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Enrichment of Cholinergic Synaptic Terminals on GABAergic Neurons and Coexistence of Immunoreactive GABA and Choline Acetyltransferase in the Same Synaptic Terminals in the Striate Cortex of the Cat

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ABSTRACT

The synaptic circuits underlying cholinergic activation of the cortex were studied by establishing the quantitative distribution of cholinergic terminals on GABAergic inhibitory interneurons and on non-GABAergic neurons in the striate cortex of the cat. Antibodies to choline acetyltransferase and GABA were used in combined electron microscopic immunocytochemical experiments.

Most of the cholinergic boutons formed synapses with dendritic shafts (87.3%), much fewer with dendritic spines (11.5%), and only occasional synapses were made on neuronal somata (1.2%). Overall, 27.5% of the postsynaptic elements, all of them dendritic shafts, were immunoreactive for GABA, thus demonstrating that they originate from inhibitory neurons. This is the highest value for the proportion of GABAergic postsynaptic targets obtained so far for any intra- or subcortical afferents in cortex. There were marked variations in the laminar distribution of targets. Spines received synapses most frequently in layer IV (23%) and least frequently in layers V–VI (3%); most of these spines also received an additional synapse from a choline acetyltransferase-negative bouton. The proportion of GABA-positive postsynaptic elements was highest in layer IV (49%, two-thirds of all postsynaptic dendritic shafts), and lowest in layers V–VI (14%). The supragranular layers showed a distribution similar to that of the average of all layers. The quantitative distribution of targets postsynaptic to choline acetyltransferase-positive terminals is very different from the postsynaptic targets of GABAergic boutons, or from the targets of all boutons in layer IV reported previously. In both cases the proportion of GABA-positive dendrites was only 8–9% of the postsynaptic elements. At least 8% of the total population of choline acetyltransferase-positive boutons, presumably originating from the basal forebrain, were also immunoreactive for GABA. This raises the possibility of cotransmission at a significant proportion of cholinergic synapses in the cortex.

The present results demonstrate that cortical GABAergic neurons receive a richer cholinergic synaptic input than non-GABAergic cells. The activation of GABAergic neurons by cholinergic afferents may increase the response specificity of cortical cells during cortical arousal thought to be mediated by the basal forebrain. The laminar differences indicate that in layer IV, at the first stage of the processing of thalamic input, the cholinergic afferents exert substantial inhibitory influence in order to raise the threshold and specificity of cortical neuronal responses. Once the correct level of activity has been set at the level of layer IV, the influence can be mainly facilitatory in the other layers.

Key words: acetylcholine, immunocytochemistry, visual cortex, GABA, arousal

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The cholinergic innervation of the cortical mantle by the basal forebrain (Lehmann et al., '80; Wenk et al., '80; Johnston et al., '81; Mesulam et al., '83; Rye et al., '84; Ingham et al., '85; Henderson, '87; Fisher et al., '88) plays an important role in the activation of cortical systems (Singer, '79; Buzsáki et al., '88; Vanderwolf, '88). Receptors for acetylcholine (ACh) modulate the way in which cortical neurons respond to other inputs and thus have influence on diverse cortical activities ranging from synaptic plasticity (Greuel et al., '88; Metherate and Weinberger, '89) and the responses to sensory thalamic input (Sillito and Kemp, '83; Sato et al., '87a,b; Lamour et al., '88; Müller and Singer, '89) to higher integrative functions such as learning and memory. The impairment of the cholinergic input to the cortex under pathological conditions such as Alzheimer's disease may contribute to cognitive deficits (Whitehouse et al., '81; Bartus et al., '82; Berger and Gaspar, '88). The mechanism of action of ACh is therefore of major interest.

The changes in neuronal responsiveness evoked by ACh are mediated predominantly by muscarinic receptors Krnjević and Phillis, '63a; for review see Prince and Huguenard, '88; McCormick, '89), although nicotine has also been shown to facilitate neurons in vivo (Stone, '72). The application of ACh to antidromically identified (Krnjević and Phillis, '63b; Stone, '72; Lamour et al., '82) or presumed pyramidal neurons mainly produces a slowly developing facilitation of the evoked and spontaneous firing often preceded by a fast inhibitory influence (McCormick and Prince, '86). The slow depolarization is selectively blocked by low concentration of pirenzepine, which indicates that the facilitatory effect is mediated by M1 receptors (McCormick and Prince, '85). The inhibition is thought to be mediated indirectly by the fast activation of GABAergic neurons within the cortex. The blocking of this effect requires high concentrations of pirenzepine, indicating that this response might be mediated by M2 receptors (McCormick and Prince, '85). The fast activation of inhibitory cells by ACh probably results in the short latency $GABA_A$ receptor mediated inhibition of some cortical cells (McCormick and Prince, '85, '86; Müller and Singer, '89).

The physiological effects and the mechanisms of action of ACh have been extensively studied in the visual cortex of the cat. This cortical area has few if any intrinsic cholinergic neurons in this species (Stichel et al., '87). It receives cholinergic afferents in all layers (Sato et al., '87a; Stichel and Singer, '87) issued from the basal forebrain (Wahle et al., '84; Fisher et al., '88). The ionophoretic application of ACh to visually driven cortical neurons enhances these evoked responses in about 60% of units, but inhibitory influences have also been reported (Sillito and Kemp, '83; Sato et al., '87b; Müller and Singer, '89). It has been suggested that the 2 mechanisms operate synergistically to enhance the signal to noise ratio of responses (Sillito and Kemp, '83; Sato et al., '87b), although decreases in response specificity have also been observed (Sato et al., '87b; Müller and Singer, '89). It is not known to what extent the direct facilitatory and the presumably indirect, GABA-mediated (Müller and Singer, '89) inhibitory components of the cholinergic response participate in the desynchronization of cortical activity observed in the EEG (for review see Singer, '79; Buzsáki et al., '88; Vanderwolf, '88), which is thought to underlie cortical arousal.

One indicator of the relative importance of direct facilitatory and indirect inhibitory effects is the extent to which cholinergic synaptic release sites are devoted to the principal cells as compared to the GABAergic local circuit neurons. De Lima and Singer ('86) studied the synaptic connections of cholinergic terminals at the electron microscopic level in the visual cortex of the cat and provided evidence that, as in the rat (Houser et al., '85; Parnavelas et al., '86), both pyramidal and nonpyramidal cells receive cholinergic synapses. Some of the nonpyramidal cells identified in their study (de Lima and Singer, '86) were probably GABAergic and were suggested to be responsible for the short latency inhibition evoked by ACh. However, the degree of participation of the 2 major cell categories of cortical cells have not been clarified. Furthermore, the specific synaptic circuits and the cell type(s) responsible for the indirect inhibitory effect of ACh are not known. The present study was undertaken to determine quantitatively the proportion of GABAergic postsynaptic targets of cholinergic synaptic terminals. Considering the somewhat disparate results obtained in vivo concerning the physiological effect of ACh in the visual cortex, we also compared the distribution of the targets of cholinergic synapses in the different laminae. Some of the results have been published in abstract form (Beaulieu and Somogyi, '89).

Abbreviations

ACh	acetylcholine
ChAT	choline acetyltransferase
DAB	3,3′ diaminobenzidine-4HCl
GABA	gamma-amino butyric acid
GAD	glutamate decarboxylase
GVG	gamma-vinyl-GABA
NGS	normal goat serum
PB	phosphate buffer
TB	Tris buffer
TBS	Tris-buffered saline

METHODS

Fixation and preparation of the tissue

Three adult cats (2–3 kg, Park Farm, Oxford) were used. One animal received an injection of gamma-vinyl-GABA (GVG, Merrel Dow Pharmaceutical Ltd, Winnersh, U.K.) at a dose of 1,500 mg/kg (i.p.) dissolved in saline. We used this inhibitor of GABA transaminase, a degrading enzyme of GABA, in order to increase the concentration of GABA in the tissue. The 2 other cats did not receive any treatment prior to perfusion. The treated cat and one of the untreated cats were used for both light and electron microscopic examination; the other untreated cat was used only for light microscopic examination. All animals were deeply anaesthetized with chloral hydrate (40 mg/100 g initially, then supplemented if necessary until all reflexes were abolished). They were perfused through the ascending aorta first with Tyrode's solution bubbled with 95% oxygen and 5% CO_2 (2–3 min, 200–300 ml), followed by freshly prepared fixative containing 2% paraformaldehyde and 1% glutaraldehyde dissolved in 0.1 M sodium phosphate buffer (PB, pH 7.4, 4°C). During perfusion, the descending aorta was clamped. The paraformaldehyde and glutaraldehyde solution was delivered by a pump at a rate of 500 ml during the first 5 minutes, followed by 1 litre during the next 15 minutes; finally another 500 ml of the fixative solution containing no glutaraldehyde was perfused. Brains were removed from the skull and washed in cold PB (4°C). Blocks of the cerebral cortex (area 17, 18, and 19) were then dissected out and washed several times in ice-cold PB. They

were placed sequentially into solutions of 10% and 20% sucrose until they sank. Blocks were then quickly frozen in liquid nitrogen and thawed in cold PB.

Five Wistar rats (200 g) were also used for technical trials. The rats were deeply anaesthetized with sodium pentobarbitone (10–20 mg/100 g, i.p., Sagatal, May and Baker, U.K.) and were perfused in a similar way as the cats, but with a wider range of concentrations of paraformaldehyde (2–4%) and glutaraldehyde (0.1 to 1%). The effect of pronase treatment on choline acetyltransferase (ChAT) immunoreactivity in tissue fixed in the different solutions was evaluated (see below).

Sections were cut from all tissue blocks on a Vibratome at 60–80 μm thickness and placed in cold PB. Some sections from each animal were placed for 30 minutes in a solution of 1% sodium borohydride dissolved in PB at room temperature (Schachner et al., '77). The sections were washed subsequently (at least 6 times, 10 min) until bubbles from the reaction in the sections completely disappeared.

Proteolytic treatment

Proteolytic treatment has been recommended to increase the immunoreactivity of some tissue constituents (Finley and Petrusz, '82). Some of the sections were transferred to Tris buffer (TB; 0.05 M; pH 7.4) for 2 times, 20 minutes at room temperature. Two concentrations of pronase E (0.01% and 0.001% Protease, type XXV, Sigma #P-6911; diluted in TB) and different incubation times were used (1 to 30 min, at room temp.). Free-floating sections were incubated in the pronase solution with constant agitation. At the end of this treatment, the reaction was rapidly stopped with cold TB (4°C). The tissue was then washed 5 to 10 times in cold TB for 30 minutes in all.

Immunocytochemistry for ChAT

Sections of the visual cortex were transferred into Tris-buffered saline (TBS; 0.05 M TB, containing 0.9% NaCl) for 2 times, 30 minutes at room temperature, and then placed for 45 minutes in a solution of 20% normal goat serum (NGS) diluted in TBS. They were then placed for 5 minutes in a solution of 1% NGS diluted in TBS. The sections were incubated for 48 hours at 4°C in a solution of a monoclonal antibody raised to pig ChAT (Boehringer) and diluted at a final concentration of 2 μg/ml in TBS containing 1% NGS. This antibody is produced by a rat-mouse hybridoma; it has been extensively characterized and is not known to cross react with tissue constituents other than ChAT (Eckenstein and Thoenen, '82). Some sections were incubated with an antibody solution containing Triton X-100 up to a concentration of 0.5%.

The tissue was washed 3 times, 30 minutes in TBS containing 1% NGS following the primary antibody treatment. Sections were then incubated for 4 hours at room temperature in a biotinylated goat antirat IgG (Sigma) at a dilution (in TBS) varying from 1:50 to 1:100. Following 3 times, 30 minutes rinse in TB, sections were placed in an avidin biotinylated peroxidase complex solution (ABC kit; Vector) for 2 hours at room temperature. Peroxidase enzyme activity was then revealed by a reaction using 3,3′ diaminobenzidine-4HCl (DAB, Sigma, 0.05% in TB, pH 7.8) as chromogen and 0.01% H_2O_2 as substrate. Some of the sections were incubated without adding the primary or the secondary antibodies to the solution or replacing the primary antibody with other monoclonal antibodies in order to check the specificity of the method.

Some of the sections were mounted on gelatin-coated slides, dehydrated, placed in xylene 2 times, 10 minutes, and rehydrated. Following 2 rinses in PB, 2–3 drops of 2% OsO_4 were added to the solution for a period of 5 minutes to intensify the reaction end-product. Sections were then washed and dehydrated again. They were finally covered with a mounting medium under a coverslip for light microscopic evaluation. Other sections were prepared for electron microscopy, and they were washed in PB and postfixed for 30–60 minutes in 1% OsO_4 dissolved in 0.1 M PB (pH 7.4). They were washed again in PB, then dehydrated in ethanol (1% uranyl acetate was included into the 70% ethanol stage for 40 min), immersed in propylene oxide (2 times, 10 min), and finally embedded on glass slides in Durcupan ACM (Fluka) resin. Portions of interest were cut out from the slides and reembedded. The visual cortex was examined in three different parts according to lamination. Supragranular (layers I-II-III), granular (layer IV), and infragranular (layers V-VI) laminae were studied separately at the electron microscopic level. In each set of layers, 3 to 5 series of sections were analyzed.

Postembedding immunogold reaction for GABA

The presence of GABA in the tissue was determined by a postembedding immunogold method (Somogyi, '88). Production and characterization of the antiserum to GABA has been described elsewhere (Hodgson et al., '85). Serial ultrathin sections of white interference colour were cut from the tissue previously reacted for ChAT under preembedding conditions. Tissue treated with pronase at a concentration of 0.001% for 1–2 minutes (see Results) as well as nontreated material were cut for electron microscopy. The sections were mounted on Formvar-coated, single-slot nickel grids. A series of 5 to 20 grids were designed as follows. Usually, 5 sections were placed on the first grid and 3 on the second grid of the series, followed by another 5 sections, and so on.

In each series, the grids carrying 3 sections were processed for GABA immunocytochemistry. These sections were treated with 1% periodic acid and 1% sodium periodate for the etching of the resin and the removal of the osmium. After washing, grids were sequentially placed on drops of: 5% ovalbumin, rabbit anti-GABA serum (Code no. 9, diluted 1:1,000 to 1:3,000), Tris buffer containing 1% bovine serum albumin and 0.5% Tween 20 at pH 7.4, colloidal gold (15 nm) coated with goat antirabbit IgG (Bioclin, U.K., diluted 1:20 to 1:40 in the previous solution). Between these steps, grids were washed in TBS. Following the incubation, grids were washed in filtered distiled water and some of them were contrasted with a fresh solution of lead citrate (Reynolds, '63).

Evaluation

The quantitative distribution of cholinergic synapses on different postsynaptic elements was performed as follows. A grid was chosen at about the middle of the series and all vesicle-containing profiles that were ChAT positive (i.e., contained electron-dense peroxidase reaction end-product) were located. All ChAT-positive profiles on the grid were mapped irrespective of whether they were in close apposition to potential or real postsynaptic elements. The immunopositive profiles were then followed through the series of grids and photographs were taken at regular intervals. When synaptic contacts made by ChAT positive profiles

were found, they were usually photographed in every section. Once a postsynaptic target was identified, it was followed from section to section until a GABA-immunoreacted grid was reached. The postsynaptic element was then photographed in several sections irrespective of whether it was still in contact with the ChAT-positive fibre. All photographs were taken at magnifications varying from 14,000 to 21,000×, and were enlarged to about 35,000–40,000× final magnification.

Postsynaptic elements were evaluated for GABA immunoreactivity by a quantitative criterion. The sectioned area of dendrites postsynaptic to ChAT-positive terminals was measured from electron micrographs of GABA-reacted sections by a planimeter, and the unit density of immunogold particles was calculated as described previously (Somogyi et al., '86). The density of immunogold particles was also measured over synaptic boutons forming type 1, or asymmetrical synapses within the same photograph. These terminals are not thought to contain GABA; therefore the average value calculated per unit area on each micrograph was considered to represent the technical noise (background) in the procedure. Structures with gold particle density at least 3 times of the background were considered to be GABA-positive.

RESULTS

Effects of protease treatment on ChAT immunoreactivity

Proteolytic treatment was used to enhance the immunoreactivity of tissue for ChAT in material that was fixed with glutaraldehyde at a concentration sufficient to retain GABA immunoreactivity for the immunogold procedure. Pronase treatment substantially increased the density of immunoreactive axons and terminals as compared to nontreated sections from the same area of the brain. This increased reactivity resulted from 2 factors, a more intense reaction in the surface layers of the sections and the deeper penetration of immunoreactivity into the section. Usually the whole thickness of the 60–80-μm thick section was reactive in pronase treated sections, whereas only the surface layers showed immunolabeling in untreated sections.

The effects of the enzyme treatment were most pronounced in material fixed with higher concentrations of glutaraldehyde. In general, the higher the glutaraldehyde concentration, the longer enzyme treatment was needed to produce optimum results. For light microscopy the use of low (0.1%) concentrations of glutaraldehyde required 0.01% enzyme for 1–2 minutes, or 0.001% enzyme for 5–10 minutes. The use of higher concentrations (1%) of glutaraldehyde required at least 2 minutes (0.01% enzyme) or 10 minutes (0.001% enzyme) treatment. Increasing the concentration of the enzyme or prolonging the treatment time resulted in gradual degradation of the integrity of the tissue, as detected by electron microscopy. The enzyme did not affect the sections evenly probably because its penetration depended on time. Thus the surface of the 60–80-μm thick sections always had poorer fine structural preservation than the deeper levels within the same section, which may not be reached by the enzyme. Nevertheless, even the well-preserved layers of the sections showed increased immunoreactivity, probably resulting from the enhanced penetration of antibodies. This has made it possible to obtain efficiently an appropriate sample size of elements postsynaptic to ChAT-positive terminals.

Controls

Peroxidase reaction product, other than associated with erythrocytes, was not observed when the primary or the secondary antibodies were omitted from the incubation sequence either in pronase-treated or nontreated material. In addition, two other monoclonal antibodies to receptor proteins did not produce staining of fibres resembling that obtained with antibodies to ChAT. Therefore it was concluded that the peroxidase reaction end-product-containing elements in the pronase-treated and untreated tissue correspond to structures that contain ChAT.

Light microscopic observations

ChAT-positive puncta and varicosities of fibres were found in all layers of the cat visual cortex (Fig. 1), as reported previously (Stichel and Singer, '87). In general, the varicosities were small and elongated (Fig. 1). The size of the varicosities did not seem to be related to the size of the fibres. Thin fibres had large varicosities, whereas the opposite was also observed (Fig. 1). The density of the positive elements was slightly higher in layer I than in other laminae (Stichel and Singer, '87). Many ChAT positive fibres of this layer were thick main axons and had a horizontal course parallel to the pial surface. In other cortical layers, the fibres appeared randomly oriented. Large fibres were also present in the white matter, running parallel with the cortical layers. These fibers were probably axons coming from subcortical structures en route to the cortical gray matter. As reported previously (Stichel and Singer, '87), no cell bodies showed immunoreactivity in area 17 or in any of the surrounding cortical areas with the methodology used in the present study, despite the consistent and reproducible staining of fibres and varicosities in all 3 cats. The absence of ChAT-positive cell bodies in the cortex of the cat is in contrast to the staining of numerous cells in the basal forebrain of these same cats, or in the cerebral cortex of the rat in the present technical trials and shown earlier (Houser et al., '85; Eckenstein et al., '88; Lysakowski et al., '89).

Electron microscopic observations

Two different, clearly distinguishable immunocytochemical labels were present in the sections that were reacted for both ChAT and GABA immunoreactivity. ChAT immunoreactivity was demonstrated by the peroxidase method resulting in fine, granular, electron-dense material within the immunopositive profiles (Figs. 2A,C, 3A,C, 4A, 5A,B); this reaction end-product was precipitated on all intracellular organelles. In contrast GABA-immunoreactivity was visualized by discrete colloidal gold particles 12–17 nm in diameter (Figs. 2B,D, 3B,D, 4B, 5C–E). Neuronal processes were considered GABA immunopositive when the density of gold particles was at least 3 times higher than that over boutons forming asymmetrical or type 1 synaptic contacts. In the majority of cases, the density difference was much higher than 3 times, approaching infinity when the boutons forming type 1 synapses were free of gold particles. Processes were considered immunonegative if the gold density was equal or lower than that over nearby boutons forming type 1 synaptic contacts. Processes with intermediate gold density were placed into a "not known" category with regard to GABA immunoreactivity (Table 1). Treatment of the animal with the GABA transaminase inhibitor GVG did not appear to increase the proportion or change the distribu-

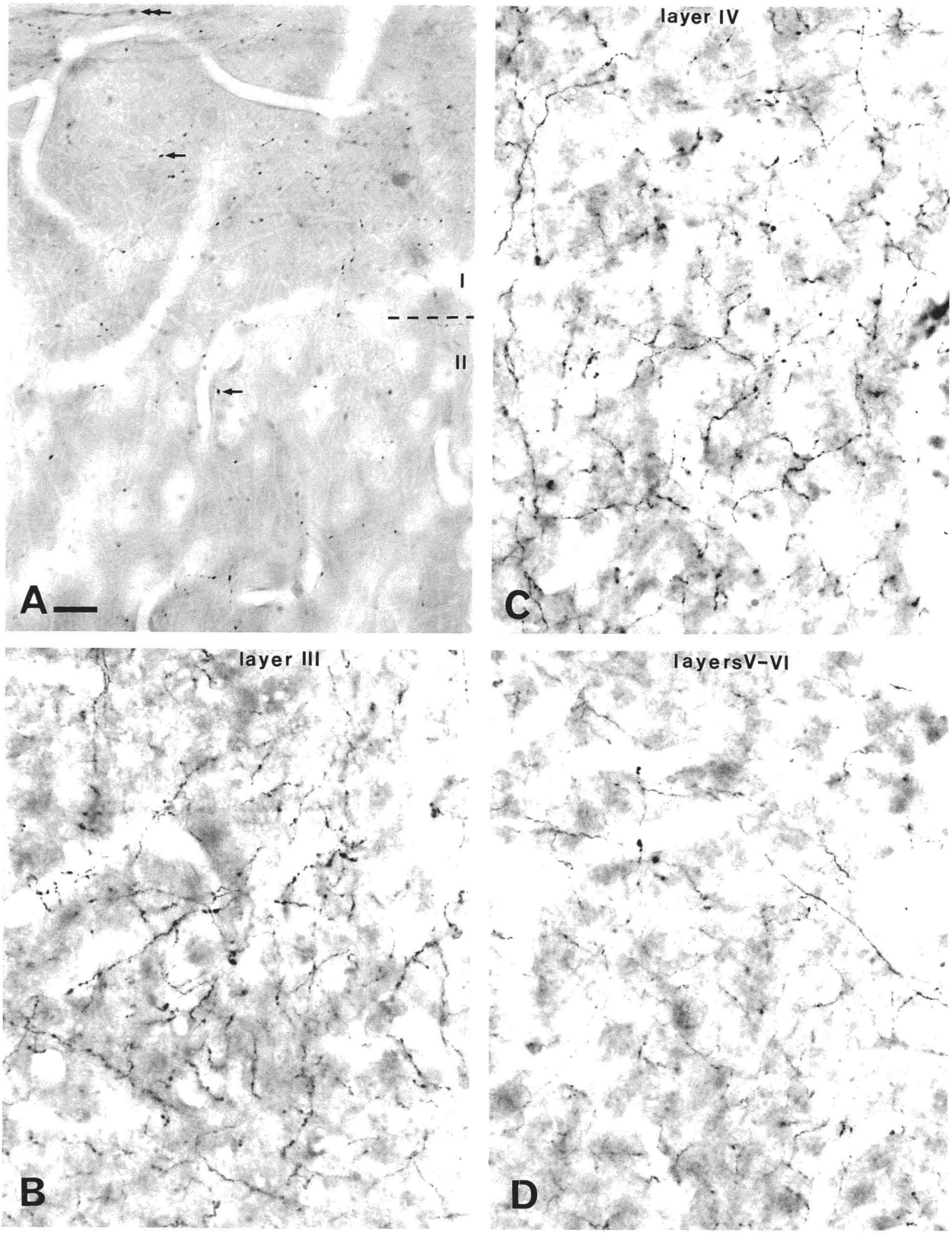

Fig. 1. Light micrographs of cat striate cortex immunoreacted for ChAT. **A.** Layers I and II shown in an osmium treated section similar to those used for obtaining electron microscopic data. Note the numerous immunopositive varicosities (e.g. arrows), and a thick fibre (double arrow) running parallel with the pial surface. **B–D.** Layers III, IV, and V–VI shown in sections treated with detergent before the immunoreaction, and not treated with osmium. Note the numerous fibres with varicosities, and the thickening of the fibres (as compared to A) due to the diffusion of the reaction product. All sections were treated with pronase. Scale applies for all figures: 20 μm.

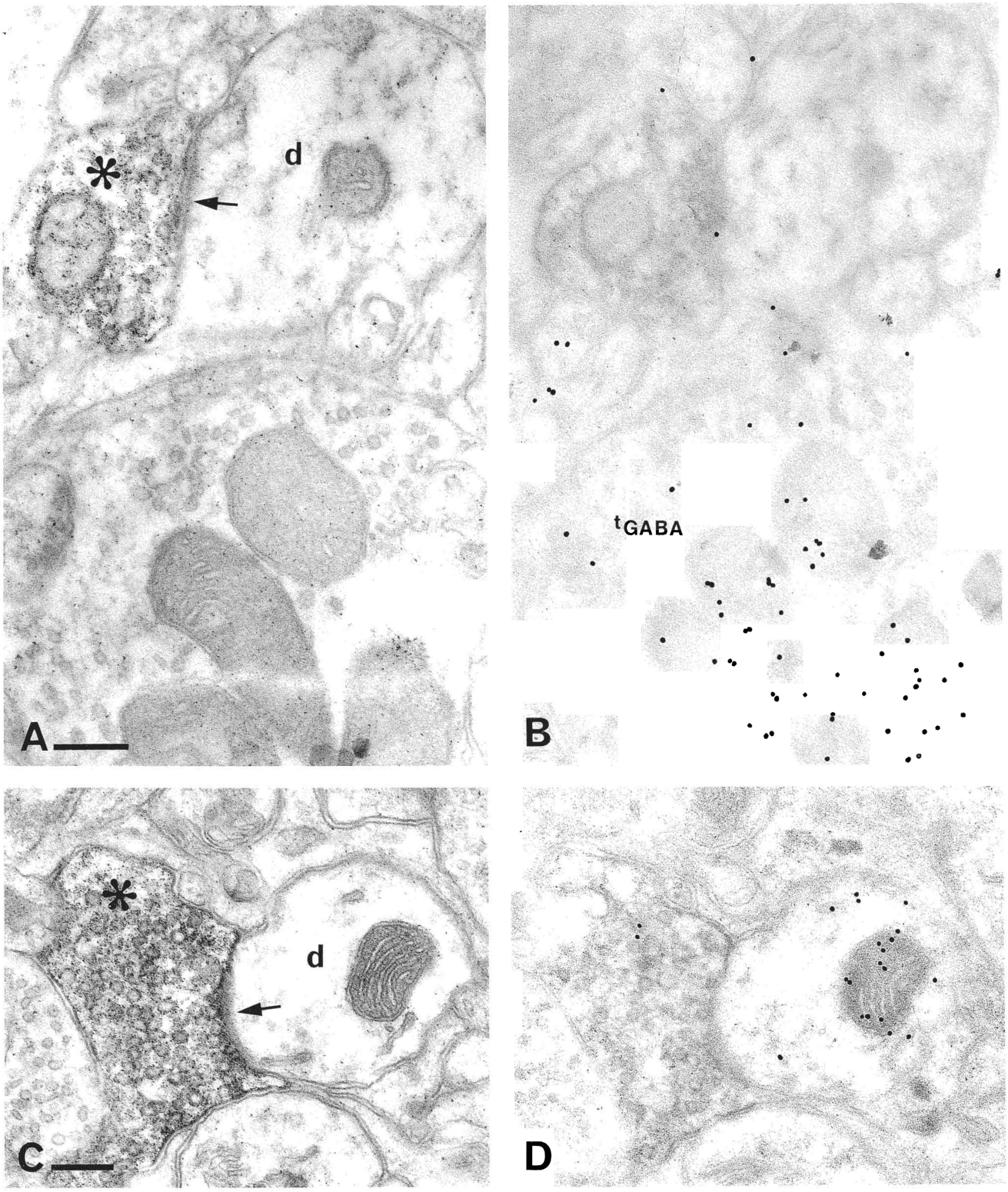

Fig. 2. Electron micrographs of ChAT immunoreactive nerve terminals (asterisks) demonstrated by the pre-embedding immunoperoxidase method in layer III of the cat's striate cortex. **A** and **C.** The terminals form synapses (arrows) with dendrites (d). **B** and **D.** Sections serial to A and C, respectively, also reacted to reveal GABA immunoreactivity by the postembedding immunogold method. In D, the selective distribution of gold particles over the postsynaptic dendrite (d in C) demonstrates that it belongs to a GABAergic neuron; in B, the postsynaptic dendrite (d in A) is immunonegative for GABA. A nearby nerve terminal (tGABA) is immunopositive. A and B. Untreated cat, tissue not treated with pronase. C and D: GABA transaminase inhibitor treated cat, pronase treated tissue. Scales: 0.2 μm.

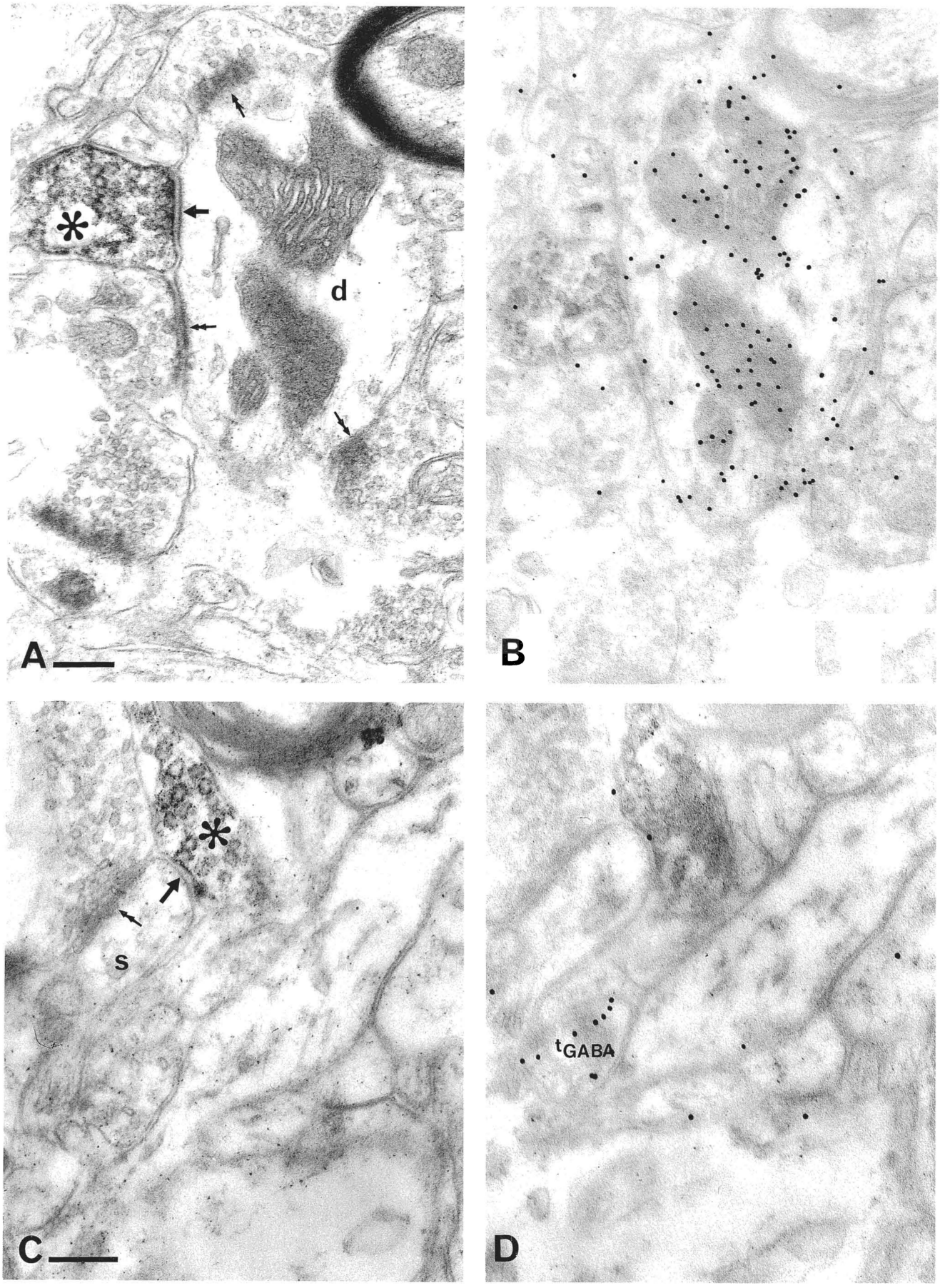

Figure 3

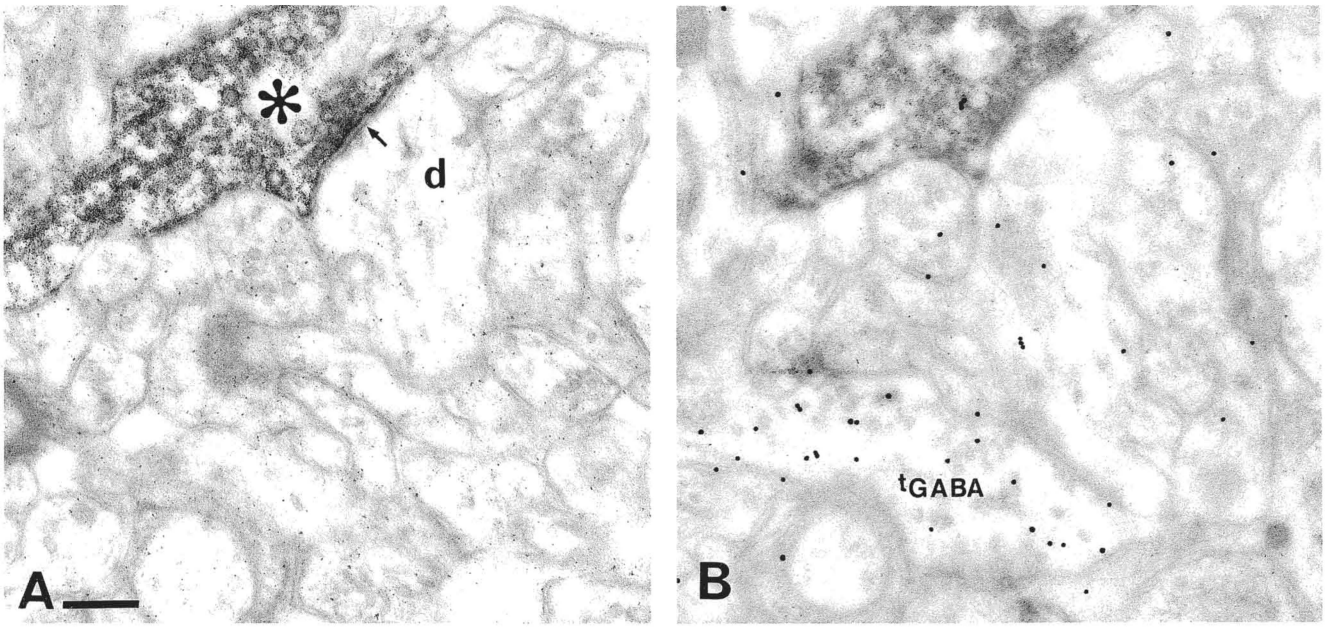

Fig. 4. Electron micrographs of a ChAT immunoreactive nerve terminal (asterisk) in layer V demonstrated by the pre-embedding immunoperoxidase method. **A.** The terminal forms a synaptic contact (arrow) with a dendritic shaft (d) identified by the presence of microtubules. **B.** Serial section also treated to reveal GABA immunoreactivity by the postembedding immunogold method. The scattered gold over the dendrite is at background level, therefore the dendrite was classified as GABA-immunonegative. A nerve terminal (tGABA) next to the same dendrite is immunopositive (tGABA). Untreated cat, tissue treated with pronase. Scale: 0.2 μm.

tion of GABA-immunopositive neuronal structures; therefore data from the treated and untreated animals were pooled.

ChAT-positive synaptic terminals were found in all laminae of the cat visual cortex (Table 1). Immunopositive terminals were usually small in size (e.g., Figs. 2, 3) and formed synapses mainly with dendritic profiles (Figs. 2, 4). Postsynaptic membrane specialization could not always be observed at the junctions even when the postsynaptic membrane was cut close to right angle (Fig. 3C). However, when the postsynaptic density was present, the dimensions of the dense material were variable (Figs. 2A, 3A, 4A). Thus the chemically identified synapses in our study could not be easily classified into the two classical groups of asymmetric and symmetric synaptic contacts, as defined previously on a morphological basis (Gray, '59; Beaulieu and Colonnier, '85).

In the striate cortex of two cats, 162 synaptic contacts established by 136 ChAT-positive, vesicle-containing varicosities were analyzed in serial sections. The majority of cholinergic terminals made only one synaptic contact. The average number of synapses per bouton was 1.2, and in this parameter there was no significant difference between cortical layers.

The postsynaptic elements of 157 synaptic contacts could be positively identified (Table 1). Postsynaptic structures to ChAT positive boutons were dendritic shafts (84.6%), dendritic spines (11.1%), and neural somata (1.2%, Table 1). Dendritic shafts were identified by the presence of mitochondria and microtubules (Figs. 2, 3A, 4A). Rarely, dendritic shafts were identified only by the presence of microtubules. In contrast, dendritic spines lacked microtubules and mitochondria (Figs. 3C, 5A), were of small diameter, and occasionally contained a spine apparatus. It could not be decided in 5 cases (3.1%) whether the postsynaptic target was a dendritic spine or a small dendritic shaft (Table 1, Fig. 6A).

The distribution of structures postsynaptic to ChAT-positive boutons varied among the different sets of laminae (Table 1, Fig. 6A). In layers I-III and V-VI, about 90% of the synapses were on dendrites, whereas in layer IV the proportion of dendritic shafts decreased. Synaptic junctions on dendritic spines followed an inverse trend, being the most numerous amongst targets in layer IV (23.4%), and decreasing to only 3.5% in the infragranular layers. Somata were very rarely postsynaptic to ChAT-positive boutons; only 1 synapse was found in layer IV and also 1 in layers V-VI.

Altogether, 154 postsynaptic targets were successfully tested for the presence of GABA. Some of the postsynaptic targets could not be tested for their GABA content because either the grid was lost or the postsynaptic element was covered by contamination or folding. In 21 of the postsynaptic dendrites, immunoreactivity was below our cutoff level

Fig. 3. Electron micrographs of ChAT immunoreactive nerve terminals (asterisks) in layer IV demonstrated by the pre-embedding immunoperoxidase method. **A** and **C.** The terminals form synapses (arrows) with a dendritic shaft (d in A) and a spine (s in C). The dendrite receives additional synapses (double arrows), and the spine also receives a type 1 synapse (double arrow). **B** and **D.** Sections serial to A and C, respectively; also treated to reveal GABA immunoreactivity by the postembedding immunogold method. The high density of gold particles over the dendrite demonstrates that it belongs to a GABAergic neuron. The spine is immunonegative, but the tip of a nearby terminal (tGABA) is immunopositive. A and B: Untreated cat, tissue not treated with pronase. C and D: GABA transaminase inhibitor treated cat, tissue treated with pronase. Scales: 0.2 μm.

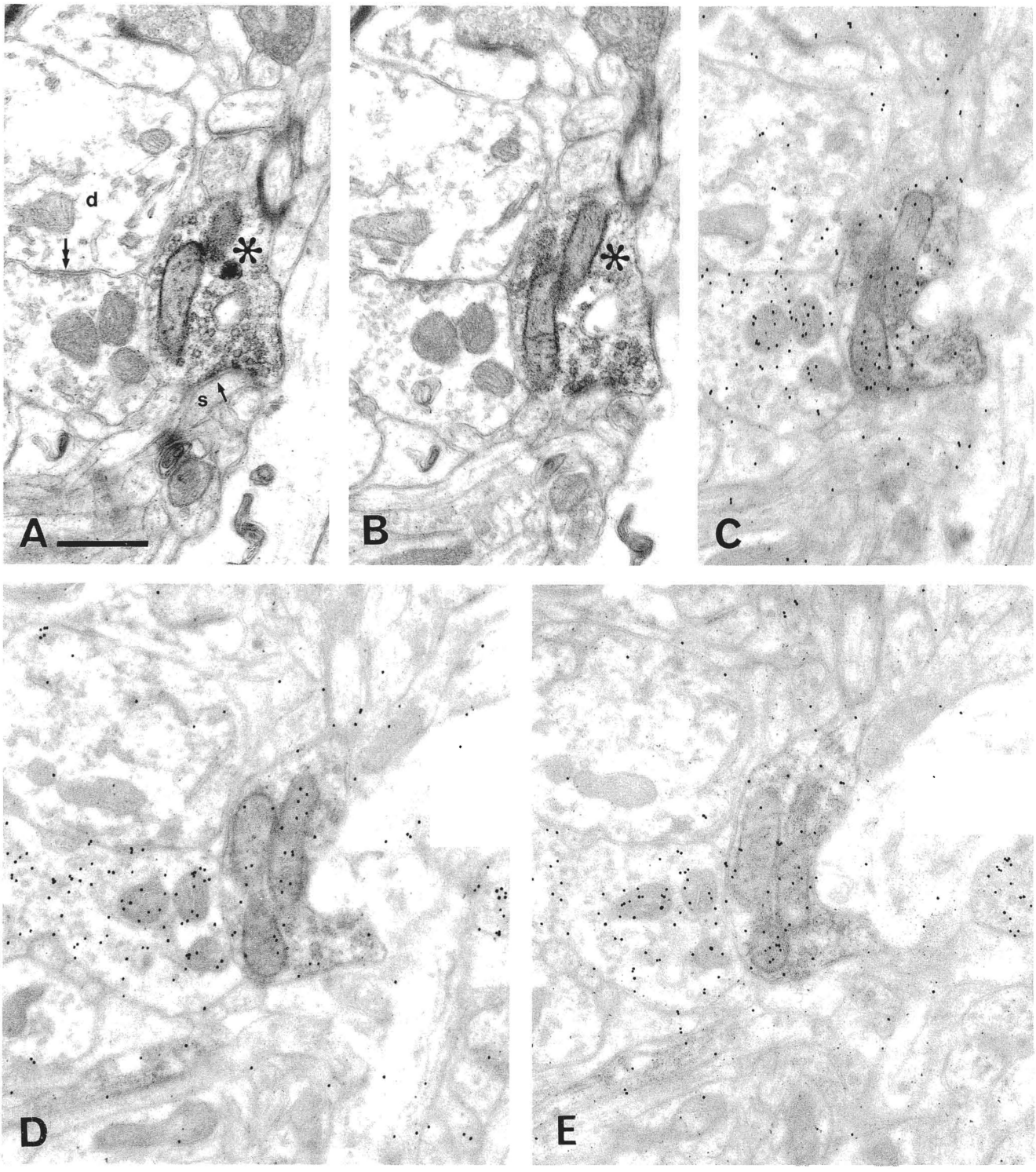

Fig. 5. Colocalization of immunoreactivity for ChAT and GABA in the same synaptic terminal shown in serial sections. Electron micrographs of a ChAT immunoreactive bouton (asterisk) in layer III demonstrated by the pre-embedding immunoperoxidase method. **A–B.** The bouton forms a synapse (arrows) with a spine (s), whereas a nearby ChAT immunonegative terminal forms a synapse (double arrow) with a dendritic shaft (d). **C–E.** Sections also treated to reveal GABA immunoreactivity by the postembedding immunogold method. The consistently high density of gold particles over both nerve terminals as well as a third one to the right demonstrates their immunoreactivity for GABA. Untreated cat, tissue not treated with pronase. Scale: 0.5 μm.

TABLE 1. Distribution of Structures Postsynaptic to Cholinergic Terminals in Layers I to VI of the Cat Striate Cortex[1]

	Postsynaptic targets of ChAT immunopositive synaptic terminals							
	dendritic shaft				spine	d. shaft or spine ?	soma	Total
Layers	GABA−	GABA+	not known	Total	GABA−	GABA−	GABA−	
I-III	25	11	12	48	5	1	—	54
IV	8	16	11	35	11	1	1	48
V-VI	41	7	6	54	2	3	1	60
I-VI	74	34	29	137	18	5	2	162
	45.7%	21.0%	17.9%	84.6%	11.1%	3.1%	1.2%	100%

[1]The majority of synaptic targets were dendritic shafts. A substantial proportion of the dendrites were immunoreactive for GABA, and therefore belong to inhibitory neurons. Some postsynaptic profiles that did not contain mitochondria could not be classified as spines or dendritic shafts and are therefore listed under the heading "d. shaft or spine?".

for positive dendrites, but above nearby boutons with type 1 synapses. These together with 8 untested dendrites formed the 17.9% category in Table 1 whose identity with regard to their origin from GABAergic or non-GABAergic neurons was not known. Technical factors could lead to an underestimation of the GABA-positive dendrite population. For example, the immunoreactivity of dendrites is generally weaker than that of the GABA-positive boutons, and the gold particles are often restricted to the mitochondria. Mitochondrial proteins probably act as a substrate for the chemical coupling of GABA by the fixative. However, this does not change the selectivity of the method. Since all mitochondria are consistently immunopositive for GABA in some dendrites, but not in adjacent ones, postsynaptic dendrites without mitochondria may be falsely classified as immunonegative for GABA. Furthermore, some populations of GABAergic cells may have less GABA in the dendrites, which is below the level detected with our method. These factors would lead to false negative results; therefore when calculating the overall proportion of GABA-positive and GABA-negative postsynaptic elements, it was assumed that the "not known" category shown in Table 1 would contain dendrites of the two populations in the same proportion as those characterised unequivocally for their GABA content. Expressed in this way, 31.5% of the postsynaptic dendrites were GABA-positive (Figs. 2D, 3B) and therefore originated from nonpyramidal inhibitory cells. This proportion of GABA-positive dendrites is much higher than that found for the overall population of those boutons establishing symmetrical synapses and containing GABA. In the latter case, only 14% of the postsynaptic dendritic shafts were GABA-positive (Beaulieu and Somogyi, '90; see also Fig. 6B). All 18 dendritic spines were successfully tested and were found to be GABA-negative (Fig. 3D), as were the 2 postsynaptic somata.

An interesting laminar difference was observed in the distribution of GABA-positive postsynaptic dendrites (Table 1, Fig. 6A); they represent 30.6% of the dendritic shafts postsynaptic to ChAT-positive boutons in the supragranular layers and only 14.6% in the infragranular layers. However, in layer IV 66.7% of dendrites postsynaptic to cholinergic terminals originated from GABAergic neurons.

Colocalization of ChAT and GABA in the same synaptic terminals

Some of the ChAT-positive terminals were found to have a high density of GABA-immunogold labeling (Fig. 5). This was not caused by the binding of the anti-GABA antibodies or the gold particles to the peroxidase reaction end-product, or cross reaction with the immunoreagents of the first reaction, because ChAT-positive terminals that had no gold particles were found in the same sections in all cases. The immunogold labeled terminals were consistently labeled from section to section and, in every positive case, at least two GABA-reacted sections were evaluated (Fig. 5). The density of gold particles over the ChAT/GABA-positive boutons was usually slightly lower than that found over GABA-positive terminals, which were ChAT-negative. This may be due to a lower concentration of immunoreactive material, although it is also possible that the peroxidase reaction end-product, or the first layer of antibodies, masked some of the antigenic sites for the GABA immunoreaction. The latter possibility is more likely since, when the DAB reaction was weaker, the gold immunolabeling was more pronounced. There was no difference in the fine structural characteristics of these double-labeled terminals and the GABA-negative population of ChAT-positive boutons (Fig. 5).

Overall, 10 (8.2%) of the 122 ChAT-positive axon terminals that were tested were also GABA-positive (Table 2). Of these, 8 were found in the normal animal and 2 in the GVG-treated animal. These boutons made a total of 10 synaptic contacts with different postsynaptic elements. The postsynaptic elements were: 8 dendritic shafts (2 of which were GABA-positive, 1 in layer IV and 1 in layer V), 1 dendritic spine, and 1 soma. Thus the synaptic contacts made by boutons immunoreactive for both ChAT and GABA follow a similar pattern of distribution to that described for the overall population of ChAT positive synapses.

DISCUSSION

Cortical targets of cholinergic synapses

The majority of neuronal elements postsynaptic to ChAT-positive synapses were dendritic shafts, confirming previous studies (Wainer et al., '84; Houser et al., '85; de Lima and Singer, '86; Parnavelas et al., '86). Many of the dendrites had characteristics of those shown to originate from spiny cells; they received few synapses, contained parallel arrays of microtubules and few, small diameter mitochondria, and they emitted occasional spines. These dendrites together with the majority of spines postsynaptic to cholinergic terminals probably originate from pyramidal and spiny stellate cells. However, we also found that a high proportion of the dendrites originated from GABAergic neurons. Although for the total cortical depth, the GABA-positive elements are only about one-third of all targets, this is *the highest proportion found for the postsynaptic elements of any type of extra- or intracortical afferents to the*

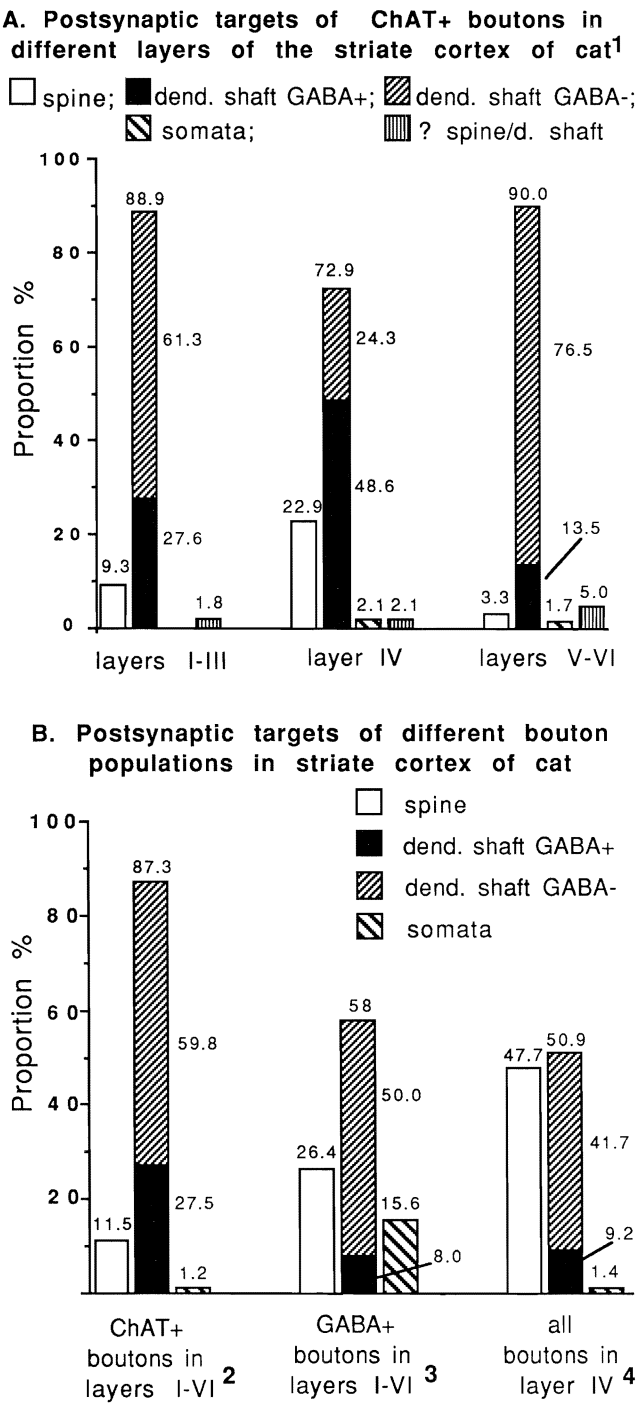

Fig. 6. **A.** Laminar distribution of targets postsynaptic to ChAT immunopositive synaptic terminals. (See FN 1 in A.) The division of all dendritic shafts into GABA-negative and GABA-positive populations is based on the results of the unequivocal reactions. As seen in Table 1, some of the dendritic shafts could not be classified with the criterion described in Methods. The immunoreaction for GABA may produce false negative results therefore the values for GABA+ targets should be considered as a minimum. **B.** Comparison of synaptic targets of characterized nerve terminal populations in the striate cortex of cat. (2) Data based on Table 1, but the unidentified targets (either dendritic shafts or spines) were distributed between the dendritic shaft and spine categories in proportion to the identified targets of the same type. (3) Data from Beaulieu and Somogyi ('90). (4) Data recalculated from Bueno-Lopez et al. ('89).

TABLE 2. GABA Immunoreactivity of Cholinergic Synaptic Boutons in the Striate Cortex of the Cat

Layers	ChAT immunopositive synaptic terminals				
	GABA immunoreacted			untested	total
	GABA+	GABA−	equivocal		
I-III	4	38	2	4	46
IV	4	25	2	6	37
V-VI	2	44	1	4	53
I-VI	10	107	5	14	136

The terminals were immunoreacted under pre-embedding conditions for ChAT using an immunoperoxidase method, and for GABA under postembedding conditions using an immunogold method. Each terminal was examined in several serial immunogold-reacted electron microscopic sections. Terminals with at least 3 times higher gold particle density than nearby terminals forming type 1 synaptic contacts were classified as GABA-positive. A few terminals had gold particle density between 1–3 times of those terminals forming type 1 synaptic contacts and were classified as "equivocal".

neocortex (for review see Somogyi, '89). The bias for GABAergic neurons in the visual cortex of the cat is clear when one makes comparisons with other populations of synaptic boutons that have been studied quantitatively. For example, among the targets of all synapses in layer IV, only 9.2% of targets, all dendritic shafts, were GABA-positive (Bueno-Lopez et al., '89, see also Fig. 6B). Similarly, among the neuronal elements postsynaptic to GABA-immunoreactive boutons in all layers of the cortex, only about 8% were also GABA-positive (Beaulieu and Somogyi, '90; see also Fig. 6B). Thus the cholinergic terminals studied here form synapses with GABAergic dendrites at least 3 times more frequently than expected on the basis of random distribution on all postsynaptic elements in the neocortex. The distribution is even more biased in layer IV, where the proportion of GABAergic targets is more than 5 times of that expected from random distribution on all postsynaptic sites. It is possible that most spines are not available as targets for cholinergic synapses; therefore one should take into account only the dendritic shafts. Dendrites form 50.9% of all postsynaptic elements to all boutons, and 18% of them are GABA-positive (Bueno-Lopez et al., '89). In contrast 66.6% of dendrites postsynaptic to cholinergic terminals in layer IV are GABA-positive, giving a 3.7 times preference of cholinergic terminals for dendrites originating from GABAergic neurons, as compared to the average bouton in layer IV.

Recently, in the rat a GABAergic projection has been demonstrated to the hippocampus from the basal forebrain that was shown to make synapses predominantly with other GABAergic neurons (Freund and Antal, '88), and a similar pathway to the neocortex has been also reported in a preliminary study (Freund and Gulyas, '89). This pathway probably has an even greater bias for terminating on GABAergic neurons than the cholinergic projection.

The values obtained above for the bias in the termination of cholinergic boutons for GABAergic cells should be considered as a minimum, since it is likely that the postembedding immunoreaction somewhat underestimates the overall population of dendrites that originates from GABAergic neurons. Nevertheless, the comparison with the targets of other populations of boutons is reasonable, because the data were all obtained with the same method. The comparisons would only be invalid if the cholinergic synaptic population was selective to particular subpopulations of GABAergic cells containing high level of GABA in their dendrites. This is an interesting possibility for the cholinergic terminals, since many of the dendrites showed charac-

teristics of previously identified basket cells (Somogyi et al., '83; Kisvarday et al., '85). However, more direct information is needed using double-labeling experiments.

Cholinergic influence on GABA-mediated inhibition

The results provide evidence for previous suggestions that a major synaptic target of cholinergic afferents in the cat's visual cortex are inhibitory local circuit neurons (de Lima and Singer, '86; Müller and Singer, '89). The preferential innervation of GABAergic cells by cholinergic synapses, if accompanied by a similar enrichment of postsynaptic receptors, can explain some of the physiological effects of acetylcholine on cortical neurons. Both extracellular (Müller and Singer, '89) and intracellular (McCormick and Prince, '86; McCormick, '89) recording demonstrated that acetylcholine can evoke inhibitory effects on visual cortical cells, which is blocked by bicuculline as well as by muscarinic antagonists. These results were interpreted as evidence for inhibition mediated by GABA released from local cortical cells activated by ACh. In the cortex it is unlikely that bicuculline would directly block nicotinic cholinergic receptors as is the case for insect neuronal somata (Benson, '88). However, cortical neurons synthesize nicotinic ACh receptors (Wada et al., '89), and a high density of nicotinic binding sites has been found in the cortex, particularly in layer IV (Clarke et al., '85), where most inhibition is elicited by ACh. Furthermore, nicotinic inhibition has been demonstrated in the rat septum (Wong and Gallagher, '89), and weak nicotinic inhibition has also been found in cortical neurons (McCormick and Prince, '85). Nevertheless, in the cortex, the available data are consistent with the view that the cholinergic input exerts its suppressive influence mainly via the GABAergic neuronal system rather than through inhibitory ACh receptors (Prince and Huguenard, '88; McCormick, '89).

Intracellular recording in vitro has indicated that, at least in the guinea pig cortex, the effect of ACh on presumed pyramidal cells and on presumed inhibitory local circuit neurons is different (McCormick and Prince, '86). Presumed pyramidal cells responded to ACh with a slow muscarinic facilitation often preceded by a fast hyperpolarization, thought to be mediated by GABA. In contrast, presumed inhibitory neurons, characterised on the basis of their biophysical properties, responded with a fast excitation, possibly mediated by M2 receptors (McCormick and Prince, '86). Similarly activated cells have also been recorded extracellularly in the cat striate cortex in vivo (Müller and Singer, '89). Thus the faster activation of presumed inhibitory interneurons is in agreement with the GABA-mediated fast inhibitory action evoked by exogenously applied ACh in presumed pyramidal cells. However, these experiments do not explain why such inhibitory effects are not detected in presumed GABAergic neurons, which, at least in the cat, receive as much GABAergic input as pyramidal cells. Since direct local GABAergic input has been demonstrated to GABAergic neurons, particularly in layer IV (Kisvarday et al., '85; Somogyi and Soltesz, '86; Somogyi, '89), one would expect that the initial inhibitory effect mediated by GABA should also be detected in cortical GABAergic cells. One possible explanation for this lack of effect may be that the particular class of GABAergic cell mediating cholinergic inhibition does not make synapses with other inhibitory cells. One such GABAergic neuron is the chandelier cell that terminates only on pyramidal cells (for rev. see Somogyi, '89), but whether this particular type of neuron is activated by cholinergic input is not known.

Cortical activation by cholinergic afferents

There is general agreement that cholinergic afferents to the neocortex and hippocampus facilitate neuronal responsiveness (e.g., Krnjević and Phillis, '63b; Krnjević et al., '71; Singer, '79; Buzsáki et al., '88). For example, without normal cholinergic input from the basal forebrain, neurons in the visual cortex of the cat are only weakly or not at all responsive to visual stimulation (Sato et al., '87a). The direct termination of two-thirds of the cholinergic synapses on pyramidal and other non-GABA-positive cells is in line with the facilitatory effect expected of the cholinergic system and is in agreement with the predominantly facilitatory effect of ACh on about 60% of visual cortical neurons (Sillito and Kemp, '83; Sato et al., '87b; Müller and Singer, '89).

However, the enriched cholinergic input to cortical inhibitory neurons seems paradoxical for a pathway that is thought to be involved in the activation of cortex. Unfortunately, there does not seem to be direct information on the effect of basal forebrain cholinergic neurons on identified cortical GABAergic cells. An inhibitory effect of ACh on GABAergic cells would produce disinhibition in cortex and would thus provide a simple mechanism for cortical activation. In the hippocampus, a disinhibitory action of ACh has been recorded (Ben-Ari et al., '81; Krnjević et al., '81) but was thought to be mediated by a presynaptic action. An inhibitory action of ACh on cortical GABAergic cells cannot be excluded, particularly if it was mediated by receptors restricted at synaptic sites and therefore not revealed by ionophoretic application of ACh. Nevertheless the experimental results obtained with exogenously applied ACh suggest an activation of GABAergic cells (McCormick and Prince, '85; Müller and Singer, '89).

The time course of the facilitatory and inhibitory effects of ACh are probably of great significance; the inhibitory effect largely precedes the slow muscarinic facilitation (McCormick and Prince, '85, '86; McCormick, '89). Most of the fast inhibition is mediated by the local cortical neurons, but a corelease of GABA and ACh from some of the cholinergic terminals (see below) would lead to the same result because of the difference in duration of $GABA_A$ or muscarinic receptor mediated effects. The two mechanisms could serve to increase the specificity of the response of cortical cells to the incoming sensory stimuli mediated by thalamic input. In the visual cortex, the block of GABAergic inhibition by bicuculline leads to a deterioration of response specificity (for review see Sillito, '84). Thus it seems that specific response to sensory stimuli requires robust GABAergic inhibition and this may explain the biased cholinergic input to cortical GABAergic neurons. Indeed, facilitatory effects produced by ACh increase the responses to visual stimulation with little effect on the selectivity of receptive field properties such as orientation and direction tuning (Sillito and Kemp, '83; Sato et al., '87b). The maintenance of stimulus selectivity in the response, as opposed to possible rhythmic activity intrinsic to cortex and reflected in the EEG (see Buzsáki and Gage, '89), requires an efficient operation of certain inhibitory neurons. The activation of GABAergic cortical cells by the cholinergic afferents may underlie the reduction in spontaneous activity observed during arousal, together with an increase in direc-

tional selectivity in the visual cortex (Livingstone and Hubel, '81).

The basic mechanism of ACh-mediated activation may be similar in the whole of the cortical mantle. For example, cholinergic innervation of GABAergic cells has also been shown in the hippocampus (Leranth and Frotscher, '87). Although the quantitative distribution of synapses between the inhibitory and principal cells is not known, it has been suggested that the muscarinic antagonist atropine enhances the population activity in the hippocampus by preventing the normal cholinergic activation of inhibitory neurons by septal afferents (Buzsáki et al., '83).

Laminar differences in targets and influence of cholinergic synapses

Layer IV is the main recipient of thalamic input and, in this layer, the cholinergic terminals show the strongest bias for GABAergic cells (50% of targets). This is in excellent agreement with physiological studies, which demonstrate that inhibitory effects evoked by ACh are observed in up to half of the cells in layer IV (Sato et al., '87b); most of the inhibitory effects were also observed in layers III–IV in another study (Sillito and Kemp, '83). Similar agreement was found between the mainly facilitatory effect of ACh in layer V–VI (Sillito and Kemp, '83; Sato et al., '87b), and the low proportion of GABAergic targets in our study. These laminar differences indicate that in layer IV, at the first stage of the processing of thalamic input, the cholinergic afferents exert substantial inhibitory influence in order to raise the threshold and specificity of cortical neuronal responses. Once the correct level of activity has been set at the level of layer IV, the influence can be mainly facilitatory in the other layers.

GABA in cholinergic terminals

At least 8% of the total population of ChAT-positive terminals were also immunopositive for GABA. This should be considered as a minimum value for the following reasons. It is noteworthy that in all terminals reacting for both ChAT and GABA, the end-product of the ChAT reaction was moderate and no strongly peroxidase-labeled terminals were found to be GABA-positive. It is possible that when the end-product for the ChAT reaction is strong, the GABA immunoreactive sites are masked by the peroxidase reaction, producing false negative result in the immunogold procedure. This is supported by a previous report that demonstrated with a similar protocol that the immunoreactivity for GABA is stronger in the very same axon in regions that have less peroxidase end-product (Freund, '89).

The relatively high proportion of colocalized terminals raises the question of their origin. One possibility is that they originate from local cortical neurons since over half of the ChAT-positive neurons in the rat cerebral cortex were also found to be immunoreactive for glutamate decarboxylase (GAD), the enzyme synthesizing GABA (Kosaka et al., '88). However, in the visual cortex of the cat, very few ChAT-immunoreactive cells have been found after extensive experiments using a number of different antibodies (Stichel and Singer, '87). Thus, although the possibility of local cells contributing to the cholinergic innervation of the cat cortex cannot be completely excluded, this is at present less likely than the well-demonstrated subcortical sources.

The basal forebrain represents the major source of cholinergic input to the cerebral cortex (Lehmann et al., '80; Wenk et al., '80; Johnston et al., '81; Mesulam et al., '83; Rye et al., '84; Ingham et al., '85; Henderson, '87; Fisher et al., '88). Several studies addressed the question of whether neurons in the basal forebrain projecting to the cortex contributed to both cholinergic and GABAergic innervation of cortex. In the rat, only 0.6 to 1% of basal forebrain neurons were found to be immunoreactive for both ChAT and GABA (Brashear et al., '86; Kosaka et al., '88). In addition, GABA-containing terminals originating in the basal forebrain have been shown to innervate mainly GABAergic neurons in the cortex of the rat (Freund and Gulyas, '89). Whether they also synthesize ACh remains to be established. In the cat, 1–2% of cortically projecting neurons were thought to contain both ChAT and GAD, and a separate population of cells containing only GAD was also reported in the basal forebrain (Fisher et al., '88). The fact that, with a conservative method, we found as many as 8.2% of all ChAT-positive terminals to be immunoreactive for GABA has several explanations. For example, axons that contain both neurotransmitters may establish more synapses in the visual cortex than GABA-negative cholinergic neurons. Another possibility is that previous studies underestimate the extent of colocalization of GABA and ACh in basal forebrain neurons, since the absence of one or the other immunocytochemical marker cannot be taken as evidence for the absence of the transmitter in the terminals.

The extent of the colocalization of ACh and GABA in the basal forebrain and in the cortex are quite different from that found in the retina, where the majority of ChAT-positive amacrine cells also express immunoreactivity for GABA (Brecha et al., '88; Kosaka et al., '88; Vaney and Young, '88). Whereas ACh is thought to be released by a calcium dependent process, GABA is released by a calcium independent, reverse transport mechanism (O'Malley and Masland, '89). Thus the release of the two neuroactive substances, and particularly the time course of action, may not be parallel under all circumstances. Whether a possible differential release can also take place in the cortex and whether the two substances act pre- or postsynaptically remain to be established.

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