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IMMUNOGOLD DEMONSTRATION OF GABA IN SYNAPTIC TERMINALS OF INTRACELLULARLY RECORDED, HORSERADISH PEROXIDASE-FILLED BASKET CELLS AND CLUTCH CELLS IN THE CAT'S VISUAL CORTEX

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Abstract—To identify the putative transmitter of large basket and clutch cells in the cat's visual cortex, an antiserum raised against GABA coupled to bovine serum albumen by glutaraldehyde and a postembedding, electron microscopic immunogold procedure were used. Two basket and four clutch cells were revealed by intracellular injection of horseradish peroxidase. They were identified on the basis of the distribution of their processes and their synaptic connections. Large basket cells terminate mainly in layer III, while clutch cells which have a more restricted axon, terminate mainly in layer IV. Both types of neuron have a small radial projection. They establish type II synaptic contacts and about 20–30% of their synapses are made with the somata of other neurons, the rest with dendrites and dendritic spines.

Altogether 112 identified, HRP-filled boutons, the dendrites of three clutch cells and myelinated axons of both basket and clutch cells were tested for the presence of GABA. They were all immunopositive. The postsynaptic neurons received synapses from numerous other GABA-positive boutons in addition to the horseradish peroxidase-filled ones. Dendritic spines that received a synapse from a GABA-positive basket or clutch cell bouton also received a type I synaptic contact from a GABA-negative bouton. A few of the postsynaptic dendrites, but none of the postsynaptic somata, were immunoreactive for GABA. The fine structural characteristics of the majority of postsynaptic targets suggested that they were pyramidal and spiny stellate cells.

These results provide direct evidence for the presence of immunoreactive GABA in identified basket and clutch cells and strongly suggest that GABA is a neurotransmitter at their synapses. The laminar distribution of the synaptic terminals of basket and clutch cells demonstrates that some GABAergic neurons with similar target specificity segregate into different laminae, and that the same GABAergic cells can take part in both horizontal and radial interactions.

The combination of intracellular recording and marking of neurons with horseradish peroxidase has greatly extended the understanding of cortical circuits.[5,10,11,25,26,29] This approach not only gives an opportunity for direct correlation of electrophysiological and structural data, but also provides a more complete picture of individual neurons than was possible with previous methods. In most cases the interpretation of the possible role of identified neurons would greatly benefit from a knowledge of their biochemical characteristics, especially their transmitters. So far, information about the transmitters of identified cells has largely been deduced from separate neurochemical, pharmacological or histochemical experiments.

This is also the case for the so-called cortical basket cells, that are thought to provide the majority of axosomatic boutons.[17,18,23,24,32,43,44,45] The existence of these neurons, which were assumed to have some selectivity for somata as synaptic target, had been based on conjecture before the intracellular filling studies combined with electron microscopy allowed the quantitative evaluation of their synaptic connections.[21,27,42] In the visual cortex of cat two types of basket cells were revealed by intracellular horseradish peroxidase (HRP) injection. The so-called "large basket cells" have axons running up to 800 μm from the soma horizontally in layers II–III, and a small radial projection to the infragranular layers.[27,42] In contrast, the neurons termed "clutch cells" terminate 100–300 μm from their somata and most of their terminals are in layer IV with a small projection to the infra- and supragranular layers.[21] Both types can be monosynaptically activated from the lateral geniculate nucleus,[27] and make 20–30% of their synapses with the somata of other neurons.[21,42]

It has been proposed that these neurons may use gamma-aminobutyrate (GABA) as their transmitter.[21,27,42] In line with previous suggestions,[7,14,17,18] this assumption was based on two lines of argument: (1) Since many axosomatic terminals have been demonstrated to contain glutamate decarboxylase (GAD),[7,14,34] the enzyme producing GABA, or GABA[37,41] itself, the axosomatic terminals of basket

Abbreviations: BC, basket cell; CC, clutch cell; GABA, gamma-aminobutyrate; GAD, glutamate decarboxylase; HRP, horseradish peroxidase; P-type dendrite, pyramidal cell-like dendrite; S-type dendrite, smooth dendritic cell-like dendrite; TPBS, Tris–phosphate-buffered isotonic saline.

and clutch cells are probably GABAergic. The problem with this assumption is that not all axosomatic terminals contain GABA or GAD, and the proportion of axosomatic boutons that originate from basket cells (i.e. neurons that have a large proportion of their termianls on somata) is not known. (2) The second argument is based on the similarity of the ultrastructural characteristics of identified basket and clutch cells on the one hand,[4,21,42] and large GABA-[8] or GAD-immunoreactive[4,14,39] somata on the other. However, it has not been possible to establish whether all large somata, with fine structural characteristics similar to basket cells, are positive for GABA or GAD, nor has it been possible to show that those which are have axons that make axosomatic synapses. Consequently it is still not known whether cortical basket cells contain GABA.

In order to overcome the inherent uncertainties of the inferential evidence, in the present study we tested directly for the presence of immunoreactive GABA in synaptic terminals of identified basket and clutch cells that have been reported previously in the cat's visual cortex.[21,27,42] This retrospective study became possible through the development of an antiserum to GABA[15,41] and the application of an immunogold procedure[37] that allows the localization of GABA in HRP-filled cells conventionally processed for electron microscopy. Some of the results have been communicated in preliminary form.[36]

EXPERIMENTAL PROCEDURES

Animals, fixation and tissue processing

Intracellularly injected cells were obtained from six adult cats used in the ongoing studies of K. A. C. Martin and D. Whitteridge.[25,26] The anaesthetic, surgical and recording procedures have been reported in detail previously.[26] At the end of the recording session the animals received an overdose of anaesthetic [Sagatal (sodium pentobarbital), May and Baker] and were perfused through the heart first with saline followed by a fixative of 2.5% glutaraldehyde, 1% paraformaldehyde (both from TAAB Laboratories, Reading) dissolved in 0.1 M sodium phosphate buffer (pH 7.4) solution. Following perfusion a block of brain was cut out and stored in 0.1 M phosphate buffer for up to 8 h before sectioning at 80 μm with a Vibratome.

The sections were washed extensively in phosphate buffer and reacted to reveal HRP enzyme activity with the *p*-phenylenediamine/pyrocatechol procedure of Hanker *et al.*,[13] with cobalt/nickel intensification.[1] Some sections of basket cell BC3 were reacted with a 3,3′-diaminobenzidine procedure. After the reaction the sections were washed in phosphate buffer and postfixed for 1 h in 1% osmium tetroxide dissolved in 0.1 M phosphate buffer, pH 7.4. They were washed again in phosphate buffer, then dehydrated in alcohol (1% uranyl acetate was included into the 70% ethanol stage for 40 min), and embedded on glass slides in Durcupan ACM (Fluka) resin. The sections containing clutch cell CC2 were also Golgi-impregnated for reasons unconnected with the present study. GABA can successfully be demonstrated in Golgi-impregnated material, as shown earlier.[37] After curing of the resin the HRP-filled cells were drawn and photographed. Parts of the resin-embedded sections containing the HRP-filled processes of the identified neurons were then removed from the slides and reembedded for further sectioning for electron microscopy.

Postembedding gamma-aminobutyrate immunocytochemistry using colloidal gold

Serial sections were cut and picked up onto Formvar-coated, single slot, gold grids. Only 3–5 sections were placed on each grid, in order to have the same boutons represented on more than one grid. The immunocytochemical method followed procedure I of Somogyi and Hodgson[37] with small modifications. Droplets of solutions for the immunocytochemical reaction were put on Parafilm in Petri dishes. The Parafilm was surrounded with wet paper tissue to avoid drying of the grids. Unless otherwise stated, the grids were floated on the droplets at room temperature, with the sections facing down. When transferred from one droplet to another the excess fluid was removed from the grids by filter paper, but the sections were never allowed to dry. All reagents and washing solutions were Millipore filtered (pore size 0.22 μm). The following steps were carried out.

(1) Pretreatment of the resin in 1% periodic acid (H_5IO_6, BDH Chemicals Ltd) for 7–10 min.
(2) Three washes in double-distilled water, by dipping of the grids several times into vials, followed by 5 min in double-distilled water.
(3) Removal of osmium in 1% sodium metaperiodate ($NaIO_4$, BDH Chemicals Ltd, freshly prepared) for 7 min, as recommended by Bendayan and Zollinger.[3]
(4) Washing as in step 2.
(5) The grids were blotted with filter paper on the side opposite to the sections. From now on unless otherwise stated the the grids were not dipped into any solution, but were just floated on the droplets. Occasionally if a grid sank in the droplet it was put into the droplets in all subsequent steps with the sections facing upwards.
(6) Two 10-min periods in Tris (10 mM)–phosphate (10 mM)-buffered isotonic saline (TPBS), pH 7.4.
(7) Blocking of non-specific reaction with a solution of 5% normal goat serum for 20 min, if IgG-coated colloidal gold was the reagent later, or with a solution of 0.5% ovalbumin, if protein-A coated colloidal gold was the reagent later.
(8) One to two min in 1% normal goat serum.
(9) Antiserum to GABA for 1–2 h (code No. 9), produced in rabbit against a GABA–glutaraldehyde–bovine serum albumin conjugate.[15] It was diluted with 1% normal goat serum at 1:1000 to 1:3000. In one experiment it was used at a dilution of 1:300. Some grids were reacted with the same antiserum diluted 1:1000 and preincubated for 4 h with GABA attached to polyacrylamide beads with glutaraldehyde as described earlier.[15]
(10) Washing in three changes of 1% goat serum diluted with TPBS, 15 min each.
(11) Three to five min in 0.5% solution of polyethylene glycol (mol. wt 15,000–20,000, Sigma), dissolved in 50 mM Tris buffer, pH 7.0. This step was carried out to avoid the mixing of high concentration salt solution in the TPBS with the protein coated colloidal gold in the next step.
(12) Goat anti-rabbit IgG coated colloidal gold (15 nm, Janssen Life Sci. Prod.), diluted usually to 1:10 to 1:40 depending on the batch, for 1–2 h. It was diluted with the same solution as used in step 11. In some experiments protein-A coated colloidal gold was used as reported earlier.[37]
(13) Three dips in double-distilled water, followed by 10 min in double-distilled water.
(14) Staining with alkaline lead citrate to increase contrast for electron microscopy.
(15) Three dips in double-distilled water and blotting with filter paper.

From each series only every second grid was incubated for immunocytochemistry. After the reaction most of the electrondensity is removed from the HRP reaction endproduct and it is difficult to locate the processes of the intracellularly filled cells. Therefore, the HRP-filled processes were first localized in sections on the non-incubated grids. Thereafter

the same process was located in the sections which were reacted to reveal GABA, using capillaries, myelinated axons and other conspicuous structures as fiducial marks.

RESULTS

Neurons analysed

Two large basket cells and four clutch cells were tested for GABA immunoreactivity (Table 1). The location of the cells, the distribution of their processes, the synaptic connections of the basket cells (BC2 and BC3 in ref. 42) and three of the clutch cells (CC1, CC2 and CC3, in ref. 21) have been reported previously. In the present study further areas of the dendritic and axonal arborizations were sectioned, from layer III in the case of the basket cells, and from layer IV in case of the clutch cells. All cells were in area 17, except clutch cell CC3 which was in area 18. Two of the cells, BC3 and CC2, were impaled intraaxonally with the microelectrode and the HRP did not spread into their somata and dendrites.

The fourth clutch cell, CC4 has not been reported previously. It was tested for GABA in the present study in order to increase the sample size, because the brains containing clutch cells CC2 and CC3 were poorly fixed and consequently the GABA-immunoreactivity of the sections was difficult to evaluate. Clutch cell CC4 had its soma in lower layer IVA of area 17. The dendritic field was restricted to layer IV with dendrites freely crossing into both the A and B sublaminae. Most of the axonal arborization was also within lamina IV except a small (less than about 3% of the boutons) radial projection to layer III, and another small (less than 10% of the boutons) projection to layers V and VI. These features, as well as the beaded appearance and size of the dense axonal arborization, were identical to those of clutch cell CC1 described in detail previously;[21] therefore they will not be illustrated here.

GABA-immunoreactive structures

Immunoreactivity was evaluated by visual comparison of the surface gold density over cellular profiles. A profile was regarded immunopositive for GABA if the surface density of gold within its plasma membrane was several times that over other profiles in the vicinity (Figs 1–6). Such comparisons can only be made within sections on the same grid, because both the absolute and relative density of gold strongly depends on the conditions of the immunoreaction. Increasing the concentration of the primary antiserum and/or the concentration of colloidal gold usually results in a higher density of gold particles, but also leads to higher background labelling (e.g. Fig. 5A). Variations also occur between different experiments even when the same reagents are used with the same tissue block. Nevertheless, as demonstrated in the present study by reacting the processes of the same identified cells under different conditions, the same neurons are always immunoreactive whenever the reaction is successful. When the serum was preincubated before the immunoreaction with GABA attached to a solid phase carrier, immunoreactivity was abolished and only a few scattered gold particles were found on the sections.

As demonstrated earlier, in the visual cortex there is good agreement in the distribution of GABA and GAD[41] as well as between results obtained with the immunogold and other procedures.[37] In the present study GABA immunoreactivity was found in somata, dendrites, myelinated and unmyelinated axons and in boutons. The GABA-positive boutons were found to make type II, symmetrical synaptic contacts, whenever the synapse could be identified (Figs 2A, 5A, 6B, E). A high proportion of myelinated axons was strongly GABA-positive (Fig. 3D), including some of the largest diameter axons in cortex (Fig. 6C).

The subcellular distribution of GABA, as revealed by the colloidal gold, is difficult to assess with the perfusion fixation used in the present study. GABA could be translocated and fixed to organelles where there is a high concentration of basic proteins carrying amino groups. These could serve as binding sites for the GABA–glutaraldehyde complex. In all

Table 1. Summary of intracellularly horseradish-peroxidase-filled neurons tested for GABA immunoreactivity, and the distribution of their postsynaptic targets

Cell	Abbreviated name	Total no. of boutons tested	Synaptic targets				Previously reported features (ref. nos)
			Somata	Dendritic shaft	Spine	Unidentified	
Large basket cell	BC2	6	1	3	1	1	27, 42
Large basket cell (axon only)	BC3	38	17	13	3	5	27, 42
Clutch cell	CC1	21	4	13	1	4	21
Clutch cell (axon only)	CC2	6	1	6	—	—	21
Clutch cell	CC3	19	2	12	5	3	21
Clutch cell	CC4	22	7	6	3	6	—

Some boutons made synaptic contact with more than one structure, therefore the total number of targets may be more than the total number of boutons. The physiological and detailed morphological properties of some of these cells has been reported previously and the references are given in the last column. All postsynaptic elements were followed in serial sections, but even so, for some of them it could not be determined whether they were dendritic spines or small dendritic shafts. These, together with cases when a synaptic contact could not clearly be seen, are listed as unidentified.

GABA-positive structures mitochondria had the highest density of gold (e.g. Figs 2B, 3A, 6B, E), followed by the areas containing synaptic vesicles in the boutons (Figs 2B, D, 3D, 5A).

Gamma-aminobutyrate-immunoreactivity of horseradish peroxidase-filled basket and clutch cells

The incubation in sodium metaperiodate greatly reduces the electron density of the HRP reaction endproduct (Figs 1–6), probably by removing the osmium. This makes the localization of the HRP-filled processes and synaptic contacts difficult, therefore unreacted serial sections are indispensable for the efficient collection of a reasonable sample of postsynaptic elements (Figs 1B, C, 2, 3B–E, 4). The reduction of electron density was observed in both the 3,3′-diaminobenzidine and the *p*-phenylenediamine/pyrocatechol-reacted HRP-filled processes. In lightly filled cells all traces of the HRP reaction disappeared and the processes could only be identified from their location (Fig. 2).

The boutons ($n = 112$) of all seven cells were immunoreactive for GABA. There was great variation between the reactivity of the individual cells just as there was variation in the general reactivity of the tissue. This is probably explained by differences in fixation, because the two most poorly fixed animals containing cells CC2 and CC3, reacted poorly (Fig. 4), and showed also the highest background deposition of gold. In the same reaction there was little variation between the boutons of an individual cell, but those sections of a bouton that contained many mitochondria usually exhibited higher gold density (compare e.g. Figs 1C and D). We examined boutons with different postsynaptic targets from each cell to see if there were differences in GABA immunoreactivity, but axosomatic (Figs 1, 2A, B, 5A, B), axodendritic (Figs 3D, E, 4C, D, 6A, B) and axospinous (Figs 2C, D, 3B, C, 4A, B) boutons were equally immunoreactive.

The dendrites of clutch cells were also immunoreactive for GABA (Fig. 6C–E). Ten dendritic segments from CC1, three from CC3 and one from CC4, were studied. Both proximal and distal dendrites were GABA-positive, most of the gold being deposited over mitochondria (Fig. 6D and E). Basket cell dendrites and the somata of HRP filled cells were not studied.

Both clutch and basket cells have myelinated axons.[21,42] For three of the clutch cells and basket cell BC3, myelinated axon segments could be shown to react for GABA (Fig. 5D), as revealed by comparing them with neighbouring axons. The axons of the other cells were not examined.

Postsynaptic targets of basket and clutch cells

Neuronal somata, dendritic shafts, dendritic spines and an axon initial segment (contacted by CC1, not shown in Table 1), received synapses from the GABA-immunoreactive, HRP-filled terminals (Table 1). The proportion of these different target structures, as detected in the present study, is not representative for the two types of cells. This is a consequence of the difficulty in following the HRP-filled terminals in the GABA-reacted sections where the boutons lose their electrondensity. Boutons near conspicuous or easily identifiable structures, such as neuronal somata, were more easily found and this distorts the sample of postsynaptic elements. In our previous studies by following every bouton in the plane of the section we obtained random samples.[21,42]

Neuronal somata postsynaptic to layer III basket cells BC2 and BC3 were pyramidal in shape, received only type II synapses and had prominent apical dendrites (Fig. 1), and were probably pyramidal cells. They received 1–5 synapses from the HRP-filled axon, and in addition also had numerous other GABA-positive synaptic boutons contacting them. The somata postsynaptic to clutch cells in layer IV were similar, but most of them were smaller than the layer III cells and had thinner proximal dendrites. They also received GABA-positive synapses in addition to the identified clutch cell boutons (Fig. 5A). None of the postsynaptic somata were immunoreactive for GABA, supporting our earlier conclusions[21,42] that basket cells mainly terminate on pyramidal cells, while clutch cells mainly terminate on spiny stellate, star pyramidal and pyramidal cells of layer IV.

The postsynaptic dendrites could be divided into pyramidal-like (type P) and smooth dendritic-like (type S) categories on the basis of ultrastructural criteria described previously.[21,42] The S-type dendrites usually contain more mitochondria,[21,42] granular endoplasmic reticulum and free ribosomes, they receive more synaptic contacts[21,42] and their cytoplasm is more electron dense. Only seven such dendrites were amongst the postsynaptic targets of clutch cells (Fig. 6A, B) and one was postsynaptic to basket cell BC2. All but one of them were GABA-positive. None of the 45 type P dendrites postsynaptic to basket and clutch cells were GABA-positive. Both type P and type S dendrites received synapses from GABA-positive terminals other than the HRP-filled one.

All dendritic spines receiving synapses from the GABA-positive boutons of basket and clutch cells also recieved an additional synapse from a GABA-negative bouton that made a type I synaptic contact (Figs 2C, D, 3B, C, 4).

DISCUSSION

Specificity of the immunoreaction

The antiserum was raised against GABA coupled to bovine serum albumin with glutaraldehyde and it probably recognizes the condensation product of glutaraldehyde and GABA.[15] Since glutaraldehyde was a major component of the fixative in the present study the serum specificity is unlikely to differ from that established in previous tests.[15,41] In those studies it was shown that the serum did not recognize a large

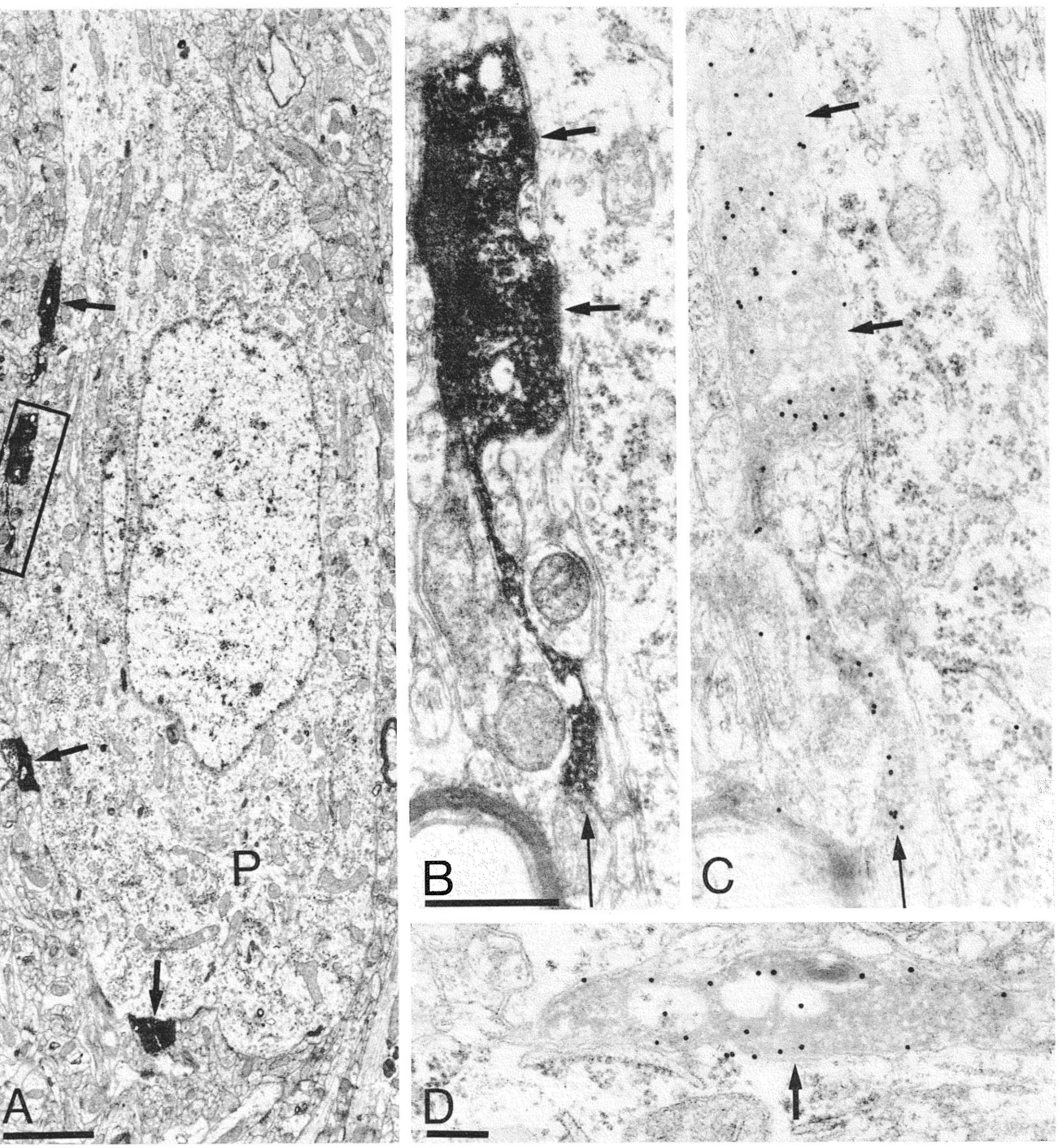

Fig. 1. (A) Electron micrograph of a large pyramidal cell (P) in layer III, receiving synaptic contacts (arrows) from several HRP-filled boutons (black) of a basket cell axon (BC3 in text). (B) The framed area in (A) is shown at higher magnification to demonstrate the type II synaptic contacts (short arrows) established by two boutons. (C) A serial section of the same boutons as shown in (B) was incubated to reveal GABA immunoreactivity which is demonstrated by the concentration of electrondense gold particles over the terminals and the preterminal axon [long arrows in (B) and (C)]. (D). The immunoreactivity of the uppermost HRP-filled bouton in (A) is shown in a serial section as it makes a type II synaptic contact. Note the "bleaching" effect of periodate treatment, which renders the reaction endproduct and myelin less electrondense in this, and in all subsequent figures. Scales: (A) 2 μm; (B) and (C), same magnification, 0.5 μm; (D), 0.2 μm.

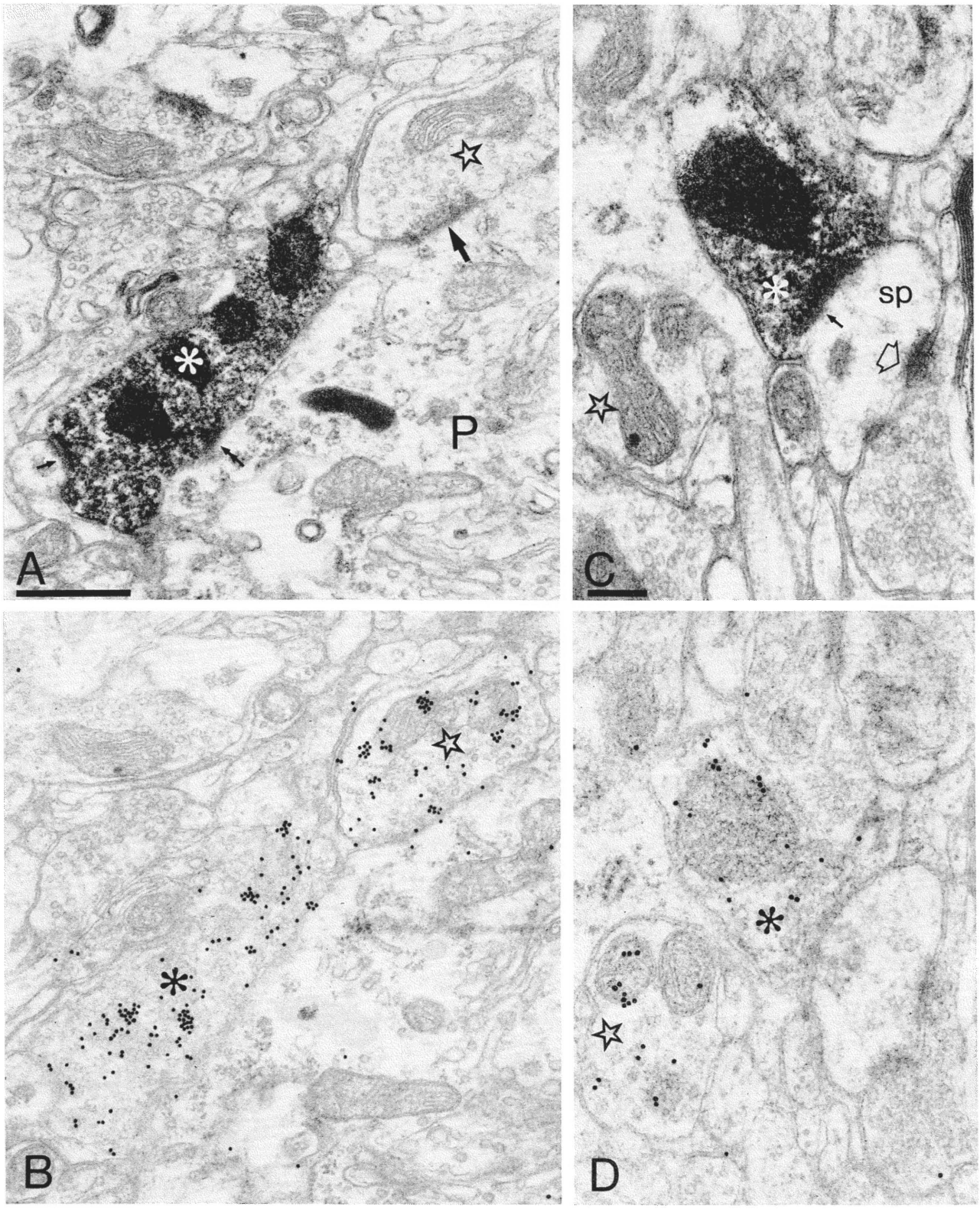

Fig. 2. Immunogold demonstration of GABA in synaptic terminals (asterisks) of a layer III large basket cell (BC2 in text). (A), (B), (C) and (D) are serial sections of the same boutons respectively. This cell was lightly filled with HRP, therefore the reaction endproduct is no longer recognizable in (B) and (D) after the immunoreaction. (A) and (B) The HRP-filled bouton makes a synapse with a spine (small arrow) and also contacts the soma of a pyramidal cell (double arrow) at a site where a synaptic specialization appeared in subsequent sections. The pyramidal cell (P) also receives a synapse (large arrow) from another GABA-immunoreactive bouton (star). (C) and (D) A spine (sp) is shown to receive synapses from both the GABA-positive basket cell bouton (arrow) and a non-immunoreactive terminal making a type I contact (open arrow). A nearby bouton (star) is also GABA-immunoreactive. Scales: (A) and (B), same magnification, 0.5 μm; (C) and (D), same magnification, 0.2 μm.

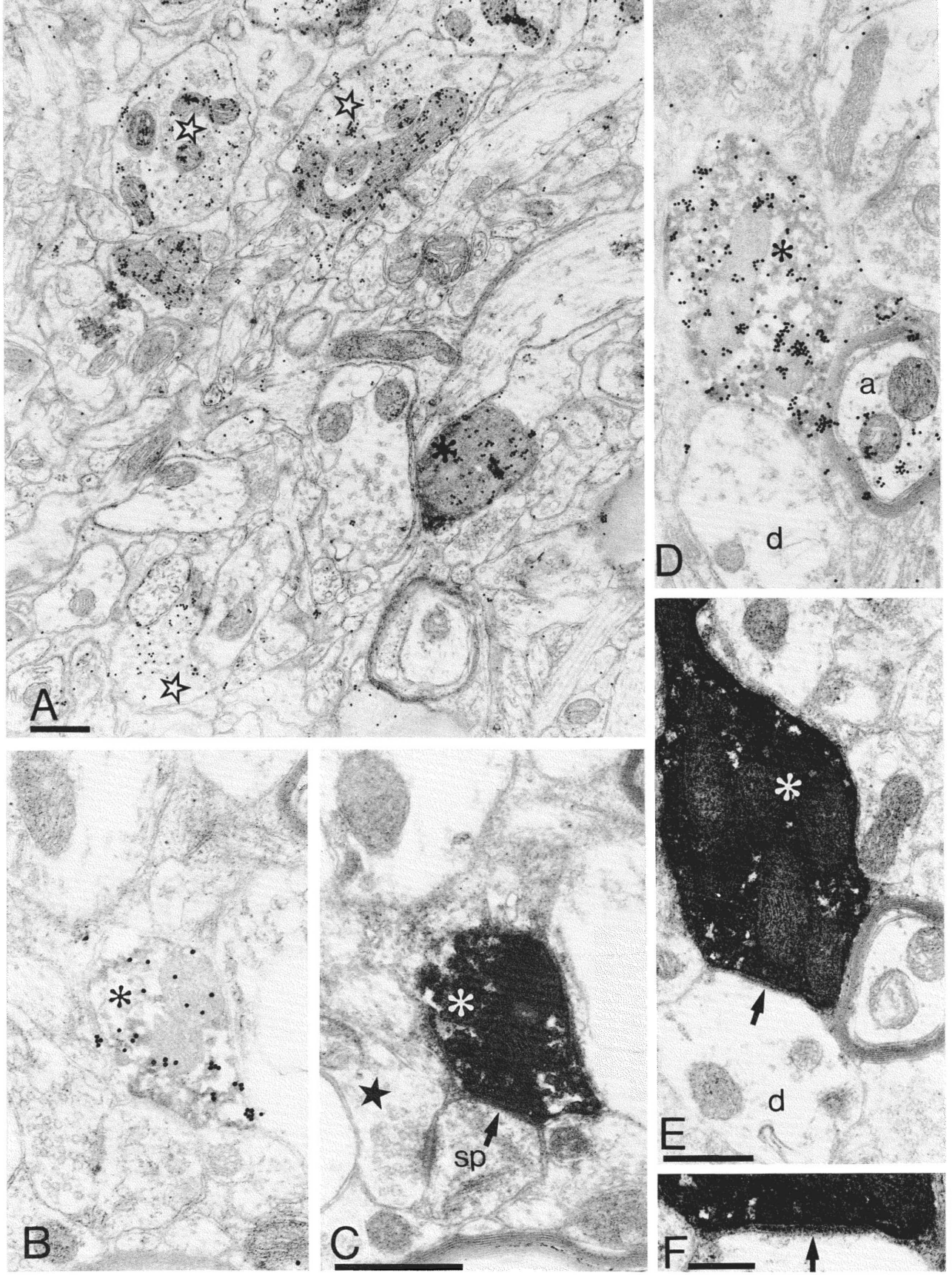

Fig. 3. (A) Electron micrograph of large GABA-immunoreactive boutons (e.g. stars) in layer IV. One of them (asterisk) is identified as originating from a clutch cell (CC4 in text) by the electrondense HRP reaction endproduct. (B) and (C) Serial sections of a bouton (asterisk) of clutch cell CC1 making a type II synapse with a spine, that also receives a type I synapse from another bouton (star). Only the clutch cell bouton is immunoreactive for GABA as shown by the accumulation of colloidal gold in B. (D) and (E) Serial sections of a bouton (asterisk) of clutch cell CC1 making a synapse (arrows) with a dendrite (d). The bouton and an axon (a) are immunoreactive for GABA as seen in (D). (F) The synaptic specialization (arrow) is shown at higher magnification. Scales: (A), (C) and (E), 0.5 μm; (B) and (C), same magnification; (D) and (E), same magnification; (F), 0.2 μm.

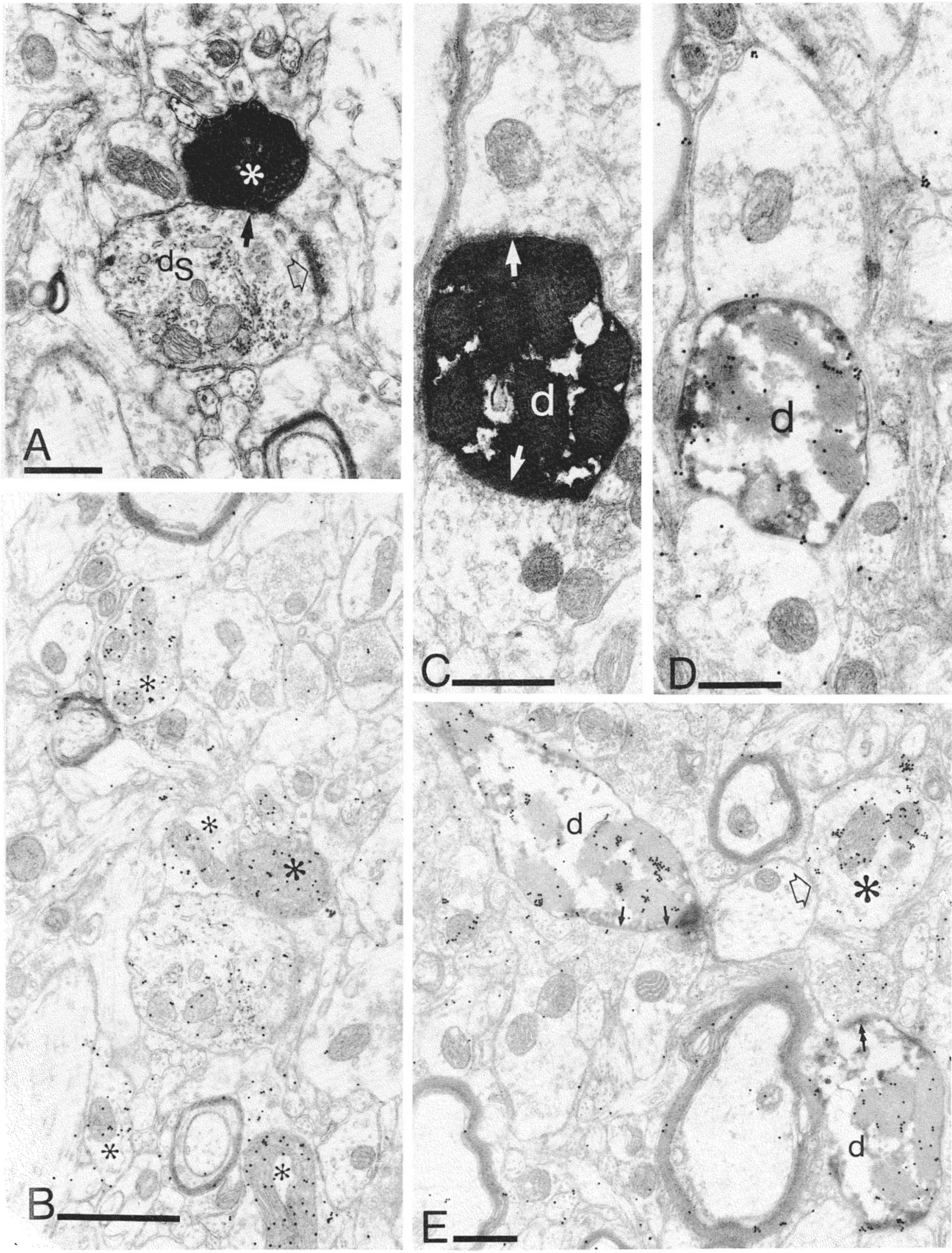

Fig. 6.

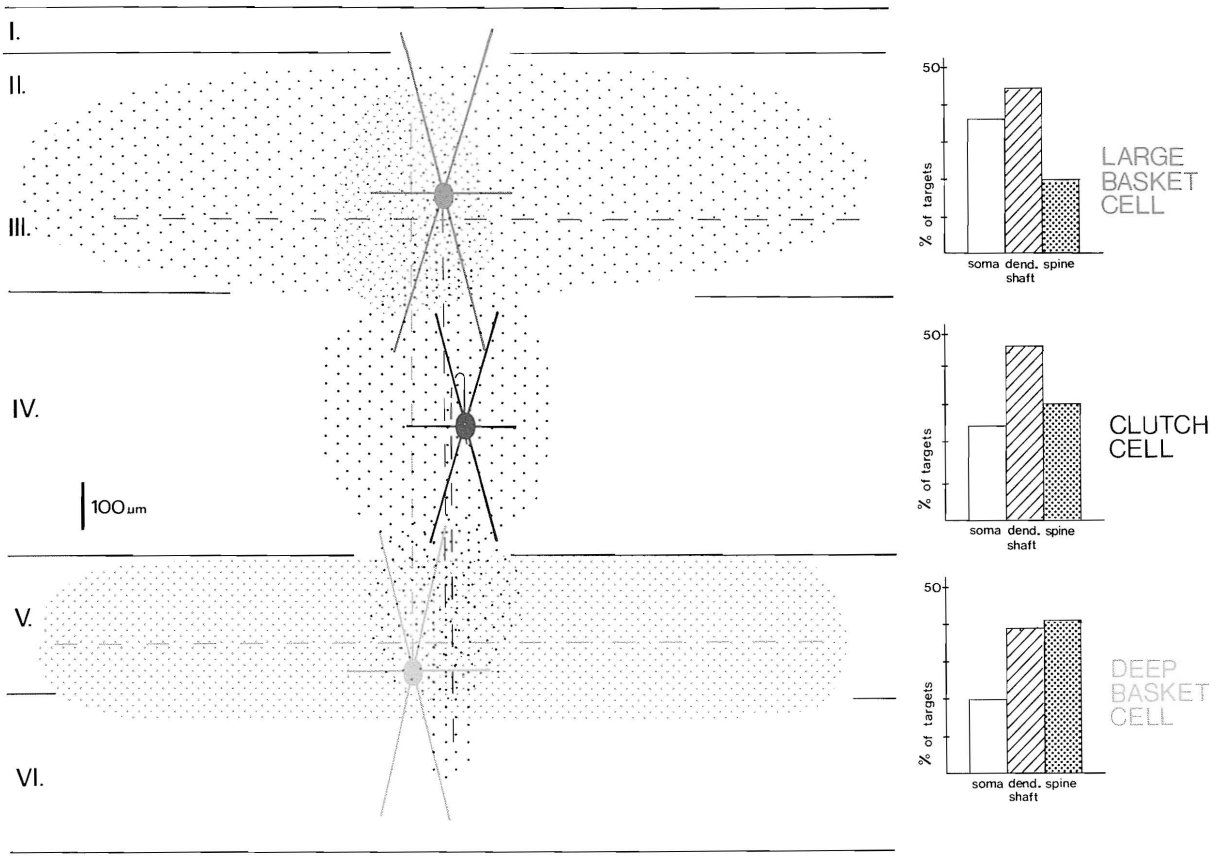

Fig. 7. Schematic drawing summarizing the laminar distribution of axonal (dotted, and broken lines) and dendritic (solid lines) arborizations of different types of GABA-positive basket cells in the striate cortex of cat. On the right the distribution of postsynaptic target elements is summarized. The extent of the arborizations is based on previously published work for the supragranular "large basket cell"[27,42] and "clutch cell".[21,27] The data for the "deep basket cell" are based on a GABA-immunoreactive neuron that was HRP-filled in a kitten[36] (also Kisvarday, Martin, Friedlander and Somogyi, unpublished observations). Note that in addition to the tangential projections, all three types of cell have a radial axonal projection in the column of the parent cell. Data for the distribution of postsynaptic targets were pooled from the termination of large basket cells BC2 and BC3 in layer III,[42] and from the termination of clutch cells CC1 and CC2 in layer IV.[21] For the deep basket cell targets were pooled from layers V and III. The rare postsynaptic axon initial segments are not shown. Note that, although all three types of cell have a substantial proportion of their synapses on the somata of other neurons, the majority of their targets are dendritic shafts and spines. The diagram is based on the analysis of a small number of neurons and several aspects of it require further studies. For example the tangential axonal projections may have preferred directions, and the translaminar projection may be absent in some basket cells. There may also be laminar differences in the proportion of different postsynaptic targets for the same cell.

Fig. 6. (A) and (B) Electron micrographs of serial sections through a bouton (large asterisk) of clutch cell CC1 contacting (arrow) a dendrite (d_S) with characteristics of smooth dendritic neurons. The dendrite is also contacted by other boutons, one of them making a type I synaptic contact (open arrow). The dendrite, the HRP-filled bouton and several other boutons (small asterisks) are immunoreactive for GABA, as shown by the accumulation of gold in B. (C) and (D) Serial sections through a dendrite (d) of clutch cell CC1. The dendrite is immunoreactive for GABA, as shown in (D), but neither of the two large boutons from which it receives synapses (arrows) show immunoreactivity. (E) A varicose dendrite of clutch cell CC1 shows GABA immunoreactivity and receives synapses from two immunonegative (arrows) and one immunopositive (double arrow) bouton. A GABA-positive bouton (asterisk) contacts an immunonegative dendrite (open arrow). Note the high density of mitochondria in the clutch cell dendrites. Scales: (A)–(E), 0.5 μm.

synaptic elements. The few postsynaptic dendrites that were labelled for GABA were all of the S-type, supporting our classification and suggestion that they originate from smooth dendritic cells. Perikarya stain strongly for GABA in the visual cortex of cat,[9,41] and they were also numerous in our sections reacted with the immunogold method. Thus it is unlikely that the lack of staining of postsynaptic somata in the present study represents false negative reaction.

Nevertheless it should be emphasized that negative results in immunocytochemistry are not proof of the absence of the antigen. In our case some varieties of GABA neurons may contain GABA levels below the sensitivity of the method, or it is possible that only some GABAergic neurons have immunoreactive GABA in their dendrites. Also some of the postsynaptic dendrites may belong to smooth dendritic cells using other transmitters than GABA, although the number of these is probably low in cortex.

Identifying putative transmitters in physiologically characterized neurons

The retrospective localization of GABA in electrophysiologically characterized cells HRP-filled using intracellular electrodes and processed for electron microscopy, was possible because the epitope(s) recognized by the antiserum were not changed during the processing. Other methods have also been used to localize the putative transmitter of physiologically characterized neurons in the brain. Dopamine has been localized in intracellularly recorded mesencephalic neurons by fluorescence microscopy, following the elevation of dopamine level by intracellular injection of drugs.[12] Fluorescence microscopy was also used to determine the chemical nature of intracellularly recorded noradrenergic and serotoninergic neurons.[2] Immunocytochemistry was employed to reveal neurophysin, enkephalin and arginine vasotocin immunoreactivity in magnocellular paraventricular neurons.[33] In both of the latter studies injected fluorescent dyes were used to locate the recorded cells. HRP-filling has the advantage that it gives not only a very detailed image of the cell, but also the processes can be followed over dozens of sections covering several millimetres in the brain. The specimens are permanent, and the electron microscopic detail is superior to that obtained by fluorescent dyes.[33] The main limitation of our method is that many epitopes, especially proteins, are sensitive to osmium treatment and embedding, and will be lost during processing of the tissue. However, GABA is not the only putative neurotransmitter that can be localized with this method; glutamate immunoreactivity has been successfully demonstrated in material similar to that used in the present study.[40] Glutamate is also a putative transmitter in the cortex and its remains to be established if it is also present in anatomically and/or physiologically distinct classes of neurons.

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