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Retrograde transport of γ-amino[^{3}H]butyric acid reveals specific interlaminar connections in the striate cortex of monkey

(cortical inhibition/GABA uptake/uptake inhibition/Golgi impregnation/nonpyramidal neuron)

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ABSTRACT **Several lines of evidence suggest that γ-aminobutyric acid is an inhibitory neurotransmitter in the cerebral cortex. To study the intracortical projection of neurons that selectively accumulate this amino acid, we injected radioactive γ-aminobutyric acid into the upper layers of the striate cortex of monkeys along tracks at an oblique angle to the pia. Sections from the injected area were then processed by a combination of autoradiography and Golgi impregnation to reveal the distribution of labeled neurons and their morphological characteristics. Labeled neurons always occurred around the injection site in each layer. In addition, a consistent radial pattern of perikaryal labeling was observed in layers IVc–VI below the injection track in layers I–IVa. The closer the injection track was to the pia the deeper the peak density of labeled cells appeared. After injection in layers IVa and the lower part of III, the highest number of labeled neurons was in layer IVc; after injection in the upper part of layer III, most labeled neurons were in layer V; and, after injection in layers I and II, the proportion of labeled neurons increased in the lower part of layer V and in layer VI. All these neurons in the infragranular layers are presumably labeled by retrograde axonal transport via the labeled fiber bundles that extended from upper to lower layers. Thirty-four Golgi-stained neurons of various types were also examined for retrograde labeling. Two were labeled, and both were aspiny stellate cells in layer V. The arrangement of these putative GABAergic neurones, with axons that ascend from lower to upper layers in a regular pattern and arborize locally, would enable them to mediate inhibition within cortical columns and between neighboring columns.**

There is substantial evidence that γ-aminobutyric acid (GABA) is a neurotransmitter in the cerebral cortex (1–5). In particular, it has been shown that GABA-mediated inhibition enhances the specialized receptive field properties of many neurons in the visual cortex (6–12). Two methods have been used to study the distribution and structure of neurons responsible for GABAergic inhibition in the cortex; (*i*) autoradiographic demonstration of selective high-affinity uptake of [^{3}H]GABA and (*ii*) immunohistochemical localization of the GABA-synthesizing enzyme glutamic acid decarboxylase (GluDCase) (13–20). Both methods reveal neuronal perikarya and nerve terminals but not their linking axons; i.e., we do not know which cell bodies in any lamina give rise to nerve terminals mediating GABAergic inhibition in that or any other lamina. Immunohistochemical demonstration of GluDCase reveals a regularly patchy nonuniform horizontal distribution in the monkey cortex (17), but it is also important to understand the connections of GABAergic neurons across and between layers because neurons whose receptive field properties are influenced by GABA-mediated interactions are organized in columns orthogonal to the layers and such that neurons in a column have similar properties (21). Recently, by exploiting the selective uptake and retrograde transport of [^{3}H]GABA (22, 23) after its injection into the deep layers of the monkey striate cortex, we found a population of neurons in cortical layer II and the upper part of layer III that sends projections vertically through all layers of the cortex (18). Here, we report the laminar distribution of GABA-accumulating neurons after injection of [^{3}H]GABA into the upper layers and provide evidence for the nonpyramidal nature of some of these neurons.

MATERIALS AND METHODS

Treatment of Animals. Two adult male monkeys (one *Macaca mulatta*, one *Macaca fascicularis*) were used. One of them (A) had been used in behavioral tests of memory and its fornix had been sectioned surgically 6 months before the present experiment. The other (B) had a high titer of antibody to *Herpes* simiae and could be used only in acute experiments. There was no reason to assume that their visual cortices were abnormal. They were sedated with ketamine hydrochloride (intramuscularly at 10 mg/kg; Ketalar, Parke, Davis) and anesthetized with sodium pentabarbitone (intravenously, Sagatal; May and Baker, Dagenham, England). Then, the occipital lobe was exposed, and the striate cortex of both hemispheres was injected at several sites with [^{3}H]GABA (0.33 mM; 60 Ci/mmol; 1 Ci = 37 GBq; Radiochemical Centre) in Krebs bicarbonate solution. Injections were delivered using glass micropipettes (tip diameter, 30–50 μm) penetrating at various oblique angles to the surface. Three injections parallel with the lunate sulcus were analyzed in the present study. They involved cortex corresponding to an eccentricity of 2–4° from the fovea. The pipette was advanced 7 mm from the pia and either 0.1 μl (2 μCi; injection 1, animal A) or 0.05 μl (1 μCi; injections 2 and 3, animal B) of [^{3}H]GABA was injected at each of 6 (no. 1) or 10 (nos. 2 and 3) sites, 1 or 0.5 mm apart, respectively, as the capillary was gradually withdrawn.

One injection track (no. 2) ended in layer IVc, and the other two passed obliquely from the pia to the bottom of layer III. Two further injections, one in each animal, were made using the same GABA solution together with the competitive GABA uptake inhibitor *cis*-1,3-aminocyclohexanecarboxylic acid (100 mM; ACHC). After various survival times (injection 1, 35 min; injection 2, 50 min; injection 3, 30 min), the animals were perfused with fixative (18).

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Abbreviations: GABA, γ-aminobutyric acid; GluDCase, glutamic acid decarboxylase; ACHC, *cis*-1,3-aminocyclohexanecarboxylic acid.

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Histology. Slices of injected cortex were processed for Golgi impregnation, gold toning, and autoradiography as described (18, 19). Plastic-embedded 1-μm-thick sections were dipped in Ilford K5 emulsion, exposed for 15–87 days, developed with Kodak D19B, fixed, and stained. From Golgi sections, selected gold-toned neurons were drawn and photographed, and 1-μm-thick sections of the perikarya were tested for labeling using autoradiography. To study the pattern of labeled perikarya, distribution diagrams were constructed as shown in Fig. 1.

RESULTS

Labeled neurons occurred around the injection track in each layer from I to IVc when the layer was injected (Figs. 1 and 2). They could be seen using bright-field illumination. The proportion of neurons that were labeled was the same irrespective of the amount of radioactivity around the track but, in sections having higher radioactivity, the area containing labeled cells was larger. The inclusion of ACHC in the [^{3}H]GABA solution either completely prevented perikaryal labeling or confined it to the immediate vicinity of the capillary track, where all cells were labeled to the same degree as the neuropil.

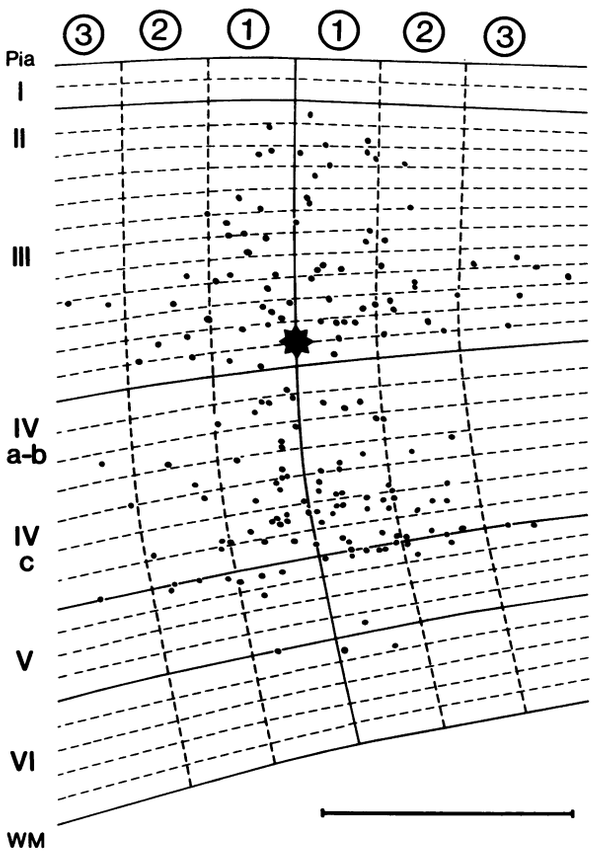

FIG. 1. Tracing of [^{3}H]GABA-accumulating neurons (dots) in a single 1-μm section cut perpendicular to the pia and approximately perpendicular to the injection track (asterisk). This illustrates the procedure used for compiling the data shown in Fig. 2. The amount of cortical shrinkage varied among sections, and the thickness was not consistent throughout a given track. To compare different sections, the divisions were standardized. First, the borders between layers III, IV, V, and VI were drawn from the corresponding Golgi sections using the classification of Lund and Boothe (24), and then the cortex was divided horizontally so that a given layer always contained the same number of bins with the same serial number. As the distribution of cells appeared to be symmetrical around a line (solid, vertical) perpendicular to the pia and passing through the center of the injection site, labeled neurons on the two sides and in the same bin were pooled. One lateral subdivision (vertical broken lines) had a width of 12% of the distance from pia to white matter (WM). All cells further than the second vertical line were plotted in graphs 3 in Fig. 2.

In addition to the neurons around the injection track, a regular pattern of perikaryal labeling was observed radially below and above the injection track and this pattern depended on the laminar position of the injection site (Fig. 2). Groups of neurons accumulating [^{3}H]GABA lay outside the area of the spread of the isotope in layers IVc–VI when the labeled GABA had been injected into layers I–IVa. These neurons were presumably labeled by retrograde transport after the selective uptake of [^{3}H]GABA at their terminals because the closer the injection was to the pia the deeper the peak density of labeled cells in the cortex beneath (Fig. 2). In addition, densely labeled and vertically disposed fiber bundles, probably representing the axons of these neurons, were also present (Fig. 3 *A* and *B*). The border between layers V and IVc was sharply delineated by neuropil labeling in addition to that of cell bodies (Fig. 3*A*). The lateral extent of the area containing labeled neurons in each layer also showed a regular pattern. The most widespread labeling was in the top of layer V, with neurons present up to 350 μm laterally from a point below the injection track. The band of labeled neurons in layers IVc and VI was slightly narrower, about 250 μm laterally in layer IVc (Fig. 3*A*). In layers IVb and VI, labeled neurons were present only directly below the injection track. When layer IVc was injected, the distinct group of labeled neurons in layer II described earlier (19) appeared directly above the injection track (Fig. 2*A*). When layer II was injected with spread to layer I, a very wide labeling pattern was found in these layers (Fig. 2*F*) but, as this injection site was very near the point where the capillary penetrated the pia, the widespread labeling may reflect spread of [^{3}H]GABA on the pial surface. Apart from this latter case, the lateral distribution of labeled perikarya probably reflects the tangential spread of the axons of labeled neurons in the upper layers where the injection was delivered.

Autoradiography itself gives little information about the type of neuron being labeled. We therefore processed for autoradiography Golgi-stained gold-toned neurons of identified types. A total of 3 spiny stellate cells in layer IVc, 14 nonpyramidal cells with smooth dendrites in layers IVc–VI, and 17 pyramidal cells of various types in layers V and VI, all from the area where perikaryal labeling occurred below the track, were impregnated and examined for uptake of [^{3}H]GABA. The two cells that were labeled were aspiny stellate neurons, both in the upper part of layer V. As the labeling of the neuropil is strong in layer V (Fig. 3*A*), labeled perikarya were identified by comparison with nearby nonlabeled perikarya, not by comparing them with the neuropil (Fig. 3*C*).

DISCUSSION

The specificity in the pattern of interlaminar connections revealed by [^{3}H]GABA labeling (Fig. 4) raises the question whether the neurons identified this way use GABA as a transmitter and therefore whether these interlaminar connections are probably inhibitory. The answer really depends on the reliability of uptake of exogenous [^{3}H]GABA solely or chiefly by neurons that use it as a transmitter. The results obtained with the competitive GABA uptake inhibitor ACHC show that the perikaryal labeling in our experiments occurs by an active uptake process. ACHC also prevented labeling of the perikarya by [^{3}H]GABA in a study of rat neostriatum (25). Further evidence for the specificity of [^{3}H]GABA uptake comes from separate experiments in the cerebellum (13, 26, 28) and in the olfactory bulb (29, 30) showing that the type of neuron that accumulates [^{3}H]-GABA also contains GluDCase.

In the cortex, GluDCase has been localized only in nonpyramidal cells (14, 17) and only nonpyramidal neurons have

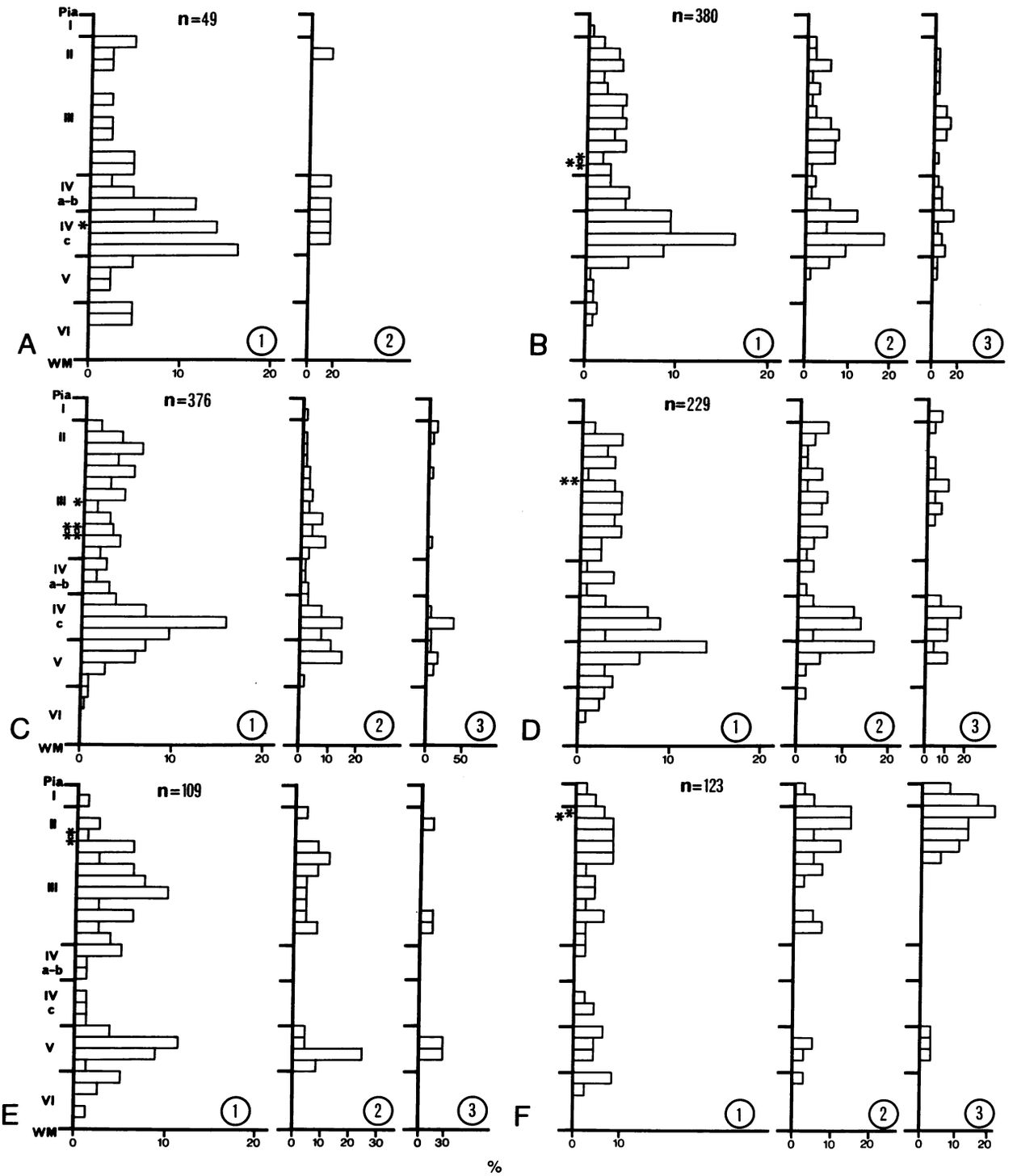

FIG. 2. Distribution of labeled neuronal perikarya after injection of [^{3}H]GABA into layers I–VI of the striate cortex. Data, collected as shown in Fig. 1, from different injections but with the tracks in approximately the same position (asterisk for each section) were pooled. The absolute number of labeled neurons depends on the volume and activity of the [^{3}H]GABA injected at that particular level and this varied along the injection track. The relative distribution of neurons, however, depends only on the position of the capillary track. Therefore, the number of labeled neurons in a bin is expressed as a percentage of the total in each lateral subdivision. Graphs 1, 2, and 3 represent the corresponding lateral subdivisions. To make parts A–F and the columns comparable, the scale of each abscissa was adjusted according to the proportion of the total (*n*) in the lateral subdivisions. (*A*) Injection of layer IVc resulted in labeling around the injection track. Injection of layers IVa and lower III gave particularly prominent retrograde labeling in the middle of layer IVc, where up to a third of the neurons were labeled. (*C* and *D*) As the injection track moved further up into layer III, the proportion of labeled neurons in the top of layer V at the border with layer IVc increased *pari passu* while that in layer IVc decreased. (*E*) Injection at the top of layer III resulted in strong labeling in the middle of layer V, and the appearance of a group consisting of few but large neurons in layer VI. (*F*) More of the large neurons were labeled when the injection was in layer II with some spread into layer I.

been found to accumulate [^{3}H]GABA (16). In the present study, two of the labeled neurons in layer V were nonpyramidal cells with smooth dendrites similar to those reported in rat cortex (19). However, it would be difficult to prove that all our labeled cells in the different layers are of this type.

Evidence for ascending intracortical projections comes from

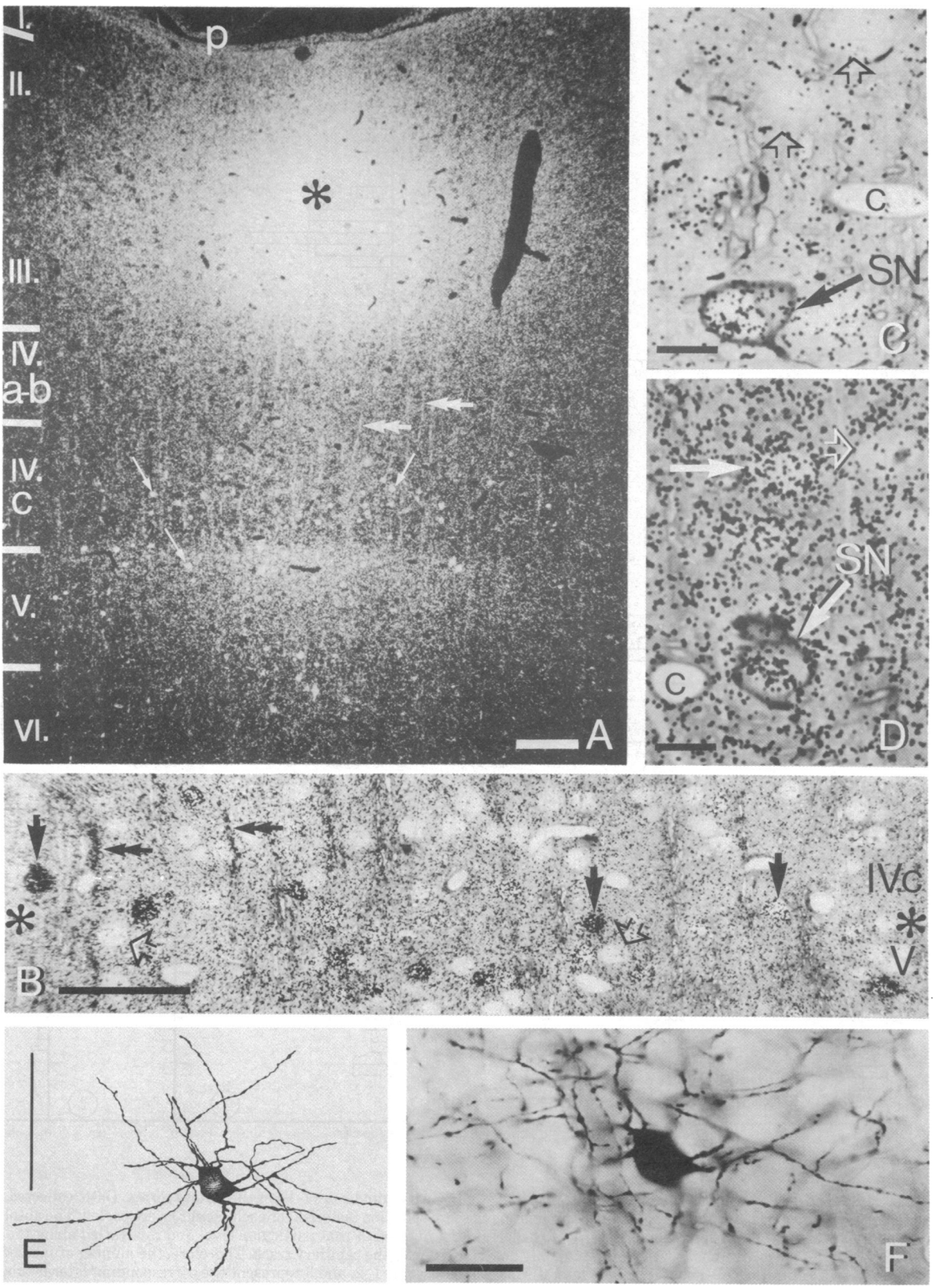

FIG. 3. Laminar distribution of labeled neurons after injection of [^{3}H]GABA into layer III of the striate cortex through a capillary that was nearly parallel with the pia (p). (*A*) Dark-field micrograph showing the injection track (asterisk), numerous labeled neurons (arrows) in layers IVc and V, and labeled vertical fiber bundles (double arrows). In addition to perikarya labeled in the top of layer V, the neuropil is also strongly labeled, delineating this layer sharply from layer IV. (*B*) Bright-field micrograph showing layers IVc and V (asterisk) marked by numerous labeled neurons (arrows). Unlabeled neurons (open arrrows) and labeled vertical fiber bundles (double arrows) are also present. (*C* and *D*) The perikarya of Golgi-impregnated gold-toned stellate neurons (SN) in layer V show accumulation of [^{3}H]GABA; unlabeled neurons (open arrows), capillaries (c), and labeled neurons (arrows) are indicated. The stellate nonpyramidal neuron with smooth dendrites seen in *D* is also shown in the drawing (*E*) and the light micrograph (*F*) taken before processing for autoradiography. (Bars: *A*, 100 μm; *B* and *E*, 50 μm; *C*, 10 μm; *F*, 20 μm).

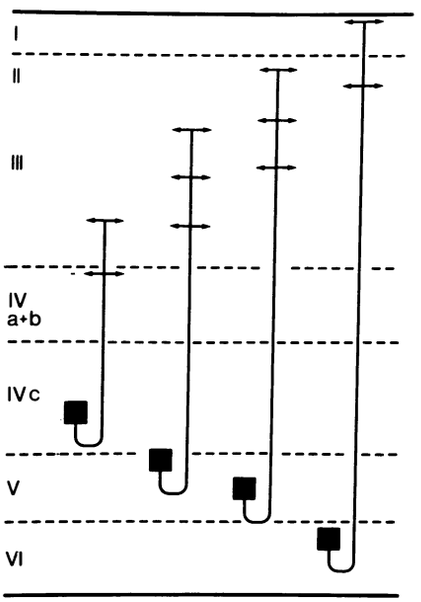

FIG. 4. Summary of the distribution of putative GABAergic neurons (■) projecting from the deep layers (IVc–VI) to the superficial layers (IVa–I). The diagram is based on the predominant contributions of the layers. Small proportions of neurons having more widespread or more restricted projections may also be present.

earlier studies using two different approaches. First, the local origin of a substantial proportion of the fiber plexi in the upper layers, particularly in layer I, was demonstrated in chronically isolated cortical slabs. It was suggested that many of the fibers originated from neurons in the deeper layers (31, 32). Second, previous Golgi studies of monkey striate cortex (24, 33) indicate that spiny stellate cells from layer IVc and small pyramidal cells from layer V project to layer III. Golgi-stained neurons of these types examined so far for [^{3}H]GABA uptake were not labeled, again indicating the selectivity of the method. It is difficult to relate the [^{3}H]GABA-labeled cells to the various types of Golgi-impregnated neurons described elsewhere because their axons are usually revealed only over short distances. Nevertheless, small stellate neurons with extensive beaded axons (34) in layer IVc may be responsible for the retrograde labeling as some send their axons to layer IVa.

The origin of the intense band of neuropil labeling in layer V (Fig. 3A) is not clear. It may result from anterograde transport of [^{3}H]GABA taken up locally by neurons in layers II and III, but could equally well indicate the local axonal or dendritic plexi of retrogradely labeled neurons. Further detailed ultrastructural studies of layer V should help to decide between these possibilities.

Because the labeling occurred vertically only around the injection track, we cannot relate our results to the tangential patches containing high concentrations of GluDCase (17). However, the difference between laminae in the lateral extent of the retrogradely labeled area indicates the spread of the axons of the labeled neurons, if we assume that most of the uptake took place in the core of the injection site. Assessed in this way, GABA-accumulating neurons in layer V have axon arbors 600–700 μm in diameter, and neurons of layer IVc about 400–500 μm in diameter distributed in layer III. This would certainly allow them to provide an inhibitory influence over small distances laterally—e.g., between neighboring physiological columns concerned with orientation tuning or ocularity.

It is not yet clear how the specific responses of visual cortical neurons are partially determined by the putative GABAergic connections shown here. Nevertheless, the precision of the ascending vertical projections suggests that they can contribute to the physiological columnar organization. It is significant that layer IVc, which receives the bulk of the geniculate input, contains a high proportion of putative GABAergic neurons, which project to upper layers where there is less direct geniculate input. Thus it is possible that this projection mediates feed-forward inhibition if activated directly by specific afferents, parallel with the presumed excitatory pathway thought to be mediated by spiny stellate cells.

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