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Glutamic Acid Decarboxylase Immunoreactivity in Layer IV of Barrel Cortex of Rat and Mouse[1]

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Abstract

The morphology and distribution of neurons and terminals that are immunoreactive to glutamic acid decarboxylase (GAD) were investigated in barrel cortex of the rat and mouse. The morphology of the GAD-immunoreactive neurons located in layer IV of the barrel field resembles that of the large, smooth stellate neurons described previously in Golgi studies. Most of the somas of GAD-positive neurons are located along the sides of the barrels. They constitute about 13 to 15% of the total neuronal population in layer IV. The spatial distribution of GAD-positive terminals in layer IV is similar to the distribution of GAD-positive somas. Very few GAD-positive neurons and terminals are found in the septal regions. This unique distribution of GAD immunoreactivity in the barrel cortex may serve as a model to study cortical inhibitory mechanisms.

In many structures, immunocytochemistry provides one of the few methods available for studying the morphology and connections of interneurons. In the mammalian neocortex, γ-aminobutyric acid (GABA) has been identified as an inhibitory transmitter (Krnjevic and Schwartz, 1967; Curtis et al., 1970; Curtis and Johnston, 1974; see Roberts, 1979, for review) and related to several morphological classes of nonpyramidal cells (Ribak, 1978; Chronwall and Wolff, 1980; Hendrickson et al., 1981; Hendry and Jones, 1981; Peters et al., 1982; Houser et al., 1983). In the present paper, we have used an immunocytochemical method to identify morphological characteristics of GABAergic neurons in the barrel cortex of both mouse and rat. The barrel cortex was chosen for study because the unique arrangements of cell somas and axon terminals in this region (Woolsey and Van der Loos, 1970; Welker and Woolsey, 1974; Pasternak and Woolsey, 1975; Woolsey et al., 1975; Harris and Woolsey, 1983) provide an opportunity to correlate neuronal morphology with immunoreactivity to specific neurotransmitters. In these experiments, an antibody raised to the specific synthesizing enzyme of GABA, glutamic acid decarboxylase (GAD), was used to identify presumptive GABAergic neurons. Several questions were addressed. First, is there any relationship between the distribution of GAD-immunoreactive neurons and the structure of the barrel fields? Second, what are the morphological features of GAD-immunoreactive neurons in barrel cortex? Finally, what is the relationship between GAD immunoreactivity and other histochemically identified characteristics of barrels, such as cytochrome oxidase activity (Wong-Riley and Welt, 1980)?

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Materials and Methods

Twenty Long Evans pigmented rats (180 to 350 gm) and Sprague-Dawley albino rats (Charles River Breeding Laboratories) and six adult black C57 mice of both sexes were used in this study. The animals were anesthetized with pentobarbital and perfused through the ascending aorta with 200 ml of saline (38°C) mixed with 1% Xylocaine followed by fixative. The brains were first perfused with 400 ml of warm (38°C) 2% paraformaldehyde in 0.1 M phosphate buffer and then further fixed with 400 ml of cold (4°C) 4% paraformaldehyde. The pH of both fixatives was in the range of 5.0 to 5.3, which decreased nonspecific immunoreactivity. The brains were stored *in situ* at 4°C overnight. They were then removed and stored in 0.1 M (pH 7.2 to 7.4) phosphate buffer. An area about 3×4 mm^2 which contained barrel fields was dissected out, fattened, and then cut in a tangential plane on a Vibratome into sections 20 to 40 μm thick.

Immunocytochemical staining. Two antibodies were used in the present experiments. First, an antiserum to GAD was used to determine the morphology and distribution of GABAergic neurons and terminals in the barrels. Second, an antiserum to neuron- specific enolase (NSE) was used to identify neurons in order to facilitate determining the proportion of GAD-immunoreactive neurons (Schmechel et al., 1984). The brain sections were processed following either the peroxidase-antiperoxidase (PAP) procedure (Sternberger, 1979) or the avidin-biotin complex procedure (Hsu et al., 1981). The antiserum to GAD (which was made from sheep) used in this study has been described previously (Oertel et al., 1981). The antiserum to NSE used in this study also has been described previously (Schmechel et al., 1978, 1984).

Sections were treated first in normal donkey serum (10%) for 30 min and then incubated overnight at 4°C in sheep GAD antiserum. The GAD antiserum was diluted 1:1500 in the 0.25 M Tris buffer with 1% normal donkey serum added. The control sections were incubated in preimmune sheep serum diluted 1:1000 in the same buffer. The GAD antibody was linked with donkey anti-sheep IgG, diluted 1:100 with 1 to 2% normal donkey serum, for 45 min. Sections were then incubated in sheep-PAP complex, diluted 1:100, for 45 min. Finally, the sections were reacted with 0.05% diaminobenzedine (DAB) in 0.05 M Tris buffer with 0.1 ml of 1% hydrogen peroxide for 15 to 20 min. The sections were mounted on gelatin-coated slides and coverslipped with Entellan or counterstained with cresyl violet and then coverslipped.

To label two different antibodies in single sections, brain sections were processed first with the sheep GAD antibody by using DAB as the chromagen. The immunoreacted sections were then processed for the second antibody (i.e. NSE). We have used 4-chloro-1-naphthol as the second chromogen (Nakane, 1968). With these combined reactions, one can easily distinguish double-stained GAD-NSE-immunoreactive neurons (brownish color) from solely NSE-immunoreactive neurons (bluish color) (Schmechel et al., 1984). Since the reaction product of 4-chloro-1-naphthol is soluble in alcohol, the sections were air-dried and then coverslipped with glycerin.

Brain sections adjacent to the immunoreactive sections were reacted for cytochrome oxidase (Wong-Riley, 1979).

Data analysis. Our analysis of the immunoreactive neurons was confined to the caudal barrels from rows A, B, and C. Most of the cell size measurements and counts of GAD- and NSE-positive neurons were obtained from B_2, B_3, C_2, and C_3 (Woolsey and Van der Loos, 1970).

Results

Distribution of GAD⁺ neurons in the barrel cortex. Of particular interest is the distribution of GAD-immunoreactive neurons in the barrel cortex. As expected, numerous GAD⁺ neurons are found in barrel cortex of both rats and mice. However, the most striking feature of the GAD⁺ neurons in the barrel cortex is their unique distribution in layer IV. In a single tangential section, aggregates of GAD⁺ neurons are found along the walls of each barrel (see Figs. 1 and 2, *C* and *F*). Usually, a total of about 12 to 20 GAD⁺ cells are present along the four walls. Occasionally, the labeled neurons are clustered together. Within the hollow of each barrel only a few (usually two to four) GAD⁺ cells were seen in each 20- to 30-μm-thick tangential section. In contrast, the distribution of GAD⁺ neurons appears to be less well organized in other cortical layers, including layer I and the white matter.

Distribution of GAD⁺ puncta in the barrel cortex. GAD⁺ puncta also have a characteristic distribution in layer IV of the barrel cortex in the rat and mouse. Specifically, the distribution of the GAD⁺ puncta corresponds with the density of GAD⁺ neurons. That is, the densest concentration of GAD⁺ puncta is present in the sides of each barrel. The least concentration was found in the centers of the hollows and in the septal regions between barrels (see Fig. 2, *B* and *C*). As was the case for GAD⁺ somas, this unique distribution pattern for GAD⁺ puncta was found only in layer IV. A lighter and more homogeneous distribution was present above and below layer IV, and in other cortical areas outside of the somatosensory cortex.

Morphology of GAD⁺ neurons in layer IV. In our material, it was also possible to determine the morphology of the GAD⁺ neurons in the barrels. Figure 3*A* illustrates the morphology and dendritic geometry of several GAD⁺ neurons in a single 30-μm-thick section. Most of the GAD⁺ neurons are larger (soma diameter about 10 to 14 μm) than the unlabeled neurons (soma diameter about 8 to 11 μm). The distributions of those large GAD⁺ neurons (*dots*) and NSE-labeled cells (*open circles*) are illustrated in Figure 4.

The GAD⁺ neurons in the barrel walls are characterized by their eccentric dendritic fields and smooth and/or beaded dendrites oriented toward the center of the barrel hollows (see Figs. 2*F* and 3*B* for cells 1, 2, 3, and 4). Occasionally, a few spines were observed on the labeled dendrites. Rarely, a labeled dendrite was observed to extend into adjacent barrels. Often three to five branches of primary dendrites were found. Secondary and tertiary branches of labeled dendrites were frequently observed (see Fig. 3*B*). The dendritic ramifications may extend up to about 100 μm in length. Thus, a single GAD⁺ neuron may occupy a dendritic field with a width of about 250 μm. Conversely, the GAD⁺ neurons that have their somas in the barrel hollows possess radially oriented dendrites (see Fig. 3*B, cell 5*). Based on their soma size, dendritic morphology, and geometry, the majority of GAD-immunoreactive neurons in layer IV of barrels form a homogeneous class of neurons which resembles smooth stellate cells described previously by the Golgi method (Pasternak and Woolsey, 1975; Woolsey et al., 1975). A very small population of GAD⁺ neurons have dendrites which were less completely labeled in the barrels, making their identification uncertain.

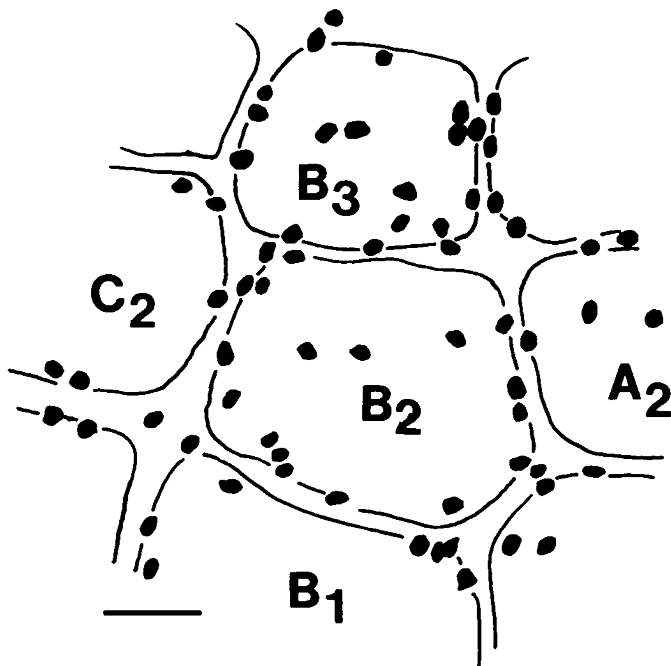

Figure 1. Drawing illustrating the location of GAD⁺ neurons in layer IV of the rat's barrel cortex. The drawing is of a tangential 30-μm thick section reacted with GAD antiserum. *Scale bar:* 100 μm.

Percentage of GAD⁺ neurons in layer IV. Because of the limited penetration ability (approximately 5 to 8 μm) of the GAD antibody, we used a double-label immunoreactive technique to estimate the percentage of GAD⁺ neurons in layer IV of barrel cortex. Since the depths of penetration by the GAD and NSE antibodies are similar, this approach allows us to estimate the relative number of GAD⁺ neurons (Schmechel et al., 1984). Within a single 30-μm-thick section through a single barrel, usually a total of about 104 to 110 cells were also labeled with NSE. Among these, about 13 to 15 cells were labeled with the GAD antiserum (see Fig. 4). Based on these results, we estimated that about 14% of the neurons in layer IV of barrel cortex are GAD immunoreactive.

Comparison of GAD immunoreactivity with cytochrome oxidase reactivity. Recently, Wong-Riley and Welt (1980) demonstrated a high level of cytochrome oxidase enzymatic activity within the barrel hollows of mice. Thus, the barrel field provides an opportunity to investigate the relationships between GAD immunoreactivity and cytochrome oxidase reactivity. In the present experiments, sections adjacent to those reacted for GAD were also reacted for cytochrome oxidase. We found that the barrel hollows exhibit a high level of cytochrome oxidase activity, whereas the septae are much less reactive (see Fig. 2, *A*, *D*, and *E*). The relative correspondence between high cytochrome oxidase activity and the hollows of barrel cortex is complementary to the distribution of GAD⁺-reactive cell bodies and terminals described in the previous sections. That is, the barrel hollows contain high levels of cytochrome oxidase reactivity, whereas the highest GAD immunoreactivity is centered along the sides of the barrels. However, there is an overlap in the barrel wall between the region containing high cytochrome oxidase activity and the one containing high GAD reactivity (see Fig. 5).

Discussion

Four main conclusions concerning the distribution of GABAergic activity in the barrel cortex can be derived from the present experiments. First, only about 14% of neurons in layer IV of barrel cortex are GAD immunoreactive. Second, there is a precise spatial distribution of GABAergic neurons that is precisely related to the organization of the barrels. Specifically, the sides of the barrels contain the majority of both the somas, dendrites, and terminals (puncta) of the GAD-immunoreactive neurons. In comparison, the hollows of the barrels contain much less immunoreactivity. Furthermore, the distribution of GAD-immunoreactive puncta corresponds closely to the distribution of GAD-immunoreactive neurons, being the heaviest along the sides of the barrels rather than in the hollows. Third, the morphology of most GAD-immunoreactive neurons in barrel cortex corresponds to that of the large, smooth stellate, or class II, cells as they have been defined in previous Golgi studies of this area (Woolsey et al., 1975). Finally, there is a complementary relationship between the distribution of GAD immunoreactivity and that of cytochrome oxidase reactivity.

Comparisons with the distributions of GABAergic activity described in previous studies. Most of the previous immunocytochemical and autoradiographic studies of the cortical distribution of GABAergic activity were concerned with the mammalian visual

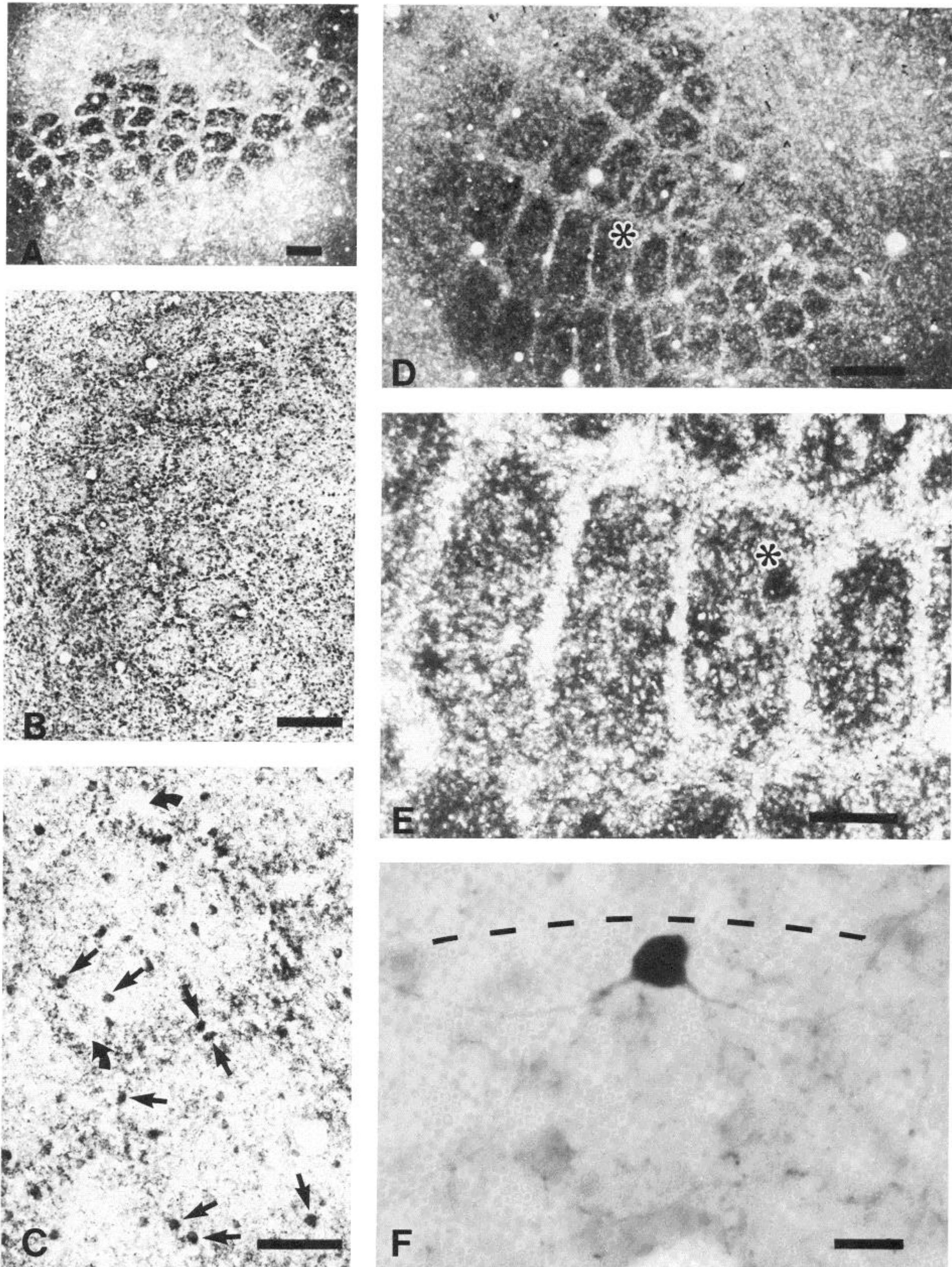

Figure 2. Photomicrographs of tangential section from layer IV barrel cortex that were reacted either with cytochrome oxidase or GAD antiserum. *A*, Section from the rat's barrel cortex reacted with cytochrome oxidase. *B*, The section adjacent to the one in *A*, reacted with the GAD antiserum and then counterstained with cresyl violet. *C*, A higher power photomicrograph showing the GAD immunoreactivity in the rat's barrel cortex. The *straight arrows* mark some of the labeled GAD^+ neurons. Note that the majority of GAD^+ neurons are located on the sides of the barrels. GAD^+ puncta (fine grains) are closely associated with the labeled GAD^+ neurons. The *curved arrows* point to the septal regions, which contain virtually no GAD immunoreactivity. *D* and *E*, Low and high power views, respectively, of tangential section from mouse barrel cortex that was stained with cytochrome oxidase. Note the uneven and patchy activity within each barrel. *, the location of a blood vessel in the same barrel in *D* and *E*. *F*, High power photomicrograph of a GAD^+-immunoreactive neuron in the rat's barrel cortex. Note the eccentric dendritic branches directed centrally. The *dashed line* demarcates the border of the wall of a barrel. *Scale bars: A* and *B*, 250 μm; *C* and *E*, 100 μm; *D*, 200 μm; *F*, 15 μm.

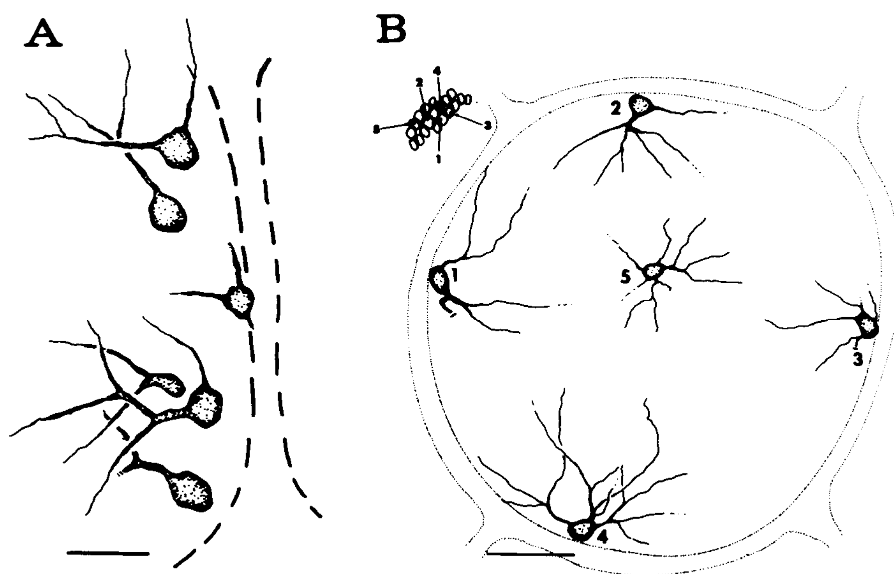

Figure 3. Camera lucida drawing of GAD$^+$ neurons in layer IV of the rat's barrel cortex. *A*, Composite line drawings of GAD$^+$ neurons from a single tangential section (30 μm) of the barrel field. *Scale bar:* 25 μm. *B*, Composite camera lucida drawings of five GAD$^+$ neurons. Note that the GAD$^+$ neurons located near the wall of the barrel have their dendritic processes pointed toward the center of the barrel, whereas GAD$^+$ neurons located in the hollow of the barrel have their dendritic processes oriented in all directions. The location of each GAD-positive neuron is indicated in the *inset* on the *left upper corner* of *B*. *Scale bar:* 50 μm.

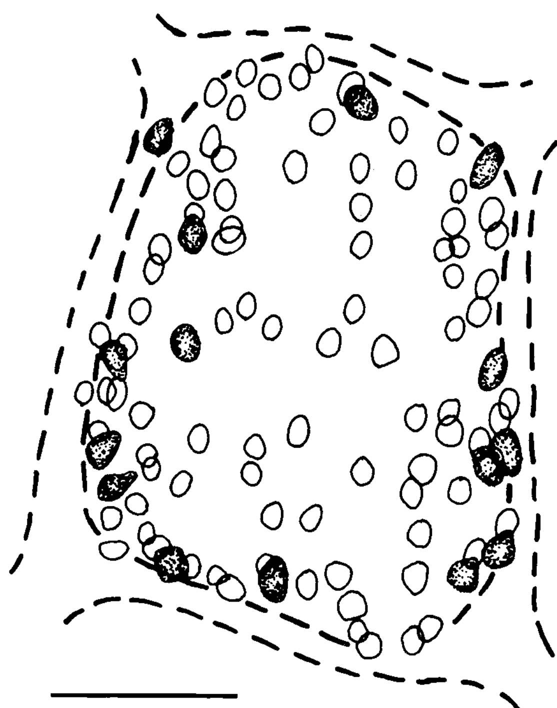

Figure 4. Camera lucida drawing of a single barrel from the rat (C_3 in row C, which has been reacted with both GAD and NSE antisera. The *dashed lines* mark the sides of each barrel. The GAD$^+$ neurons are marked with *small dots* on their somata, whereas the NSE-labeled neurons are marked as *open circles*. Note GAD$^+$ neurons, which are also labeled with NSE, are larger than the neurons in layer IV labeled only with the NSE antiserum. *Scale bar:* 100 μm.

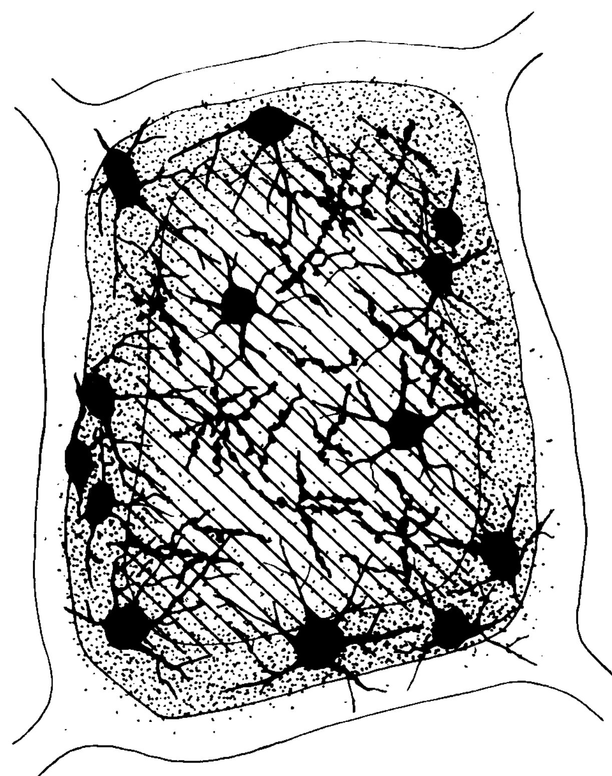

Figure 5. A summary scheme to illustrate the difference in the distribution of different neurochemicals in a single barrel. The *solidly labeled* neurons are GAD$^+$ cells. The *small dots* represent the distribution of GAD$^+$ puncta. The *diagonal lines* represent the region of high cytochrome oxidase activity. The catecholamine fibers are marked as beaded processes with a "sunburst" appearance and are located in the hollow of the barrel.

cortex (Ribak, 1978; Hendrickson et al., 1981; Fitzpatrick et al., 1983; Freund et al., 1983; Somogyi et al., 1981, 1983, 1984). Those studies demonstrated that, although GABAergic neurons are found in all layers of visual cortex, they are more abundant in layer IV.

Recently, Hendry and Jones (1981), House et al. (1983), and Harandi et al. (1983) studied the laminar distribution of presumed GABAergic neurons in the monkey and cat somatosensory cortex. As was the case for the visual cortex, GABAergic neurons were found in all layers of somatosensory cortex and were more common in layer IV. Based on the somata shape and the limited branching patterns of the "primary" dendrites that could be identified in their material, Houser et al., (1983) suggested that GABAergic neurons belong to several classes of nonpyramidal cells, including large basket, bitufted, and multipolar neurons. However, detailed knowledge of the dendritic branching patterns and the axonal arborizations of the GABAergic neurons is essential for classifying these cells and for determining their functional contribution to cortical organization. A step toward identifying such detailed neuronal morphology was taken by Freund et al. (1983), Somogyi et al. (1984), and Martin et al. (1983), who combined Golgi impregnations or intracellular horseradish peroxidase labeling with either immunocytochemistry or triti-

ated GABA autoradiography. They suggested that the "basket" cells in the cat's visual cortex and bitufted neurons in the monkey visual cortex use GABA as a transmitter.

The main contributions of the present study depend on the higher sensitivity of the immunocytochemistry in the cortex of the rat and mouse as compared to other species. The consequent labeling of distal dendrites as well as the somas and more proximal processes allowed us to compare the morphology of GAD-immunoreactive cells with those described previously in Golgi studies. The results suggest that the GAD immunoreactivity in layer IV of the barrel cortex is related to a distinctive class of neurons. These neurons resemble the class II cells—large, smooth stellate cells—of Woolsey et al. (1975). That is, both exhibit large round somas, smooth dendrites, and from four to seven primary dendrites. The present study also suggests that the GABAergic neurons are mainly located in the sides of the barrels and send dendrites into the barrel hollows, features which are also characteristic of large, smooth stellate cells. In contrast, the GAD^+ neurons located in the other cortical layers of barrel cortex processed many different morphologies, even in a single layer. A more complete description of these other GAD^+ neurons will be presented elsewhere (Lin et al., 1985).

Relationships between the distribution of GABAergic activity and other features of barrel field organization. The distribution of GAD immunoreactivity is related to the distribution of other putative transmitters. For instance, although the barrel hollows have little GAD activity, they contain a very dense concentration of catecholamine-fluorescent fibers. In contrast, the septal areas have minimal catecholamine activity (Lidov et al., 1978). High levels of cytochrome oxidase (Wong-Riley and Welt, 1980), succinic dehydrogenase (Killackey and Bedford, 1979), acetylcholinesterase (Kristt, 1979), 2-deoxyglucose uptake (Durham and Woolsey, 1978), and mitochondrial enzyme (Dietrich et al., 1981) activity have been reported previously in the barrel hollows. High levels of cytochrome oxidase activity and GAD activity overlap only in a small border near the barrel wall (see Fig. 5). The correlation between the distribution of cytochrome oxidase and GAD activity may be different in other cortical areas. For example, Hendrickson et al. (1981) reported that more than 80% of GAD^+ immunoreactivity overlaps the cytochrome oxidase activity. Furthermore, the reactivity of cytochrome oxidase has also been suggested to be associated with ocular dominance columns (Horton and Hedley-Whyte, 1984) and color blobs (Livingstone and Hubel, 1984) in the visual system.

Finally, it has been suggested that the high levels of the substances found in the hollows are associated with the thalamocortical fibers that terminate there. Since the GABAergic neurons send the majority of their dendrites into the barrel hollows, they are in a position to receive a major direct projection from the ventrobasal complex of the thalamus (White and Rock, 1981; White et al., 1984). In future studies, such direct projections might be verified by electron microscopic demonstrations of degenerating thalamic terminals on GAD^+ dendrites following thalamic lesions.

Percentage of GABAergic neurons in somatosensory cortex. The GABAergic cell is generally recognized to be an inhibitory interneuron in the mammalian cerebral cortex; therefore, it is of particular interest to determine the relative numbers of these neurons in different cortical areas and layers. Recently, Hendry and Jones (1981) reported that about 40% of the neurons in layer IV of areas 3b, 1, and 2 of the monkey somatosensory cortex can be labeled with [^{3}H] GABA. In the present experiments, we found that only about 13 to 15% of the neurons in layer IV of the barrel field are GABAergic neurons. This estimate is similar to those of Fitzpatrick et al. (1983) and Lin et al. (1984), who estimated that GABAergic neurons constitute about 13 to 15% of the neurons in cortical layers II/III, IV, V, and VI of visual cortex in rat, cat, and monkey. The higher estimates for the relative number of GABAergic neurons in the monkey somatosensory cortex may reflect either species or technique differences. For example, we have used a double immunoreactive technique to identify the relative numbers of GAD- and NSE-positive neurons, whereas in their study, Hendry and Jones (1981) calculate the percentage of cells labeled with [^{3}H]GABA in the total cell population. However, Neale et al. (1983) reported that there was a close correspondence (about 90%) between [^{3}H]GABA uptake and GAD immunoreactivity. Consequently, the different estimates may reflect that primate somatosensory cortex does indeed have more intrinsic neurons than does the cortex in rats and mice.

Inhibitory action in sensory cortex. Strong inhibitory influences on cortical neurons have been reported previously. For instance, when bicuculline, a GABA antagonist, is iontophoretically injected into cat visual cortex, an increase of both evoked and spontaneous activity is found (Curtis et al., 1970; Rose and Blakemore, 1974; Sillito, 1973a, b, 1977). Moreover, Sillito (1975a, b, 1977) recently indicated that direction and orientation selectivity can be modified after cortical bicuculline iontophoresis. Similarly, when 3-mercaptopropionic acid, an inhibitor of GABA synthesis, is injected intravenously or iontophoretically into the cortex, orientation selectivity can be completely abolished (Sillito, 1977).

To date, there have been only a few physiological studies of the barrel cortex (Axelrad et al., 1976; Ito, 1981; Simons, 1978, 1983a, b). In particular, Simons (1983b) reported that barrel cortical neurons influence each other when two adjacent whiskers are stimulated. Specifically, suppression of a neuronal response to whisker stimulation could be elicited by prior deflection of adjacent whiskers. Thus, inhibitory side effects exist in the barrel cortex which could be mediated by those GABAergic neurons described in the present experiments.

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