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Original Articles

Antisera to γ-Aminobutyric Acid.

II. Immunocytochemical Application to the Central Nervous System[1]

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An antiserum to γ-aminobutyric acid (GABA) was tested for the localization of GABAergic neurons in the central nervous system using the unlabeled antibody enzyme method under pre- and postembedding conditions. GABA immunostaining was compared with glutamate decarboxylase (GAD) immunoreactivity in the cerebellar cortex and in normal and colchicine-injected neocortex and hippocampus of cat. The types, distribution, and proportion of neurons and nerve terminals stained with either sera showed good agreement in all areas. Colchicine treatment had little effect on the density of GABA-immunoreactive cells but increased the number of GAD-positive cells to the level of GABA-positive neurons in normal tissue. GABA immunoreactivity was abolished by solid phase adsorption to GABA and it was attenuated by adsorption to β-alanine or γ-amino-β-hydroxybutyric acid, but without selective loss of immunostaining. Reactivity was not affected by adsorption to glutamate, aspartate, taurine, glycine, cholecystokinin, or bovin serum albumin. The concentration (0.05–2.5%) of glutaraldehyde in the fixative was not critical. The antiserum allows the demonstration of immunoreactive GABA in neurons containing other nueroactive substances; cholecystokinin and GABA immunoreactivities have been shown in the same neurons of the hippocampus. In conclusion, antisera to GABA are good markers for the localization of GABAergic neuronal circuits.
KEY WORDS: Light and electron microscopy; Glutamate decarboxylase; γ-aminobutyric acid; Immunocytochemical localization; Cerebellum; Cerebral cortex; Hippocampus; Coexistence of neuroactive substances.

Introduction

Glutamate decarboxylase (GAD; EC 4.1.1.15) was purified for the first time from brain by Wu et al. (33) and an antiserum produced against this GABA-synthesizing enzyme revealed that the enzyme was selectively localized in neurons (14,15, 23,24) that from other studies were thought to use γ-aminobutyric acid (GABA) as transmitter. Subsequently several antisera were produced (16,20) and used in immunohistochemical studies for the identification and localization of GABAergic neurons. (For reviews see refs. 17 and 32). Although some controversy arose (see refs. 3 and 32), it is generally accepted that GAD is a specific marker for neurons that synthesize and release GABA (1,32). However, as has been argued in the previous article (10), the localization of GABA itself would be desirable for several reasons. Thus, we, like others (25,30), have developed antisera to GABA. The specificity of the antisera has been characterized with a model system using nitrocellulose paper (10). One of the sera has been applied in this study to the localization of GABA in tissue sections of the hippocampus, cerebral and cerebellar cortices; areas where both the function of the GABAergic neuronal system and the localization of GAD have been extensively investigated. The aims of the study were: 1) to establish fixation and processing conditions for the light and electron microscopic immunohistochemical demonstration of GABA; 2) to compare GAD and GABA immunohistochemistry for the localization of GABAergic neurons; 3) to determine whether adsorption of the sera with compounds found to react in the model system (10) affected the localization of immunoreactivity; and 4) to see if fixation and processing con-

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ditions for the immunocytochemical demonstration of GABA and other neuroactive substances, such as neuropeptides present in the same area or cell, could be found. Some of the results have been presented in preliminary form (9).

Materials and Methods

Antisera to GABA characterized in the previous study (10) have been applied to the central nervous system of rats, cats, monkeys, and humans with identical results. Conclusions on fixation and processing conditions were based on studies with a total of 46 animals. To illustrate the usefulness of the antisera in immunohistochemistry we selected material from three adult cats (Table 1).

Antisera. The antiserum to GABA (code no. GABA-7) (10) was raised in a rabbit. Rabbit antiserum to GAD was a gift from Dr. J-Y. Wu (code no. P4 10/17). The specificity of this serum has been summarized (32). Antiserum (code no. L-112) to cholecystokinin (CCK) was raised in a rabbit and was a gift from Dr. G.J. Dockray. This antiserum has been characterized and shown to be specific for the carboxy terminus of the peptide (4).

Animals. One of the cats (no. 3 in Table 1) recieved local injections of colchicine 24 hr before perfusion directly into the hippocampus and lateral gyrus of the cortex under anesthetic and surgical conditions described previously (28). All three animals were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally) and perfused through the heart first with Tyrode's solution then with fixative (Table 1). To investigate how fixation conditions affected GABA immunoreactivity, eight male rats (Porton strain) were perfused under chloral hydrate anesthesia in the same way as the cats. Each fixative listed in Table 1 was used for two rats, and an additional two rats were perfused with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. After removing the brain from the skull, 3–5 mm thick slices were immersed in the same fixative for 2–3 hr, at room temperature, then blocks of tissue, not exceeding 5 mm in any dimension, were washed for 1 day, at 4° C, in several changes of 0.1 M sodium phosphate buffer (PB; pH 7.4). Blocks were further processed in one of three ways.

1. Nonosmicated blocks for postembedding immunohistochemistry were dehydrated and embedded in Durcupan ACM (Fluka) resin. Serial, semithin sections (1 μm) were cut and mounted on slides coated with egg white.
2. Osmicated blocks for postembedding immunohistochemistry and for correlated electron microscopic examination were postfixed for 2–3 hr at room temperature in 1% OsO_4 dissolved in PB (0.1 M, pH 7.4). Subsequently they were washed in PB, dehydrated, and embedded in resin. Serial, semithin sections (0.5 μm) were cut and mounted on slides coated with egg white.
3. For preembedding immunocytochemistry, blocks were immersed sequentially in 10% then 20% sucrose till they sank, they were then frozen in liquid nitrogen, thawed, and sectioned on a Vibratome at 60 μm.

Table 1. *Fixatives and processing procedures for the animals used to illustrate the present study*[a]

Animal No.	Fixative	Figure	Method of processing
1	1% paraformaldehyde 0.5% glutaraldehyde 0.2% picric acid	1 A,B 1 C–G 2 B–E	Osmicated, postembedding Preembedding, postosmicated
2	1% paraformaldehyde 2.5% glutaraldehyde	2 A 3 A–E	Osmicated, postembedding
3	4% paraformaldehyde 0.05% glutaraldehyde 0.2% picric acid	3 F–K	Nonosmicated, postembedding

[a]All fixatives were dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Aldehydes were obtained from TAAB Labs. Equip. Ltd., (Reading, U.K.). Methods of processing are explained in more detail in the text.

Preembedding immunohistochemistry. Incubation of sections was carried out in the following sequence, at room temperature unless otherwise stated: 1 hr in 20% normal sheep serum; rinse in Tris (10 mM)–phosphate (10 mM) buffered saline (TPBS); 24 hr at 4°C in antiserum to GABA diluted 1:3000, or in antiserum to GAD diluted 1:500, or in antiserum preincubated with antigens; three 40 min washes in TPBS; 4 hr in sheep anti-rabbit immunoglobulin (IgG) fraction (Silenus Lab. Ltd., Dandenong, Australia) diluted 1:30; three 40 min washes in TPBS; overnight at 4°C in rabbit peroxidase–antiperoxidase (PAP) complex diluted 1:100; two 40 min washes in TPBS. All sera were diluted with TPBS containing 1% heat-inactivated sheep serum.

Postembedding immunohistochemistry. The slides with the semithin sections were treated for 30–40 min with ethanolic sodium hydroxide (12) to etch the resin. This was followed by three washes in absolute ethanol and two washes in distilled water. The osmium was removed by treatment with freshly prepared 1% $NaIO_4$ (2) for 7 min, followed by two washes in distilled water. Then all slides had two washes in TPBS. Thereafter the sera were overlayered onto the slides in a humid chamber at room temperature in the following sequence: normal sheep serum (20%) for 30 min, rinse in TPBS; 2 hr in antiserum to GABA, cholecystokinin, or the adsorbed sera preincubated with antigens; three 20 min washes in TPBS; 1 hr in sheep anti-rabbit IgG (see above) diluted 1:40; three 20 min washes in TPBS; rabbit PAP (Dakopatts Ltd., Denmark) diluted 1:100; two 20 min washes. All sera was diluted in 1% heat-inactivated sheep serum.

Postincubation treatments. Both Vibratome and semithin sections were reacted for peroxidase enzyme activity using 3, 3′-diaminobenzidine tetrahydrochloride (0.05%; Sigma) as substrate as described previously (8,28). They were then washed in several changes of TPBS and the Vibratome sections were postfixed for 1 hr with 1% OsO_4 dissolved in 0.1 M PB. The semithin sections were treated for 5 min with 0.01% OsO_4 to enhance the peroxidase reaction end product; they were washed in TPBS, dehydrated, and mounted in XAM neutral media.

The Vibratome sections were washed in TPBS, dehydrated, and embedded on slides in Durcupan ACM (Fluka) resin. One percent uranyl acetate was added to the 70% ethanol to enhance contrast for electron microscopy.

Specificity of the immunohistochemical reaction. Method specificity was tested by replacing the anti-GABA serum by normal rabbit serum at the same dilution. Antiserum specificity was tested by solid phase adsorption of the diluted antisera to amino acids or to peptides. The antigens were coupled to polyacrylamide beads (Biogel P200, BioRad Lab., Richmond, CA) as described previously (10, 28). The following compounds were used: GABA; β-alanine; L-glutamic acid; L-aspartic acid; D,L-γ-amino-β-hydroxybutyric acid (GABOB); taurine; glycine; CCK-8 (Sigma); [leu]-enkephalin (Sigma); bovine serum albumin (BSA; Sigma). The dilution of antisera and the proportion of adsorbent to sera were the same for all compounds. In addition L-glutamic acid (10^{-5}, 10^{-2} M) and GABA (10^{-5}, 10^{-4},

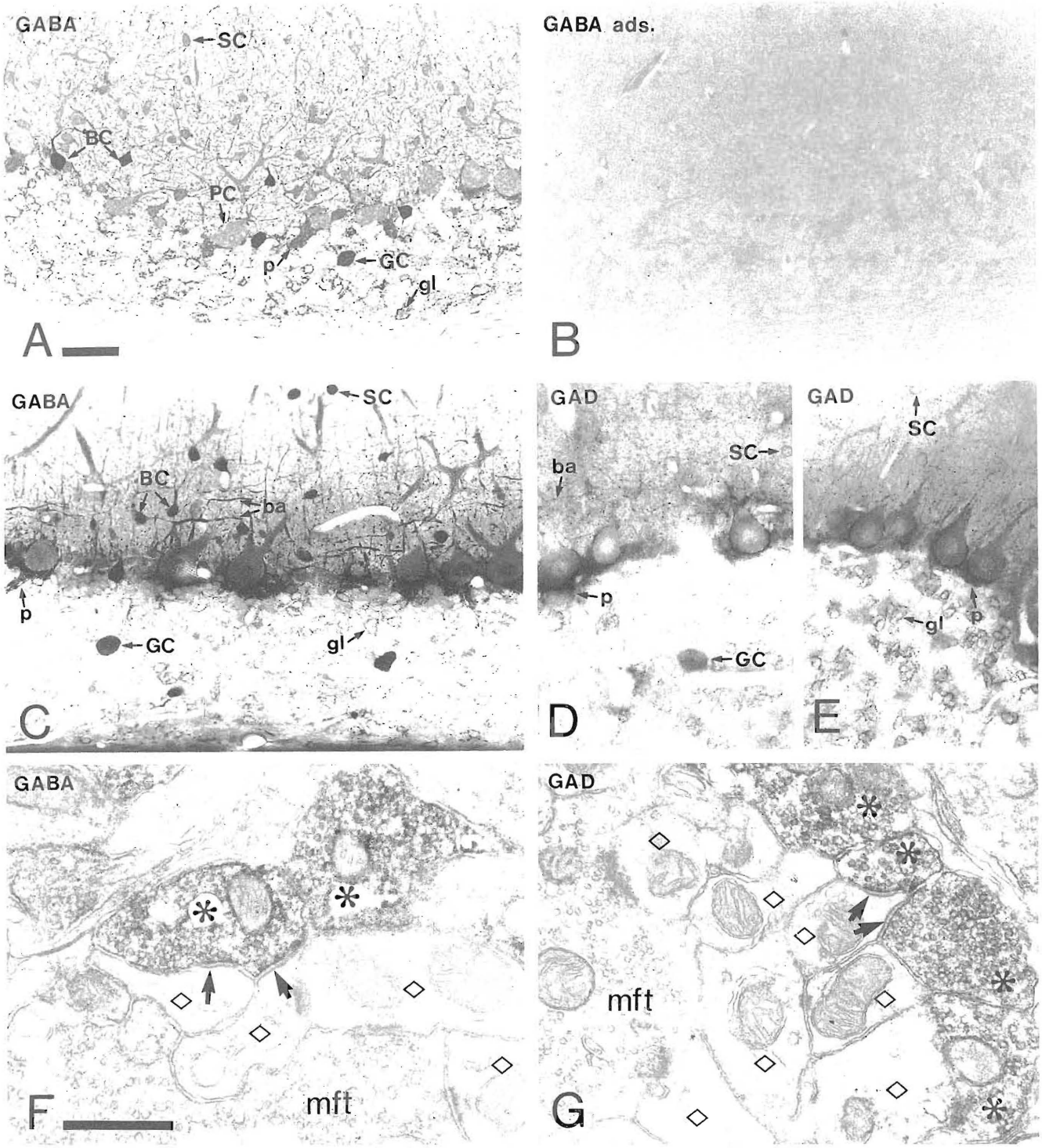

Figure 1. Cerebellar cortex of cat reacted by the unlabeled antibody enzyme method. (**A**and **B**) Serial semithin (1 μm) sections reacted under postembedding conditions, with the anti-GABA serum (**A**) or with the same serum after solid phase adsorption to GABA (**B**). Basket cells (BC) and Golgi cells (GC) are strongly immunoreactive, Purkinje cells (PC) and stellate cells (SC) reacted less strongly. GABA-immunoreactive terminals are present in all layers, but the terminals of basket cells around the Purkinje perikarya and in the pinceau (p) and the Golgi cell terminals in the glomeruli (gl) are especially strongly reacting. (**C–E**) Preembedding demonstration of GABA and GAD in sections cut on a Vibratome. The distribution of amino acid and its synthesizing enzyme are very similar, but perikarya of basket, stellate, and Golgi cells, and basket cell axons (ba) stain stronger for the amino acid than for GAD in animals not treated with colchicine. (**F** and **G**) Electron micrographs of glomeruli demonstrating GABA (**F**) and GAD (**G**) in the terminals (asterisks) of Golgi cells. The dendritic digits of granule cells (diamonds) receive synapses (arrows) from the immunoreactive terminals as well as from the mossy fiber terminal (mft). (**A–E**) Bar = 50 μm; (**F** and **G**) bar = 0.5 μm.

10^{-3}, 10^{-2} M) were also used in liquid phase for the absorption of the GABA serum. In the peptide/GABA coexistence experiment both antisera were adsorbed to the homologous as well as to the other antigen.

Results

Adsorption of the Sera to Various Compounds. Effect on Immunostaining

Liquid phase absorption of the sera with GABA (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M) or with glutamate (10^{-5}, 10^{-2} M) had a negligible effect on the immunostaining. Using solid phase adsorption the immunostaining was attenuated by β-alanine and γ-amino-β-hydroxybutyric acid (Figure 3A,E) but not by glutamate (Figure 3B), aspartate, taurine, glycine, [leu]-enkephalin, CCK (Figure 3I), somatostatin (28), or BSA. The attenuation by β-alanine and GABOB was uniform in that all structures stained less strongly and there was no selective loss of immunoreactivity observed from particular neuronal populations. The immunostaining in all the areas of the central nervous system examined was completely or almost completely abolished by solid phase adsorption of the sera to GABA (Figures 1B, 3D,J), provided that the dilution of the anti-GABA serum and the proportion of adsorbent were appropriately chosen. A cross-adsorption paradigm showed that the two sera used for the demonstration of CCK and GABA immunoreactivity in the same cell recognized different antigens (Figure 3F–K).

No immunoreactivity was observed under either pre- or postembedding conditions when the anti-GABA serum was replaced by normal rabbit serum of the same dilution.

Comparison of GABA and GAD Immunoreactivity

Cerebellar cortex. Strong GABA immunoreactivity was present in the soma, axons and terminals of Golgi, basket, and stellate cells (Figure 1A,C). The strongest reaction was found in the terminals of the Golgi and basket cells. Purkinje cell perikarya, main dendrites, and axons were moderately or weakly reacting (Figure 1A,C). In the GABA-positive neurons both the cytoplasm and the nuclei were stained. Granule cells, Bergman and other glial cells were not immunoreactive. Electron microscopic (EM) examination confirmed that the immunoreactive varicosities whose position identified them as terminals of Golgi, basket, and stellate cells were in fact synaptic boutons (Figure 1F).

GAD immunoreactivity was generally much weaker in perikarya, which is in agreement with previous studies (23,27,29) in which colchicine treatment was required to obtain strong staining of perikarya with this antiserum. GAD immunoreactivity was localized in the same types of neuron as GABA (Figure 1D,E). The axons and terminals of Golgi, basket, and stellate cells were immunoreactive, and the pattern of staining agreed with that obtained using the anti-GABA serum. Examination in the EM of synaptic boutons immunoreactive for GAD produced identical results to that obtained with anti-GABA serum (Figure 1F,G).

Striate cortex. GABA-immunoreactive neurons were present in all layers and varied in size (8–35 μm) and shape (Figure 2A). In layer I most neurons were immunoreactive, while in the other layers only a minority of the cells were GABA positive (Figure 2A). In the colchicine-injected cortex the proportion and distribution of GABA-positive cells was the same as in normal cortex. A slight increase was observed in immunoreactivity of neurons close to the colchicine injection site. Both the immunoreactive and GABA-negative cells were surrounded by immunoreactive spots. There were also punctae in the neuropile together with a dense interwoven network of immunoreactive fibers. The strongest immunoreactive structures were some large myelinated axons in layers IV and lower III. Typical pyramidal cells, bundles of apical dendrites, most myelinated axons, and glial cells were not GABA immunoreactive. Examination in the EM confirmed that the GABA-immunoreactive punctae were synaptic boutons. They formed Gray's type 2 synaptic contacts with perikarya of both pyramidal and nonpyramidal cells (Figure 2B,C,E), with dendrites (Figure 2D), axon initial segments, and to a lesser extent with dendritic spines. The immunoreactive boutons contained pleomorphic vesicles. Boutons making Gray's type 1 synaptic contacts were never GABA positive. GABA-immunoreactive neurons were very heterogeneous in their synaptic input and fine structural characteristics. Most of them received a variable number of synaptic contacts from GABA-positive terminals as well as from unstained boutons making Gray's type 1 synaptic contacts (Figure 2B,C). A large number of small unmyelinated axons and some myelinated axons were immunoreactive in the neuropile.

Since the distribution of GAD immunoreactivity has recently been described in detail in the same cortical area of the same species (8,27) it will not be repeated here. In general there was good agreement in the distribution and density of neuronal elements immunoreactive for GAD in the colchicine-injected cortex and for GABA in the present study. However, in noninjected cortex, fewer GAD-positive cell bodies were encountered.

Hippocampal formation. GABA-immunoreactive cells were examined only in the light microscope; they were present in all layers and areas. The neurons were generally of small to

Figure 2. Cat striate cortex reacted for GABA under post-(**A**) and preembedding conditions (**B–E**). (**A**) Light micrograph of a semithin section (0.5 μm) showing the lower layers (III–VI). The density and distribution of immunoreactive neurons (dark) is identical to that reported in the same tissue for GAD (8,27). Nonimmunoreactive neurons and dendrites are surrounded by dark dots representing nerve terminals. (**B–E**) Electron micrographs: (**B**) Small immunoreactive neuron in layer IV. The emerging dendrite (framed area) is also shown in **C**. (**C**) The dendrite is contacted by an immunoreactive (asterisk) and two nonimmunoreactive (triangles) boutons. (**D**) A dendrite (d) receives type I synapses (open arrows) from nonimmunoreactive boutons (triangles) and a type II synapse (solid arrow) from an immunoreactive bouton (asterisk). (**E**) An immunoreactive GABAergic neuron (N_{GABA}) and a pyramidal cell (N_{Pyr}) are shown side by side, both receiving type II synapses (arrows) from immunoreactive boutons (asterisks). (**A**) Bar = 100 μm; (**B**) bar = 2 μm; (**C** and **D**) bars = 0.2 μm; (**E**) bar = 0.2 μm.

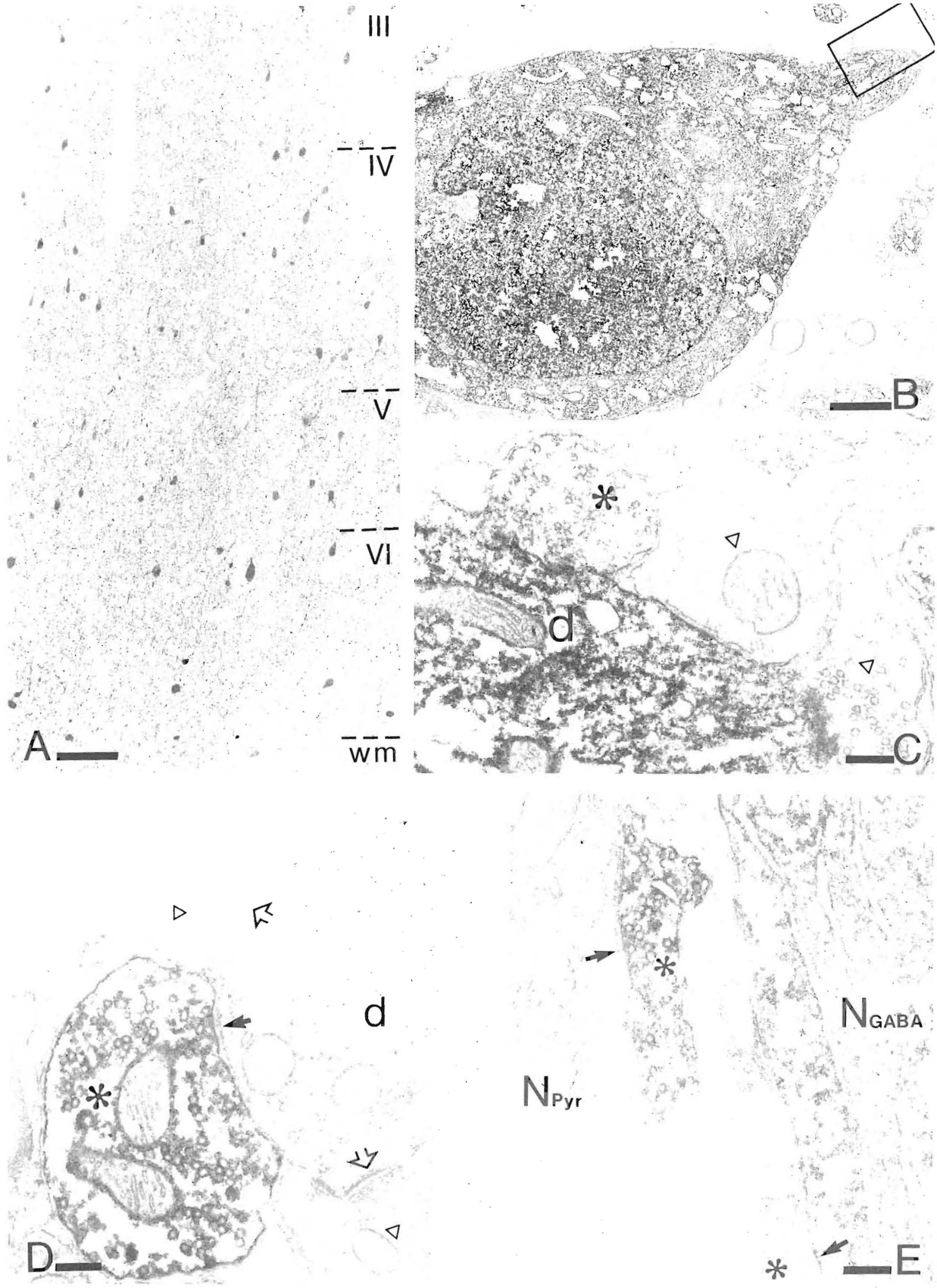

Figure 2

medium size (8–20 μm), but some large cells (30–50 μm) in the pyramidal layer and the stratum oriens were also immunoreactive. All of the stained neurons represented various types of nonpyramidal cells. Few GABA-positive cells were present n the granule cell layer and molecular layer of the dentate gyrus. Pyramidal cells and granule cells were not GABA immunoreactive, but their cell bodies, dendrites, and axon initial segments were surrounded by GABA-positive varicose fiber networks. Such varicose fibers were present in all layers. These results were in good agreement with the distribution of GAD in the colchicine-injected hippocampus of the same species (29).

Subcellular Localization of GABA Immunoreactivity

In the synaptic terminals, the reaction end product was present on the surface but not in the lumen of small clear vesicles and on all other membrane sufaces facing the cytoplasm (Figures 1F, 2C,D,E). The electron-dense core of large granulated vesicles present in some GABA-positive boutons was immunoreactive. In perikarya, the reaction end product was also attached to the surface of all intracellular organelles and was very heavily deposited in the nucleus.

The localization of GAD immunoreactivity was different in that it was not found in the nuclei of neurons.

Effect of Fixation

The cerebelli and the occipital cortices of rats fixed with the various fixatives were compared using postembedding staining of semithin (1 μm) sections. In addition, material from the three cats was also evaluated for the effect of fixation. Only very weak immunoreactivity was observed in sections of the paraformaldehyde-fixed rats (not illustrated). The glutaraldehyde-containing fixatives gave satisfactory staining. The results were similar in both rats and cats. The concentration of glutaraldehyde was not critical and good immunoreactivity was obtained with concentrations ranging from 0.05% up to 2.5%; the higher concentrations usually gave more consistent results. The speed and thoroughness of fixation seemed to be the major factor determining the immunoreactivity in the tissue. Components such as formaldehyde and picric acid were included into the fixative in most animals because they did not adversely affect GABA immunoreactivity while allowing the use of the same tissue for other histological procedures (28).

Colocalization of Neuroactive Peptide and GABA

Many neurons contain and probably release more than one neuroactive substance. In the case of GABAergic neurons it is important to establish the presence of neuroactive peptides in the same neuron. The immunoreactivity of many peptides is usually adversely affected by a high concentration of glutaraldehyde, therefore it was gratifying to find that strong GABA immunoreactivity was present in tissues fixed with the picric acid–paraformaldehyde–glutaraldehyde fixative. This fixative has a low concentration of glutaraldehyde and CCK immunoreactivity could readily be demonstrated in the same neurons that were also immunoreactive for GABA in the hippocampus (Figure 3F–K). Such neurons comprised only a minority of all the GABA-positive neurons.

Discussion

Nature of the Antigen Recognized by Antisera to GABA

Immunochemical experiments provided evidence that GABA fixed by glutaraldehyde was recognized by our antisera (10). In addition it was found that the amino acids β-alanine and GABOB, which may be present in the same brain areas as GABA, reacted weakly with the antisera. It is therefore important to establish that the immunostaining in tissue sections corresponded to the presence of GABA.

Two lines of evidence suggest that under our experimental conditions these sera reveal GABAergic neurons. First, the type, distribution, and proportion of neurons and nerve terminals stained by the anti-GABA sera in the cerebellum, cerebral cortex, and hippocampus corresponded to that revealed in our experiments when the anti-GAD serum of Wu et al. was used (32). In our previous study GABA immunoreactivity could be demonstrated in all GAD-immunoreactive neurons in the cortex and hippocampus (28). The distribution of GABA immunoreactivity also agreed well with GAD immunoreactivity obtained with other antisera (16,17,20). Since GAD is considered to be a specific enzyme marker for those neurons that use GABA as transmitter, the agreement of the staining pattern is good evidence that GABA fixed in tissue sections was localized.

This view was strengthened by the results of the adsorption experiments. On the one hand, solid phase adsorption to GABA abolished immunostaining and on the other, the adsorption to cross-reacting amino acids could not remove all the antibodies from the serum. All the GABA-immunoreactive neurons and terminals retained their immunostaining, although at a lower level. This means that in the areas tested, staining was not a result of contaminating antibodies recognizing antigens dif-

Figure 3. (**A–E**): Serial semithin (0.5 μm) sections of layer IV in the cat's striate cortex reacted under postembedding conditions with the anti-GABA serum after it had been preincubated with the following compounds attached to a solid-phase carrier: D,L-γ-amino-β-hydroxybutyric acid (**A**), L-glutamate (**B**), GABA (**D**), β-alanine (**E**), or reacted with the same serum without adsorption (**C**). Note that while adsorption to GABA abolishes staining, adsorption to the other substances, although it may weaken staining, does not change the staining pattern; the same neurons (e.g., arrows) remain immunoreactive. (**F–K**) coexistence of CCK and GABA immunoreactivities in the same neuron of the cat's hippocampus as demonstrated in 1 μm thick serial sections under postembedding conditions. The sections were incubated with: (**F**) antiserum to CCK preincubated with GABA; (**G**) antiserum to CCK preincubated with CCK; (**H**) antiserum to CCK; (**I**) antiserum to GABA preincubated with CCK; (**J**) antiserum to GABA preincubated with GABA; (**K**) antiserum to GABA. One neuron (vertical arrow) contains both substances, the other two (horizontal arrows) contain only GABA and the two sera reveal different antigens. (**A–E**) Bar = 50 μm; (**F–K**) bar = 20 μm.

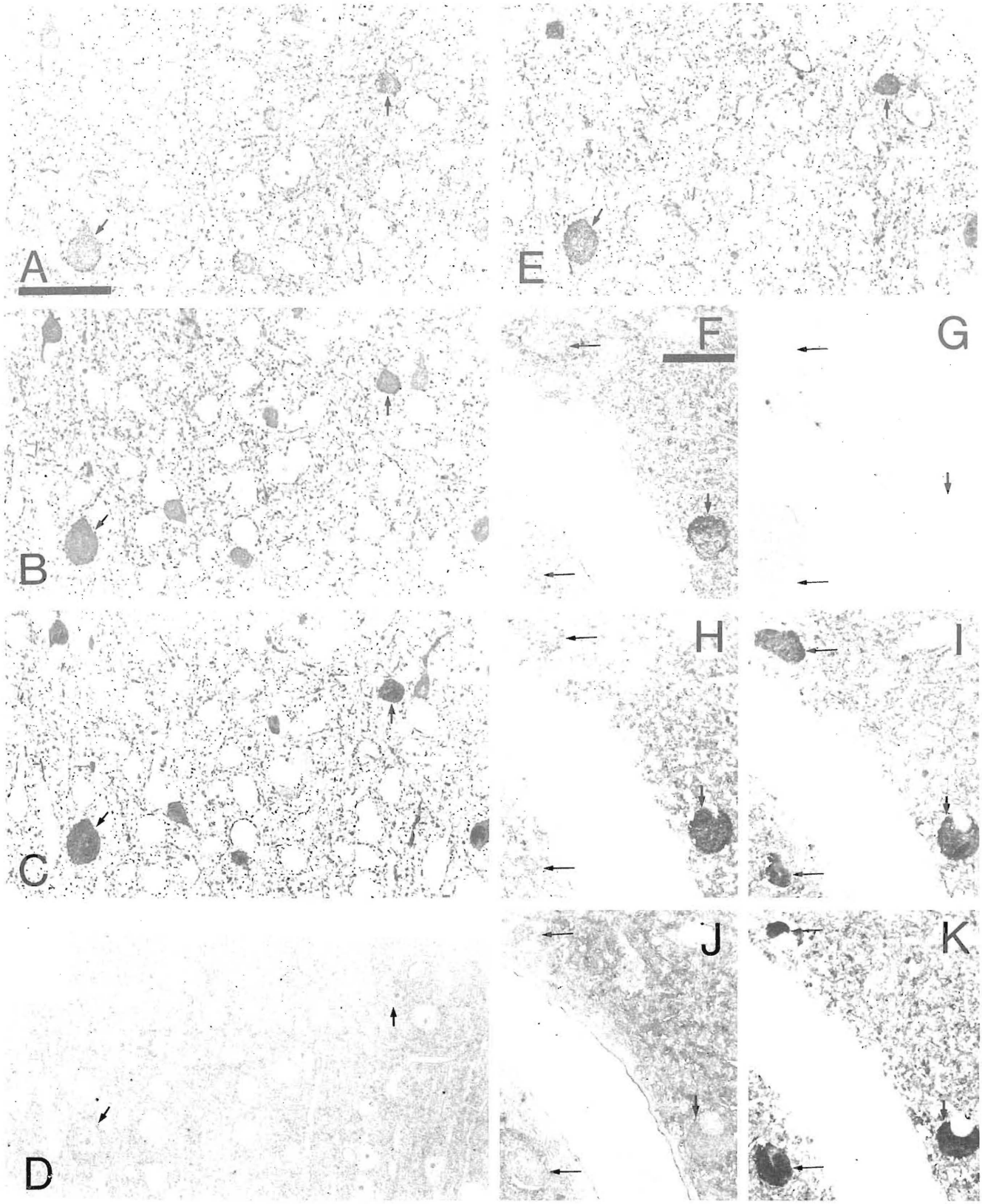

Figure 3

ferent from GABA. This is important to establish because although β-alanine and GABOB are present in much lower concentration in brain than GABA (see previous article for reference (10)), they could be concentrated in a few cells, resulting in immunostaining.

Glutamate, which is related in structure to GABA, has been shown to have a transmitter role in the brain areas we have studied (see ref. 6). Adsorption to glutamate did not appreciably affect the immunostaining in sections. Furthermore types of neuron such as pyramidal cells and cerebellar granule cells, which are believed to use glutamate as transmitter (for review see ref. 6), showed no immunoreactivity in their soma or in their terminals with the anti-GABA serum.

All neurons considered to use GABA as transmitter in the cerebellum (11,15,16,24) were immunoreactive for GABA in this study. It has been suggested that the transmitter of some stellate cells in the guinea pig could be taurine (18). The present results indicate that GABA is present in most if not all stellate cells in the cat. However, it cannot be excluded that a small subpopulation may use taurine as their transmitter or that some stellate cells may use both GABA and taurine. Whichever possibility turns out to be true, it is clear that our antisera do not cross-react with taurine in the model system (10) nor does solid phase adsorption of the sera to taurine affect the immunostaining of stellate cells in tissue sections. Purkinje cells are thought to be inhibitory (5) and use GABA as their transmitter (7). Recently however, it has also been suggested that not all of them are GABAergic (3). In the present experiments most Purkinje cells stained for GABA but the staining was usually much weaker than that of the cortical interneurons. This may indicate that different types of neurons metabolize GABA differently, resulting in a lower concentration of the amino acid in the cell bodies of some types.

Subcellular Localization

The peroxidase reaction-end product indicating GABA immunoreactivity was distributed like that commonly found with neuroactive peptides (22) as well as with small transmitters like serotonin (13,19). In all such cases the peroxidase reaction end product was attached to all intracellular organelles and was not present in the lumen of the small synaptic vesicles. The nonvesicular staining could result from the displacement and subsequent fixation of GABA to all organelles in the terminal during the processing of the tissue. In contrast, the core of the large granulated vesicles was immunoreactive for GABA as it was for GAD (29) neuroactive peptides (e.g., ref. 22) and serotonin (13).

The nuclei of GABA-positive cells stained very strongly, as in the previous GABA immunohistochemical study (30). This is in agreement with heavy labeling of nuclei in cells that selectively accumulate [^{3}H]GABA (11). The significance of this immunoreactivity is not yet clear, and biochemical experiments will be required to determine the molecular nature of the immunoreactive molecule(s).

Effects of Fixation on Immunostaining

For the autoradiographic detection of exogenously applied GABA, high concentrations of glutaraldehyde have usually been used as fixative (11). In the present experiments glutaraldehyde was a component of the fixative; low concentrations gave satisfactory results for GABA immunocytochemistry, and the immunoreactivity only slightly improved with higher concentrations. This indicates that the low concentrations (0.05–0.2%) were equally effective provided that fixation of the cells was rapid. The potency of glutaraldehyde in its ability to fix free amino acids is in accord with studies showing that this bifunctional agent is much more effective than the monofunctional agent, formaldehyde, in fixing amino acids into tissue (21).

The possibility of using such low concentrations of glutaraldehyde for the immunocytochemical demonstration of GABA proved useful when we attempted to localize neuroactive peptides in the GABA-positive cells as many of the peptides are sensitive to glutaraldehyde fixation. These results have been described elsewhere (28), hence only an example has been included here to illustrate the usefulness of the anti-GABA sera for studies of the coexistence of several neuroactive compounds in the same cell. Since GABA can also be demonstrated under conditions that are suitable for the immunocytochemistry of proteins such as GAD (28), it will also be possible to carry out immunohistochemical double labeling paradigms (31).

In the following study (26) we demonstate the use of the anti-GABA sera under conditions that allow the characterization of immunoreactive neurons in much greater detail than current methods.

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