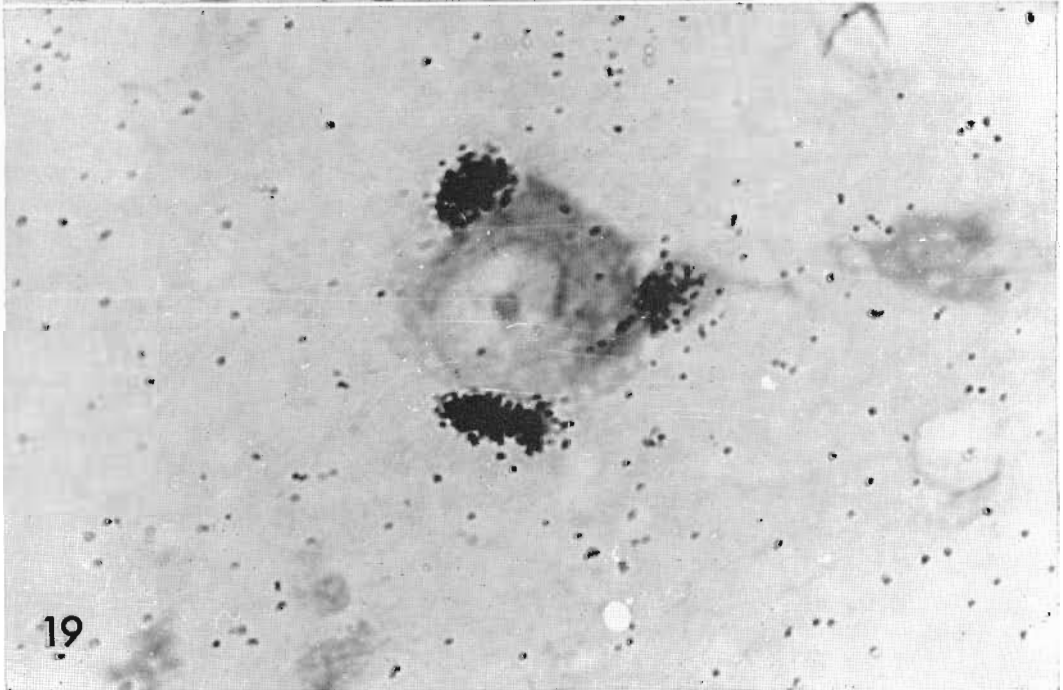
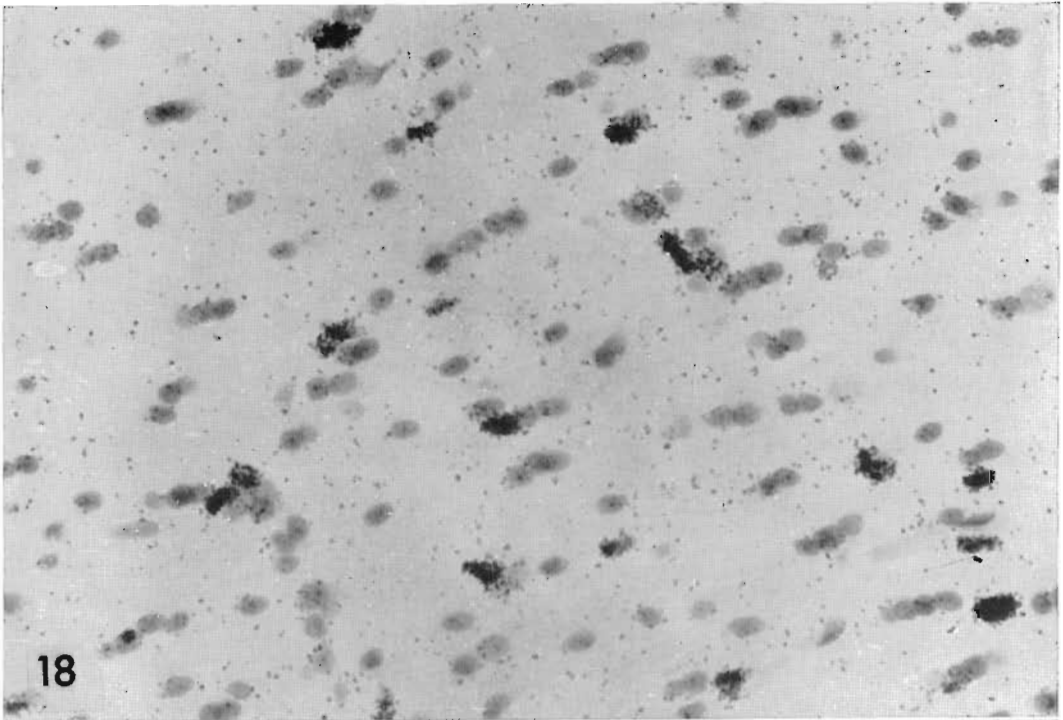


PLATE 6

EXPLANATION OF FIGURES

20-31 Examples of apparently labeled neurons in the medial surface of the lateral gyrus in cat. Cresyl violet,  $\times 1,229$ .



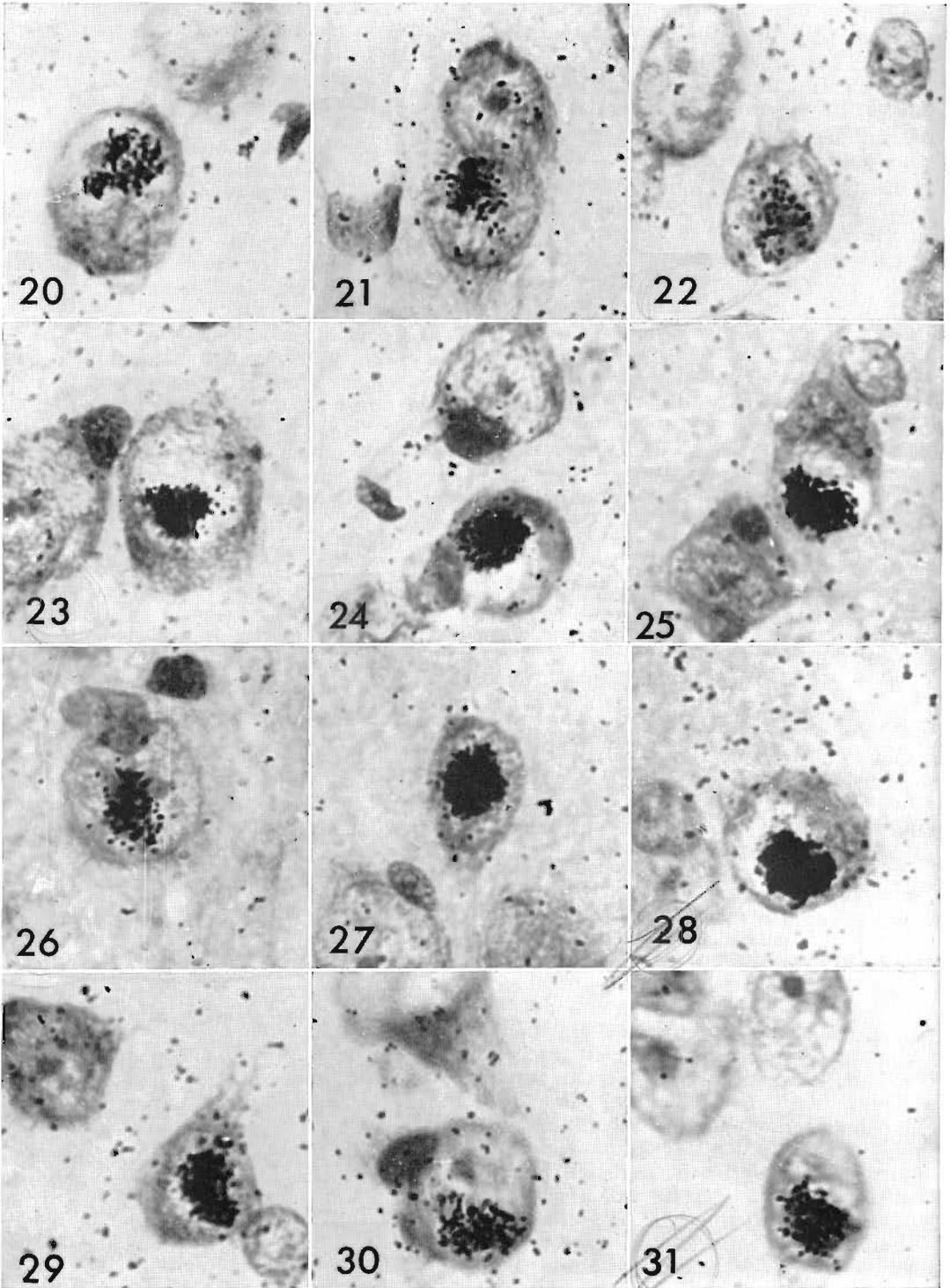


PLATE 7

EXPLANATION OF FIGURES

Apparently labeled neurons in cortex of cat.

- 32 An apparently labeled neuron (upper middle cell) together with unlabeled ones. Cresyl violet,  $\times 1,229$ . The two parallel lines, representing the nominal width of the section in a horizontal cut, show how a perineuronal glia cell (if labeled) could produce an image similar to that provided by the upper, apparently labeled neuron.
- 33 Binucleate labeled neuron. Cresyl violet, oil immersion,  $\times 2400$ .
- 34, 36 Neurons in cortex, in each picture one with two labeled nuclei. Cresyl violet,  $\times 1,229$ .
- 35 Apparently labeled neuron with an intensely labeled unidentified (satellite) cell. Cresyl violet,  $\times 1,229$ .

