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Original Articles

Antisera to γ-Aminobutyric Acid.

III. Demonstration of GABA in Golgi-impregnated Neurons and in Conventional Electron Microscopic Sections of Cat Striate Cortex[1]

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Two methods are described for the immunocytochemical demonstration of immunoreactive γ-aminobutyric acid (GABA) in the visual cortex of the cat, an area that contains several types of GABAergic neurons and requires combined methods for their characterization. The first method is illustrated by a representative example of a Golgi-impregnated and gold-toned interneuron of the "bitufted" type situated in layer VI and having an ascending axon. After recording the three-dimensional features of the cell, semithin (0.5 μm) sections of the perikaryon were cut and GABA was demonstrated in the cell body by the unlabeled antibody enzyme method. While immunocytochemistry was used to determine the probable transmitter of the neuron, Golgi-impregnation of the same cell was used to identify its neuronal type. Since aldehyde–osmium fixation was used, further electron microscopic (EM) analysis of the neuron's synaptic connections was possible. The second procedure demonstrated GABA in EM sections of aldehyde–osmium-fixed cortex using protein A–gold as an immunocytochemical marker. Immunoreactivity was found in certain neurons, dendrites, axons, and boutons forming type II synaptic contacts that from previous studies have been thought to be GABAergic. Thus ultrastructural analysis using optimal conditions can now be supplemented with the identification of the transmitter in the same section.

KEY WORDS γ-Aminobutyric acid; Postembedding immunocytochemistry; Electron microscopic application of protein A–gold; Golgi impregnation; Visual cortex of cat; Identification of interneurons; GABAergic interneurons.

Introduction

The immunochemical (12) and immunohistochemical (26) characterization of antisera produced by immunization with γ-aminobutyric acid (GABA) coupled to a carrier has been described in the preceding articles. It was concluded that the immunoreactivity was produced by GABA fixed into the tissue section and the distribution of GABA immunoreactivity agreed well with that of glutamate decarboxylase (GAD) in the cerebellar and cerebral cortices, and in the hippocampus. In this article we explore the usefulness of these antisera in localizing GABAergic neurons in complex neuronal networks.

Some areas of the central nervous system contain more than one type of GABAergic neuron, as defined by their input, output, and neurochemical properties. One such area is the cerebellar cortex where there are at least four types of GABAergic neurons, all immunoreactive for GAD (17, 23), that can be identified on the basis of their position and morphological characteristics. The situation is more complicated in other areas such as the neostriatum and cerebral cortex where only a combination of methods can identify particular types of neurons. Two methods have been developed and are in current use. Both use rapid Golgi impregnation, one of the best and most widely employed procedures for the morphological characterization of neurons. In the first method the selective accumulation of exogenously applied [^{3}H]GABA (24) or [^{3}H]muscimol (8) demonstrated in the impregnated neurons by autoradiography serves as the criteria to establish that they used GABA as transmitter. In the second method the immunocytochemical demonstration of GAD within the Golgi-impregnated neuron (6, 25) or in its identified terminals (7) is taken as evidence for the GABAergic nature of the neuron. Both methods have several drawbacks (see Discussion) and require considerable experience so that their general use is limited.

This was one of the reasons that prompted us to develop

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antisera to GABA (12) that we hoped would allow the use of processing conditions more amenable to combined procedures (see Discussion) but that would simultaneously decrease the immunoreactivity of proteins such as GAD. In the previous study (26) we demonstrated that GABA can readily be visualized in osmium-treated sections, a treatment required for the rapid-Golgi procedure. In the first part of the present study we demonstrate that GABA immunoreactivity can also be visualized in Golgi-impregnated and gold-toned neurons.

Electron microscopic (EM) analysis of synaptic microcircuits has contributed greatly to our understanding of the structure of the brain. During such an analysis it would be useful if neuronal processes of interest could be characterized with respect to their neurotransmitter(s). Most material is usually not processed for immunocytochemistry of substances that may become of interest after the tissue has been fixed and embedded, hence the demonstration of such a substance requires postembedding immunocytochemistry on plastic-embedded thin sections. Most brain specimens are routinely fixed with high concentrations of aldehydes and osmium, which give the greatest fine structural detail. A method that allows the demonstration of transmitters in such EM sections could have widespread application. In the second part of this article GABA immmunoreactivity is demonstrated in routine ultrathin sections with the protein A–colloidal gold method. A preliminary report of some of these results has appeared (11).

Materials and Methods

Two procedures are described; the GABA–Golgi method for the demonstration of GABA in Golgi-impregnated neurons, and the GABA–gold method for the demonstration of GABA in conventional osmium-treated EM sections. Both have been successfully applied to different areas of the brains of rats, cats, monkeys, and humans. The visual cortex of the cat was chosen to illustrate both methods because of the wealth of information available on GABAergic neurons in this area and the need for more sophisticated techniques for their characterization.

GABA–Golgi Method

Animals and preparation of tissue sections. Two cats were anesthetized and perfused with a fixative containing 2.5% glutaraldehyde (TAAB Lab. Equip. Ltd. Reading, U.K.) and 1% depolymerized paraformaldehyde (TAAB Lab. Equip. Ltd.) dissolved in O.1 M sodium phosphate buffer, pH 7.2–7.4. After removing the brain, blocks of the lateral gyrus containing area 17 were dissected and processed by the rapid-Golgi impregnation procedure as described previously (6). The sections were then gold-toned, dehydrated, and embedded in Durcupan ACM (Fluka) resin on slides (6). Golgi-impregnated and gold-toned neurons were selected and drawn with the help of a drawing tube, photographed, and reembedded into polyethylene capsules for further sectioning (25). Semithin (0.5 μm thick) serial sections were cut from the perikarya of the Golgi-impregnated neurons and dried on a hotplate onto slides coated with egg white. Care was taken to have at least three sections of the perikaryon, including the nucleus of the cell, on different slides and to keep as much as possible of the cell and its processes in the block for further EM studies.

Immunocytochemistry. The slides with the semithin sections of the identified Golgi-impregnated and gold-toned neurons were kept overnight at 56° C. The resin was etched using ethanolic sodium hydroxide (15) for 20–30 min. Subsequently they were washed in three changes of absolute ethanol for 5 min each. This was followed by two 5 min washes in distilled water and a 7 min treatment with 1% freshly prepared sodium metaperiodate ($NaIO_4$) to remove the osmium tetroxide from the sections. Following two washes in distilled water, the slides were kept in Tris (10 mM)–phosphate (10 mM) buffered isotonic saline (TPBS), pH 7.4, two changes for 10 min each.

Thereafter, the sections were incubated in a humid chamber at room temperature using the unlabeled antibody–enzyme method (28), with the following sequence of reagents layered over the slides: 20 min in 20% heat inactivated, normal sheep serum; rinsed in TPBS; 2 hr in primary antisera to GABA (code no. GABA-7 or GABA-9) or the same sera after adsorption, all diluted 1:2000; three 10 min washes; 40 min in sheep anti-rabbit immunoglobulin G (IgG) fraction (Silenus Lab. Ltd. Dandenong, Australia) diluted 1:30; three 10 min washes; 1 hr in rabbit peroxidase–antiperoxidase complex (PAP; Dakopatts Ltd., Denmark) diluted 1:100. All sera were diluted with TPBS containing 1% heat inactivated normal sheep serum.

Following incubation the slides were reacted for peroxidase enzyme activity using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) as substrate, the reaction end product was intensified with OsO_4, and following dehydration, the sections were covered with XAM neutral mounting medium (BDH, Poole, U.K.) in accord with previously described procedures (27).

GABA–Gold Method on EM Sections

Preparation of tissue sections. Other cortical blocks from the same cats were postfixed for 2 hr in 1% OsO_4 dissolved in 0.1 M phosphate buffer (PB, pH 7.4), washed in 0.1 M PB, dehydrated, and embedded in Durcupan ACM resin. To enchance contrast 1% uranyl acetate was added to the 70% ethanol. Serial EM sections with light yellow interference color were cut and mounted in pairs on Formvar- (Ladd Res. Industries, Burlington, VT) coated single slot 2 × 1 mm) grids. Every second pair of sections was placed on Ni grids and processed for immunocytochemistry; the rest were put on Cu grids and were contrasted with lead citrate (20).

Immunocytochemistry. Without etching of the resin and removal of the osmium no immunoreactivity could be seen. Therefore we experimented with several methods and finally adopted two that provided satisfactory results. In procedure GABA–gold I, the grids were floated on drops of 1% periodic acid (H_5IO_6) for 4 min, followed by three washes in distilled water, and 6 min in 1% freshly prepared $NaIO_4$. Periodic acid and sodium metaperiodate have been recommended for removal of osmium (1) and this combination gave the best result in our hands. After washing in three changes of distilled water and two changes of TPBS the grids were processed for immunocythochemistry. To avoid contamination they were not dried either during the above procedure or during the subsequent steps.

In procedure GABA–gold II, the grids were placed on Parafilm with the sections facing up and they were covered for 6 min with a few drops of sodium ethanolate diluted 1:1 with absolute ethanol. Sodium ethanolate usually was made 2 days earlier by placing sodium hydroxide pellets into absolute ethanol (15). the Parafilm with the grids was kept in a petri dish saturated with ethanol vapor to inhibit the formation of crystals on the surface of sodium ethanolate droplets. Following etching of the resin, the grids were removed, taking care to avoid their contamination by crystals from the surface of the ethan-

olate droplet, and washed quickly in three changes of absolute ethanol and dried. To avoid contamination the grids were not dried again until the end of the incubation. The grids were floated on droplets of 1% freshly prepared $NaIO_4$ for 7 min. Subsequently the grids were washed in three changes of distilled water and two changes of TPBS and processed for immunocytochemistry by floating the grids on drops of Millipore-filtered (0.22 μm pore) reagents placed on Parafilm. The sequence was the following: 3% normal sheep serum or 1% ovalbumin for 10 min.; rinse in TPBS; antiserum to GABA (code nos. GABA-7 and GABA-9) diluted 1:1000 or 1:2000; three rinses in TPBS followed by 10 min in TPBS; protein A-coated colloidal gold diluted as described below; two rinses in TPBs, followed by 10 min in TPBS; three rinses in distilled water; then the grids were dried. Colloidal gold (diameter ca 16 nm, (5)) was coated with protein A (Pharmacia) (13, 22) and diluted in TPBS containing 0.05% polyethylene glycol (carbowax, mol wt 15,000–20,000; Sigma) to give an absorbance between 0.250 and 0.300 at 540 nm wavelength using a 1 cm path length.

GABA–Gold Method on Semithin Sections

Some semithin sections were reacted with a protein A–gold–silver intensification procedure. After incubation in the primary antiserum and washing, the sections were covered with protein A-coated colloidal gold (see below) for 2 hr and washed in TPBS. They were then reacted by a silver intensification method (2) in which the silver lactate was replaced by an equivalent concentration of silver nitrate. This method is useful in the preliminary testing for immunoreactivity for a large number of specimens prior to the more laborious cutting and reacting of EM sections.

Immunohistochemical Controls

For the GABA–Golgi procedure controls included: i) the full sequence with the anti-GABA serum being replaced by normal rabbit serum of the same dilution; ii) a sequence omitting the anti-GABA serum; iii) the full sequence but the anti-GABA serum (either GABA-7 or GABA-9) was incubated overnight with GABA coupled to polyacryalmide beads as described in the previous article (12).

Controls for the GABA–gold procedure included the incubation of EM sections on grids in a sequence omitting the anti-GABA serum, or using GABA-absorbed anti-GABA serum as described in control variant iii.

Results

Golgi Impregnation

The perikarya of Golgi-impregnated, gold-toned neurons were transparently grey due to the metallic gold deposited along the plasma membrane (4). When such a neuron was cut into semithin sections the gold appeared as a grey rim along the plasma membrane, leaving the interior of the neuron, including the nucleus, free of deposit (Figure 1D,F). This allowed the visualization of immunoreactivity within the cell. The processes of the cell were darker, thin processes being black. All fine detail, including dendritic spines, fine axon collaterals, and boutons, could be traced in the light microscope. The neuron illustrated in Figure 1 was selected from a sample of 47 GABA-immunoreactive neurons identified by Golgi impregnation. It was situated in layer VI and was drawn from a single 90 μm thick section. The perikaryon was of a spindle shape with its long axis oriented radially. Two groups of dendrites emerged from the poles of the soma, giving the cell the characteristic "bitufted" or "bipolar" appearance. They were covered sparsely by spines and appendages, and the soma emitted a few spines. The initial segment of the axon originated from the upper dendritic shaft and branched several times within the first 50 μm of its course into ascending branches that further divided. The axon formed an ascending array of eight collaterals crowded with boutons and emitting bouton-laden side branches. The ascending collaterals could not be followed beyond layer IV, since they were cut at the surface of the section, as were many of the side branches. Although this meant that the reconstruction of the neuron was not complete, nevertheless, the Golgi impregnation made it possible to follow the neuron over 1 mm dorsoventrally and over 600 μm laterally.

GABA Immunoreactivity in Golgi-impregnated and Gold-toned Neurons

Both antisera gave an equally good reaction (Figure 1). Immunoreactivity could readily be demonstrated in the semithin sections (0.5 μm) cut from the thick (90 μm) Golgi-impregnated section (Figure 1C,E). This meant that none of the numerous chemical treatments in the Golgi-impregnation and gold-toning steps affected the antigen. GABA-immunoreactive neurons, including their nucleus, but not the nucleolus, were homogeneously dark brown. The same density of peroxidase reaction end product was also present in the perikaryon of the gold-toned neuron (Figure 1C,E). In some other gold-toned neurons (not illustrated) the immunoreactivity was attenuated in the cytoplasm if the gold deposit was very dense. Since the nucleus usually was not penetrated by the Golgi precipitate and consequently had no gold deposit, its GABA immunoreactivity was the same in impregnated and nonimpregnated neurons. The peroxidase reaction end product in the perikarya of the gold-toned neurons was not the result of a chemical reaction nor was immunoreactivity induced in any other way by the Golgi procedure as evidenced by the fact that the perikarya of gold-toned pyramidal neurons, which are not GABAergic, were never immunoreactive for GABA (Figure 1C). The neuropil was full of small immunoreactive blobs representing the boutons, axons, and possibly dendrites of GABAergic neurons. The perikarya of both GABA-positive and negative neurons were surrounded by similar immunoreactive varicosities (Figure 1C,E). The density, distribution, and type of GABA-immunoreactive structures was no different from that described in the previous article (26).

Demonstration of GABA Immunoreactivity by the Silver Intensification of Protein A–gold Labeling

After immunoreaction the colloidal gold that was selectively deposited over the GABA-positive structures could not be detected in the light microscope. Following silver intensifi-

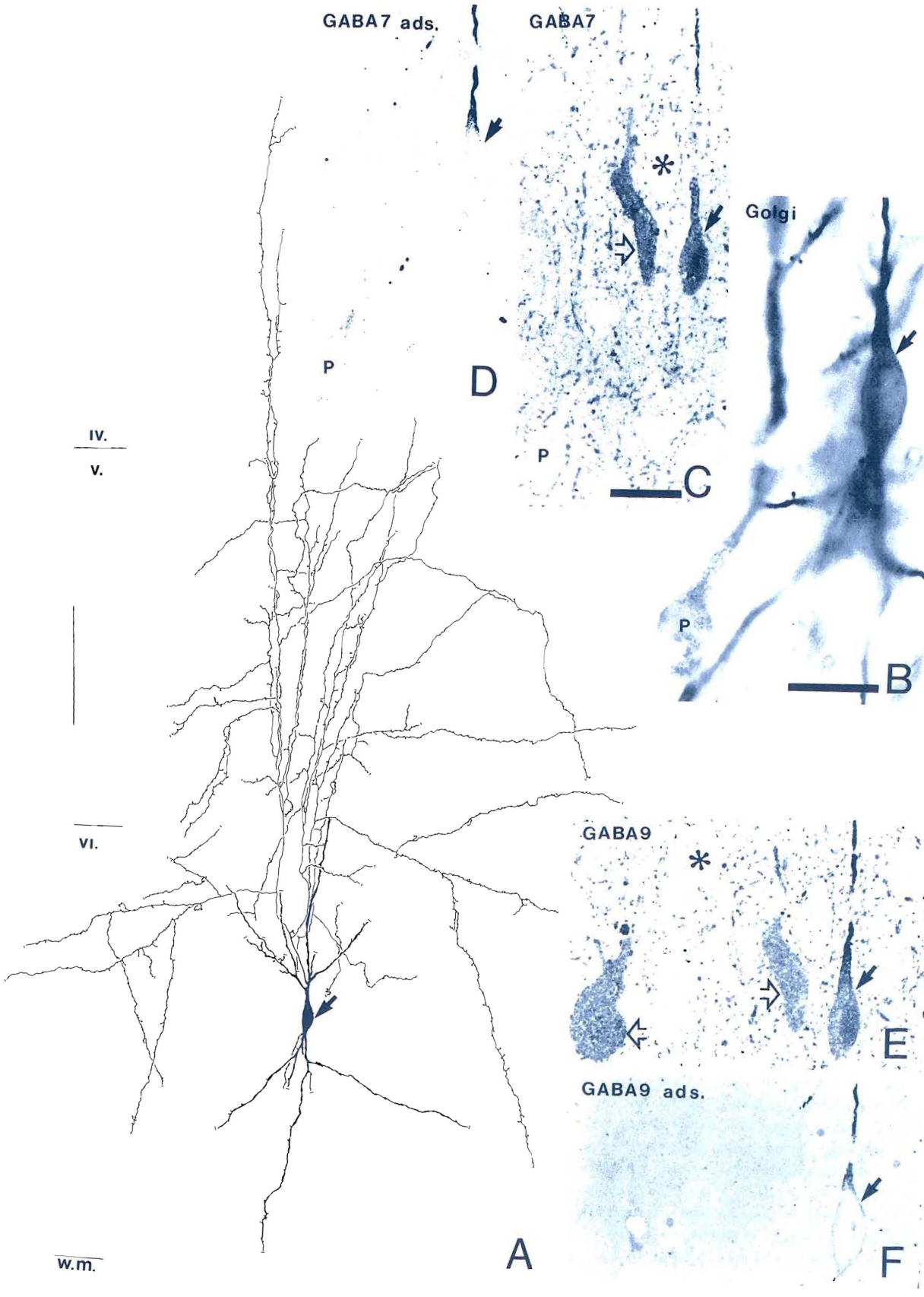

Figure 1

Figure 1. (A) Camera lucida drawing of a Golgi-impregnated "bitufted" neuron in layer VI of the striate cortex of cat. The impregnation allowed the tracing of the extensive, ascending axon arborization. (B) Light micrograph of the same neuron (arrow) and a nearby impregnated pyramidal cell (P) as seen in the 90 μm thick section after gold toning. (C–F) Serial semithin (0.5 gmm) sections cut from the same thick section shown in B and reached by the unlabeled antibody–enzyme method with two different anti-GABA sera (no. 7 in C, and no. 9 in E) or with the same sera following solid phase adsorption to GABA (D and F). Note that the perikaryon of the "bitufted" neuron (arrow) is immunoreactive along with two other neurons (open arrows in E). Many neurons (e.g., asterisks), including the gold-toned pyramidal cell (P in C and D), are surrounded by GABA-immunoreactive boutons that are also present in the neuropil. Following adsorption only the thin rim of gold marks the impregnated neurons. (A) Bar = 100 μm; (B) bar = 20 μm; (C–F) bar = 20 μm.

cation, however, the GABA-positive neurons became visible, turning grey or black as a result of silver deposition (Figure 2A). The GABA-positive boutons around perikarya and in the neuropil were also black. The pattern was no different from that obtained by the unlabeled antibody–enzyme method (26).

GABA Immunoreactivity in Conventional EM Sections

Labeling of neuronal profiles with colloidal gold particles corresponded to that predicted from light microscopy of silver-intensified sections (Figure 2A). The most frequently immunolabeled elements were synaptic boutons, establishing Gray's type 2 (9) synaptic contacts (Figures 2B–F, 3B,C). The postsynaptic elements were pyramidal cell perikarya (Figure 3B,C), nonpyramidal cell perikarya, identified by their afferent Gray's type I synaptic contacts, non-pyramidal and pyramidal cell dendrites (Figure 2B–F), dendritic spines, and axon initial segments. The gold particles, representing GABA-immunoreactive sites, were accumulated over the synaptic vesicles and mitochondria (Figure 2D,E). Mitochondria were labeled only in immunoreactive profiles (Figures 2D,E; 3B,C). Many axons were also labeled by gold, these included large and small myelinated axons, which were very heavily labeled and most numerous in layer IV. A minority of the neuronal perikarya, consisting entirely of non-pyramidal cells, were also heavily labeled (Figure 3A); the gold was distributed homogeneously over the whole neuron, including the dendrites originating from it. Some isolated dendritic profiles in the neuropil were also labeled. Overall the types of immunoreactive elements matched those observed in the preembedding immunoreacted material. By reacting serial EM sections of the same area it was found that the same gold-labeled profile was equally immunoreactive in all sections (Figure 2D,E). Pyramidal cells, their dendrites, boutons forming Gray's type 1 synaptic contacts, and glial cells were not labeled. These elements had gold particles scattered over them at a much lower density than labeled profiles. The structural detail of EM sections treated in the procedure using periodic acid/sodium metaperiodate treatment was superior to that observed in sections treated with sodium ethanolate/sodium metaperiodate. The sodium ethanolate etching of sections was difficult to control and there was variation from experiment to experiment. On the other hand this procedure usually resulted in lower background staining. The use of alternate sections for immunocytochemistry and normal ultrastructural observation proved invaluable in identifying the synaptic connections of immunoreactive elements.

Specificity of the Immunohistochemical Reaction

In the GABA–Golgi procedure no peroxidase reaction end product, indicating immunoreactivity, was observed when: i) the anti-GABA serum was replaced with normal rabbit serum of the same dilution; ii) the anti-GABA serum was omitted; iii) prior to the reaction the anti-GABA serum was incubated overnight with GABA coupled to polyacrylamide beads (Figure 1A,F). In the GABA–gold procedure when the anti-GABA serum was preincubated with GABA coupled to polyacrylamide beads, or when the anti-GABA serum was replaced by normal rabbit serum of the same dilution, only scattered gold particles were found over the tissue. Accumulation of gold was occasionally observed over empty resin, both under the above conditions and when the unadsorbed anti-GABA serum was used. This represents a nonimmunoreactive attachment of gold to resin that we cannot explain.

Discussion

The GABA–Golgi Method

The direct demonstration of immunoreactive GABA in a Golgi-impregnated neuron showed that some GABAergic neurons in layer VI have an axon ascending into superficial layers, and at least some of these neurons have the "bitufted" or "bipolar" dendritic arborization. One neuron was used to illustrate the method but other examples (unpublished) show that its features are representative of a class of cells defined by their position and both dendritic and axonal arborization. In the cat visual cortex this is the second identified GABAergic neuron type besides the axo-axonic cell (7) that terminates specifically on the axon initial segments of pyramidal neurons. Strong indirect evidence also exists that a third neuronal group, the various basket cells, also use GABA as a transmitter (see discussion in refs. 7 and 10). The transmitters of scores of other types of cortical neurons described in previous Golgi studies (e.g., refs. 19, 29, and 30) are not known, but the procedure discussed here offers the possibility to determine which ones are GABAergic. The method in our hands has proved superior to all previous immunohistochemical or other combined procedures for the morphological and neurochemical characterization of neurons.

Immunohistochemistry for GAD (10,17,21) or GABA (26) does reveal isolated perikarya and boutons; however, in a system like the cortical network where several types of neurons, differing in connectivity, use the same transmitter, an additional marker, in this case gold particles, has to be used to connect the boutons and processes to the parent soma. We have previously developed two procedures for tackling the

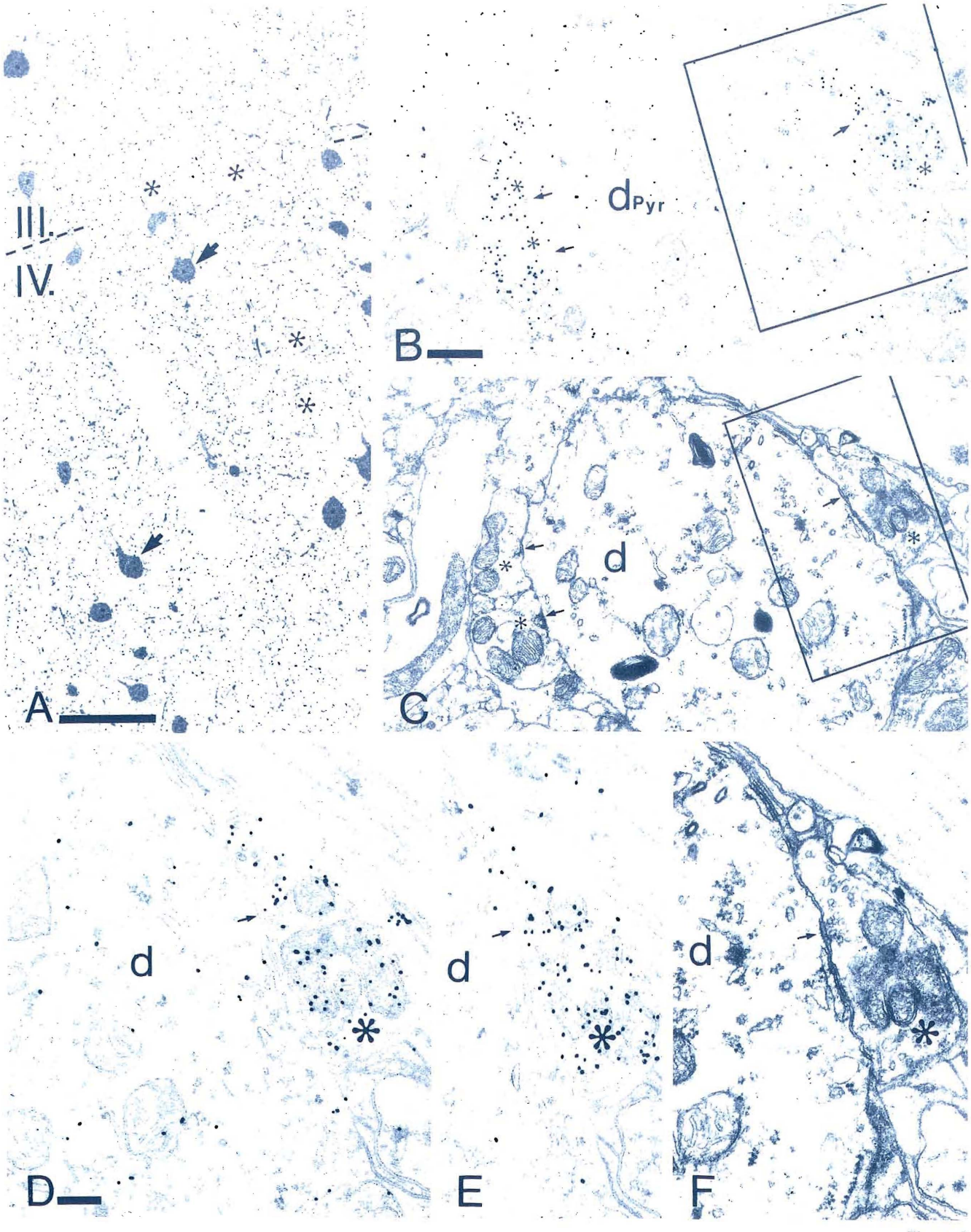

Figure 2

Figure 2. (**A**) Semithin (0.5 μm) section of the cat's striate cortex at the border of layers III and IV reacted for GABA by the protein A–gold–silver intensification method. Immunoreactive neurons are black, others (e.g., asterisks) are surrounded by immunoreactive dots representing GABAergic nerve terminals. (**B** and **C**) Serial EM sections of a pyramidal cell basal dendrite (d_{Pyr}) receiving synaptic contacts (arrow) from three boutons (asterisks). The section in **B** was reacted to reveal GABA by the protein A–gold method I. The accumulation of gold over the boutons demonstrates that they are GABAergic. Scattered gold over the pyramidal cell dendrite probably represents background. Boxed areas in **B** and **C** are shown at higher magnification in **D** and **F**, respectively. (**E**) Serial section of the same bouton showing that the GABA immunoreactivity can be demonstrated from section to section. Only the section shown in **C** and **F** was stained with lead citrate. (**A**) Bar = 50 μm; (**B** and **C**) bar = 0.5 μm; (**D–F**) bar = 0.2 μm.

same task; in both of them the Golgi impregnation of the neurons served to characterize the dendritic and axonal arborizations, i.e., to identify the neuronal types. In the first method the selective accumulation of exogenously applied [^{3}H]GABA and its subsequent autoradiographic demonstration in the impregnated neuron was used to identify the putative transmitter (24). This procedure had the disadvantage that it relied on an exogenous marker injected into the tissue and we have found recently (14), at least in the monkey visual cortex, that not all of the neurons accumulating [^{3}H]GABA, were immunoreactive for GABA. This raises the possibility that some of the [^{3}H]GABA accumulating cells do not store and use endogenous GABA as a transmitter. The other limitation of the method was that the Golgi method randomly impregnates less than 1% of the cells present. The probability of impregnating a neuron that accumulated [^{3}H]GABA in the small radio-labeled area was therefore low. These problems are overcome by the present procedure as it reveals the endogenous transmitter, and any impregnated neuron can be tested for immunoreactivity anywhere in the section as well as in gold-toned Golgi material fixed and embedded years ago.

The second combined method was based on immunocytochemical demonstration of GAD and the subsequent Golgi impregnation of the same neuron (25), and provided direct immunocytochemical evidence for the GABAergic nature of the axo-axonic cell (7). One limitation of the procedure was the poor penetration of some antisera into the 70–90 μm thick Vibratome sections used in the immunocytochemistry. This was overcome in the present method by using 0.5 μm thick sections, hence eliminating penetration barriers. The other limitation was again the low chance of the random Golgi impregnation coinciding with the immunoreactivity, which was overcome by the GABA–Golgi method as all impregnated neurons could be tested for immunoreactivity.

It should be emphasized, however, that the section–Golgi-impregnation procedure combined with preembedding immunohistochemistry (7,25) can be used to demonstrate afferent, neurochemically characterized, synaptic connections of identified neurons. The GABA–Golgi method demonstrates the transmitter in the impregnated neuron, but whether boutons making synapses on the same neuron are also GABAergic cannot be established because the immunocytochemistry is carried out at the light microscopic level. The combination of the GABA–Golgi method with the GABA–immunogold method could solve this limitation. Another advantage of the section–Golgi–immunocytochemistry combination is that it can be applied to any antigen that can be demonstrated in the preembedding methods. With the present postembedding combination we have so far succeeded in demonstrating only GABA, probably because the other antigens, mainly peptides and proteins, that we have tried are more sensitive to the processing conditions of the Golgi impregnation.

The great advantages of the present method over so-called "Golgi-like" immunohistochemical staining are that: i) it provides superior detail, and reveals the processes of the neuron over a larger area; ii) it shows the relationship of the neuron to other neurons that are morphologically and possibly neurochemically different; and iii) since optimal EM fixation is used, the synaptic connections of the gold-toned processes can be identified at the EM level (4).

The GABA–Immunogold Method

As suggested by several reviews (3,22,31), the postembedding immunogold techniques have a promising future in the analysis of complex neuronal networks. This is because the demonstration and interpretation of synaptic connections between neurons requires both electron microscopic resolution with good structural preservation and the identification of transmitters mediating the interaction. To our knowledge this is the first direct demonstration of a non-peptide transmitter in conventional osmium-treated EM sections. The quality of the fine structurel detail, especially with the strategy of using alternate serial sections, is comparable to that considered optimal for the central nervous system (18). The method has tremendous potential, because if it can be applied to other antigens, as has already been shown in the periphery and in the neuronendocrine cells (for review see refs. 3, 22, and 31), then double labeling experiments, using different sizes of gold for each antigen, can unravel the connectivity underlying complex neurochemical interactions. Another advantage is that we have been able to use specimens embedded several years ago. Thus the immunocytochemical analysis of such unique specimens as intracellularly filled and electrophysiologically characterized neurons embedded in EM resin (16) can now be carried out retrospectivevly. The postembedding immunogold method can also be combined with the preembedding demonstration of other antigens using, e.g., the unlabeled antibody–PAP method (28).

The potential of the two procedures has been demonstrated in the visual cortex and future studies will show how widely these techniques can be applied to other areas. In our hands both methods have worked reliably in different brain areas of several species. If the immunocytochemical demonstration of GABA proves to be a good example, it is very likely that in a short time speculations based on tentative fine structural criteria for the neurochemical and functional identity of synaptic connections will be tested directly.

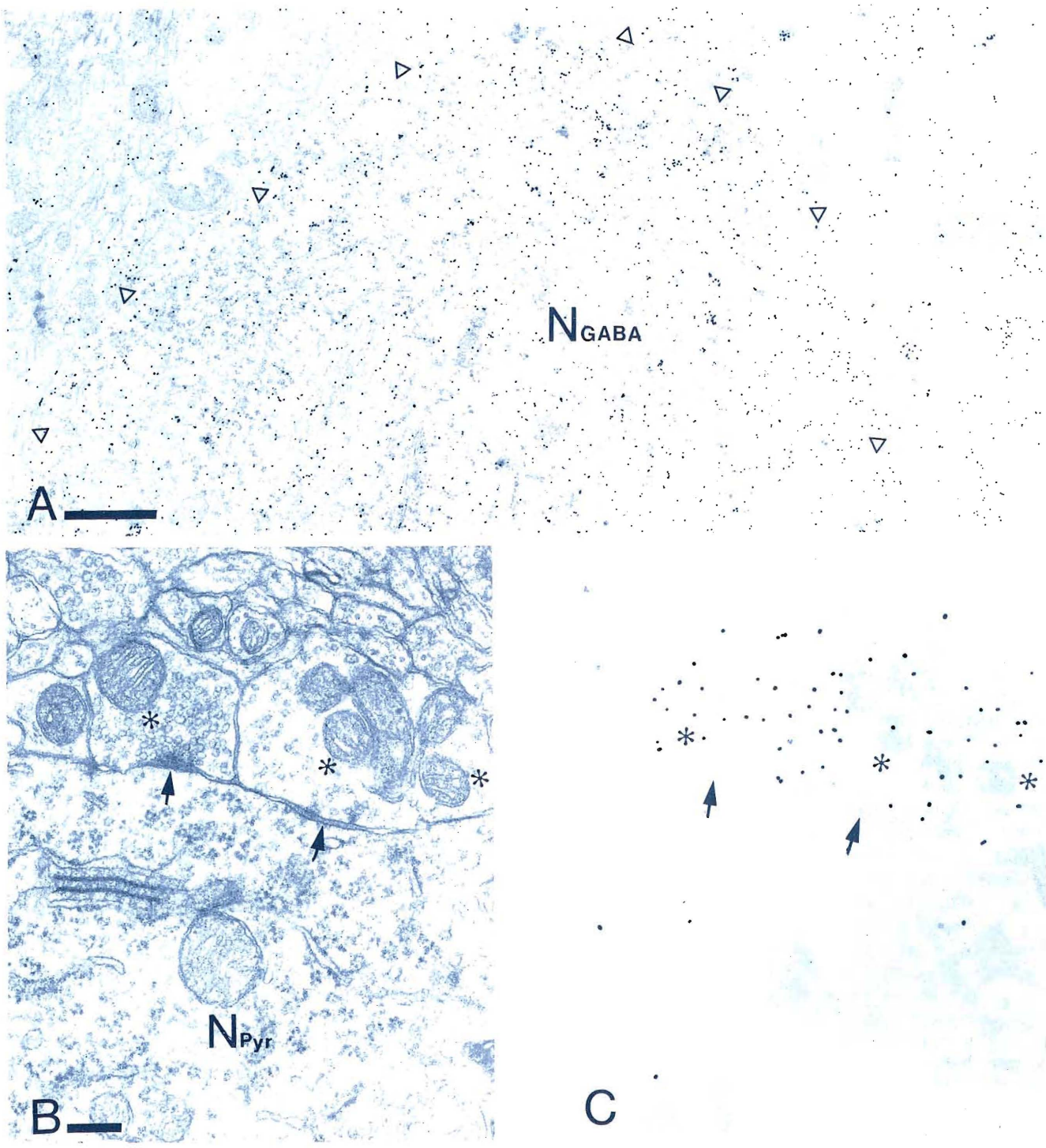

Figure 3. (**A**) Immunoreactive neuron (N_{GABA}) in layer III of the cat's striate cortex visualized by the protein A–gold method I. Triangles indicate the boundary of the neuron. Accumulation of gold outside this neuron represents specific staining of elements of the neuropil. (**B** and **C**) Serial sections of boutons (asterisks) making type II synaptic contacts (arrow) with a pyramidal cell (N_{Pyr}) in the striate cortex of cat. Section shown in **C** was reacted with the protein A–gold post-embedding procedure II. The selective accumulation of gold particles over the boutons demonstrates that they are GABAergic. The gold particles over the pyramidal cell soma probably represent background staining. Section shown in **B** was not reacted for GABA but was stained with lead citrate. (**A**) Bar = 1 μm; (**B**and **C**) bar = 0.2 μm.

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Literature Cited

1. Bendayan M, Zollinger M: Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J Histochem Cytochem 31:101, 1983.
2. Danscher G: Localization of gold in biological tissue. A photochemical method for light and electron microscopy. Histochemistry 71:81, 1981
3. De Mey J: A critical review of light and electron microscopic immunocytochemical techniques used in neurobiology. J Neurosci Meth 7:1, 1983
4. Fairen A, Peters A, Saldanha J: A new procedure for examining Golgi impregnated neurons by light and electron microscopy. J Neurocytol 6:311, 1977
5. Frens G: Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature: Physical Sci 241:20, 1973
6. Freund TF, Somogyi P: The section-Golgi impregnation procedure 1. Description of the method and its combination with histochemistry after intracellular iontophoresis or retrograde transport of horseradish peroxidase. Neuroscience 9:463, 1983
7. Freund TF, Martin KAC, Smith AD, Somogyi P: Glutamate decarboxylase-immunoreactive terminals of Golgi-impregnated axo-axonic cells and of presumed basket cells in synaptic contact with pyramidal cells of the cat's visual cortex. J Comp Neurol 221:263, 1983
8. Goebel DJ, Pourcho RG: Morphological and neurochemical characterization of individual retinal neurons: a combined Golgi and autoradiographic technique. J Neurosci Meth 6:295, 1982
9. Gray EG: Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. J Anat 93:420, 1959
10. Hendry SHC, Houser CR, Jones EG, Vaughn JE: Synaptic organization of immunocytochemically identified GABA neurons in the monkey sensory-motor cortex. J Neurocytol 12:639, 1983
11. Hodgson AJ, Erdei A, Penke B, Chubb IW, Somogyi P: Antiserum to GABA: characterization and immunocytochemical use on cat, monkey, and human brain to identify GABA-ergic neurons. Soc Neurosci Abstr, in press
12. Hodgson AJ, Penke B, Erdei A, Chubb IW, Somogyi P: Antisera to γ-aminobutyric acid. I. Production and characterization using a new model system. J Histochem Cytochem 33:229, 1985
13. Horisberger M, Rosset J: Colloidal gold, a useful marker for transmission and scanning electron microscopy. J Histochem Cytochem 25:295, 1977
14. Kisvárday ZF, Cowey A, Smith AD, Hodgson AJ, Somogyi P: Relationship between GABA immunoreactive neurons and neurons accumulating [^{3}H]-GABA or [^{3}H]d-aspartate in striate cortex of monkey. Trab Inst Cajal Invest Biol LXVV:55, 1984
15. Lane BP, Europa DL: Differential staining of ultrathin sections of Epon-embedded tissues for light microscopy. J Histochem Cytochem 13:579, 1965
16. Martin KAC, Somogyi P, Whitteridge D: Physiological and morphological properties of identified basket cells in the cat's visual cortex. Exp Brain Res 50:193, 1983
17. Oertel WH, Mugnaini E, Schmechel DE, Tappaz ML, Kopin IJ: The immunocytochemical demonstration of gamma-aminobutyric acid-ergic neurons—methods and application. In Cytochemical Methods in Neuroanatomy. Edited by V Chan-Palay, SL Palay. Alan R Liss, Inc, New York, 1982, p 297–329
18. Peters A, Palay SL, Webster HdeF: The Fine Structure of the Nervous System: The Neurons and Supporting Cells. Saunders, Philadelphia, 1976
19. Ramon y Cajal S: Histologie du Systeme Nerveux de l'Homme et des Vertebres. Maloine, Paris, 1911
20. Reynolds ES: The use of lead citrate at high pH as an electron opaque stain in electron-microscopy. J Cell Biol 17:208, 1963
21. Ribak CE: Aspinous and sparsely-spinous stellate neurons in the visual cortex of rats contain glutamic acid decarboxylase. J Neurocytol 7:461, 1978
22. Roth J: The preparation of protein A-gold complexes with 3 nm and 15 nm gold particles and their use in labelling multiple antigens on ultra-thin sections. Histochem J 14:791, 1982
23. Saito K, Barber R, Wu J-Y, Matsuda T, Roberts E, Vaughn JE: Immunohistochemical localization of glutamic acid decarboxylase in rat cerebellum. Proc Natl Acad Sci USA 71:269, 1974
24. Somogyi P, Freund TF, Halász N, Kisvárday ZF: Selectivity of neuronal [^{3}H]-GABA accumulation in the visual cortex as revealed by Golgi staining of the labeled neurons. Brain Res 225:431, 1981
25. Somogyi P, Freund TF, Wu J-Y, Smith AD: The section-Golgi impregnation procedure. 2. Immunocytochemical demonstration of glutamate decarboxylase in Golgi-impregnated neurons and in their afferent synaptic boutons in the visual cortex of the cat. Neuroscience 9:475, 1983
26. Somogyi P, Hodgson AJ, Chubb IW, Penke B, Erdei A: Antisera to γ-aminobutyric acid: II. Immunocytochemical application to the central nervous system. J Histochem Cytochem 33:240, 1985
27. Somogyi P, Hodgson AJ, Smith AD, Nunzi GM, Gorio A, Wu J-Y: Different populations of GABA-ergic neurons in the visual cortex and hippocampus of cat contain somatostatin- or cholecystokinin- immunoreactive material. J Neurosci, in press
28. Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG: The unlabelled antibody-enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315, 1970
29. Szentágothai J: Synaptology of the visual cortex. In Handbook of Sensory Physiology. Edited by R Joung. Springer, Heidelberg, 1973, p 270–321
30. Szentágothai J: Local neuron circuits of the neocortex. In The Neurosciences. Fourth Study Program. Edited by FO Schmitt, FG Worden. MIT Press, Cambridge, MA, 1979, p 399–409
31. Van den Pol AN: Colloidal gold and biotin-avidin conjugates as ultrastructural markers for neural antigens. Q J Exp Physiol 69:1, 1984