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GABAergic Input to Cholinergic Forebrain Neurons: An Ultrastructural Study Using Retrograde Tracing of HRP and Double Immunolabeling

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ABSTRACT

Amygdalopetal cholinergic neurons in the ventral pallidum were identified by combining choline acetyltransferase (ChAT) immunohistochemistry with retrograde tracing of horseradish peroxidase (HRP) following injections of the tracer in the basolateral amygdaloid nucleus. Although ChAT-positive terminals were identified in the ventral pallidum, they were never seen in contact with either immunonegative or ChAT-positive amygdalopetal neurons. In material, in which immunostaining against glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA was combined with retrograde tracing of HRP from the basolateral amygdaloid nucleus, GAD-positive terminals were seen to contact immunonegative amygdalopetal neurons. In addition, when sections of the rostral forebrain were processed, first to preserve and identify the transported HRP, and then were sequentially tested for both ChAT and GAD immunohistochemistry with the immunoperoxidase reaction for both tissue antigens, GAD-immunopositive terminals were seen to make synaptic contacts with cholinergic amygdalopetal neurons. These results suggest that amygdalopetal, presumably cholinergic, neurons receive GAD-positive terminals. In separate experiments using immunoperoxidase for ChAT and ferritin-avidin for GAD labeling, we confirmed the presence of GAD-containing terminals on cholinergic neurons. In addition, cholinergic terminals were seen in synaptic contact with GAD-positive cell bodies. These morphological studies suggest that direct GABAergic-cholinergic and cholinergic-GABAergic interactions take place in the rostral forebrain.

Key words: cholinergic neurons, basal forebrain, electron microscopy, double antigen labeling

The topographical organization and terminal distribution of the cholinergic projection neurons in the basal forebrain have been described in considerable detail over the past few years (Sofroniew et al., '82; Armstrong et al., '83; Mesulam et al., '83a,b; Rye et al., '84; Amaral and Kurz, '85; Carlsen et al., '85; Wainer et al., '85; Záborszky et al., '86). In the rat, the ascending cholinergic projection system originates in a widely dispersed, more or less continuous collection of aggregated and nonaggregated cells that invade many basal forebrain areas, but especially the septum, diagonal band of Broca, ventral pallidum, sublenticular substantia innominata, and peripallidal region.

Using a fluorescent retrograde marker combined with choline acetyltransferase (ChAT) immunohistochemistry, we have recently demonstrated that a significant number of the cholinergic cells in the ventral pallidum project to the basolateral amygdala (Carlsen et al., '85). It has also been shown that cholinergic cells in the ventral pallidum

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TABLE 1. Summary of the Immunocytochemical Procedures in the Experimental Animals

	Animals with HRP injections in the amygdala				
	A	B	C	D	Intact animals (E)
HRP visualization	DAB	DAB	DAB	DAB	–
1st immunocytochemical protocol	–	Anti-ChAT Antirat IgG ABC complex DAB	Anti-GAD Antisheep IgG ABC complex DAB	Anti-ChAT Antirat IgG ABC complex DAB	Anti-ChAT Antirat IgG ABC complex DAB
2nd immunocytochemical protocol	–	–	–	Anti-GAD Antisheep IgG ABC complex DAB	Anti-GAD Antisheep IgG ↓ ↓ Ferritin-avidin ABC complex DAB

are embedded in a dense GABAergic terminal network. In the present study, a double antigen label technique (Leranth et al., '85) was used in combination with HRP retrograde tracing (Záborszky and Leranth, '85) to investigate whether GABAergic axons establish synaptic contact with cholinergic amygdalopetal neurons. Part of this work has been published in a preliminary form elsewhere (Záborszky et al., '84b).

MATERIALS AND METHODS

Surgery and perfusion of the animals

Twenty-three male Sprague-Dawley rats, weighing 200±25 g, were used in this study. Surgical procedures and perfusion of the animals were performed during deep anesthesia (50 mg/kg Nembutal). In 19 animals, 0.06 μl 20% HRP (Sigma type VI) in 2% dimethylsulfoxide (Keefer, '78) was injected stereotactically into the basolateral amygdala (coordinates from Paxinos and Watson, '82: A-P=3.0 mm, L=4.6 mm, V=7.2 mm) through glass micropipettes attached to a Hamilton syringe. The tip diameter of the pipettes was 50 μm and the HRP injections were made over a period of 30 minutes. The pipette was left in place for another 10 minutes after finishing the injection to minimize diffusion of the enzyme along the pipette track. Twenty-four to 48 hours after the injection, the animals were perfused by saline followed by a solution containing 4% paraformaldehyde, 0.08% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.3) (Somogyi and Takagi, '82). Small tissue blocks containing the ventral pallidum were immersed in glutaraldehyde-free fixative overnight at 4°C. After several rinses in PB, 40-μm Vibratome (Lancer) serial sections were cut and collected in four compartments containing ice-cold buffer. Alternate sections were processed as follows (Table 1).

HRP visualization (Table 1A)

To visualize the retrograde tracer HRP, sections containing the rostral forebrain and the injection site in the amygdala were treated with a freshly prepared solution of 50 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB), 200 mg beta-D-glucose, 40 mg ammonium chloride, and 0.3 mg glucose oxidase (Sigma type VII) per 100 ml of 0.1 M PB for 60–90 minutes at room temperature (modified from Itoh et al., '79). In three animals, the retrogradely transported HRP was visualized with the regular DAB reaction (0.06% DAB in 0.002% H_2O_2 in 0.05 Tris-HCl buffer, pH 7.6, 10 minutes).

Combined retrograde HRP tracing and ChAT immunohistochemistry (Table 1B)

In order to preserve both the reaction product from the transported HRP and the peroxidase-labeled tissue antigen, we used a sequential protocol as described recently (Oldfield et al., '83; Záborszky and Leranth, '85). Briefly, sections were processed as above to localize the transported HRP, after which an immunohistochemical technique was used for ChAT staining. After finishing the HRP reaction, the wet sections of the ventral pallidum were mounted from PB on glass slides and subsequently coverslipped in order to photograph the retrogradely labeled cells. Following rinses in several changes of PB, the sections were collected in vials containing 10% sucrose in PB. The vials were placed in liquid nitrogen until the fluid was frozen, after which the contents were thawed to room temperature. Immunostaining was carried out with the avidin-biotin-peroxidase technique according to Hsu et al ('81). Following application of the rat anti-ChAT monoclonal antibody (Eckenstein and Theonen, '82) at a 1:10 dilution in PB containing 0.25%, lambda carrageenan (Sigma) and 0.1% sodium azide for 36–48 hours at 4°C, sections were incubated in the following solutions at room temperature: (1) biotinylated antirat IgG (Vector Labs., 1:100 dilution in PB, 2 hours), (2) the ABC complex (Vector Labs., 1:500 dilutions in PB, 2 hours, and (3) DAB as described above.

Combined retrograde HRP tracing and GAD immunohistochemistry (Table 1C)

Following the DAB-glucose oxidase reaction, photography, and freeze-thawing of the sections containing the ventral pallidum, sections were processed for glutamic acid decarboxylase (GAD) immunoreactivity with the ABC technique of Hsu et al. ('81). Application of sheep anti-GAD (prepared by Oertel et al., '81) at 1:2000 dilution in 0.25% lambda carrageenan-PB for 48 hours at 4°C was followed by incubation at room temperature in biotinylated anti-sheep IgG (Vector Labs., dilution 1:100, in PB, for 2 hours). The sections were then incubated in the ABC complex and the DAB solution as described above.

Combined retrograde HRP tracing and ChAT and GAD immunohistochemistry using DAB for both antigens (Table 1D)

In this experiment, sections were processed sequentially for HRP visualization and ChAT and GAD immunohistochemistry (see Table 1). Although the heavy punctate GAD immunoreactivity covered the area of the ventral pallidum, continuous light microscopic monitoring allowed the detection and localization of retrogradely labeled cholinergic neurons for subsequent processing for electron microscopy.

Combined ChAT and GAD immunohistochemistry using two different markers (DAB and ferritin) for the two antigens (Table 1E)

Forebrain sections from two normal animals were processed for ChAT immunohistochemistry by using the ABC

technique of Hsu et al. ('81). After finishing the DAB reaction and a thorough rinsing in PB, sections were incubated in sheep anti-GAD and biotinylated antisheep IgG as described above. The sections were then divided into two groups, *a* and *b*. Sections in group *a* were treated with Ferritin Avidin D (Vector Labs., 1:80 dilution in 0.1 M PB, 12 hours, 4°C). Since in this case the GAD staining is not visible at the light microscopic level, sections in group *b* were incubated in the ABC complex and in DAB in order to make sure that the second antibody staining was successful.

Controls

Controls for those experiments in which either anti-ChAT or anti-GAD was used included omission of primary antisera (ChAT or GAD) or the use of preimmune serum or normal sheep serum (for GAD), but processing with the corresponding linking antibodies, the ABC complex and DAB (controls 1, 2; Table 2). Cross-reactivity between the individual immunoreagents, or between the two secondary antibodies in the double-immunoperoxidase experiments, was tested by processing sections in the full immunohistochemical sequence with one or both primary antibodies deleted and with anti-ChAT antibody replaced with PB and anti-GAD antibody replaced with normal sheep serum, preimmune serum, or PB (controls 3–5). Controls for the combined immunoperoxidase-ferritin experiments included four different protocols (controls 6–9). Sections from intact animals were processed only for ChAT or GAD immunoreactivity. ChAT was visualized with the ABC technique and DAB, while GAD was identified with the ferritin-avidin technique (controls 6 and 7). In controls 8 and 9, sections were first incubated to visualize ChAT immunoreactivity with the ABC complex and DAB. Following these steps, the anti-GAD antibody (No. 8) or the biotinylated antisheep IgG (No. 9) was replaced with PB prior to the ferritin-avidin reaction. Finally, sections from two animals, in which the micropipette was introduced without injecting the HRP, were incubated only with DAB. See Table 2 for a detailed protocol of the control experiments and the evaluation of the material.

Embedding for electron microscopy

Sections were postfixed in 1% OsO_2 for 30 minutes, dehydrated, stained *en bloc* with 1% uranyl acetate at the 70% ethanol stage, and flat embedded in Spurr's low-viscosity embedding media (EMS, Fort Washington, PA) between aluminium foil and a coverslip (Leranth and Feher, '83) to keep them flat and to allow light microscopic examination and photography. Plastic-embedded sections from the ventral pallidum containing previously photographed cells were mounted on a cylindric Araldite block for electron microscopic examination. Ultrathin sections were cut on an LKB Ultratome and viewed with a Zeiss EM 109 or a Hitachi HU 12 electron microscope with or without lead citrate staining.

Immunofluorescence and light microscopic evaluation

In order to compare the topography of the cholinergic cells with that of the ventral pallidum in the same section, a double immunofluorescence technique was used. Briefly, sections were incubated in a solution containing both rat anti-ChAT (1:10) and sheep anti-GAD (1:1,000) antibodies and then in a mixture of rhodamine isothiocyanate (RITC)-conjugated rabbit antirat IgG (1:50, Capell Labs) and fluorescein isothiocyanate (FITC)-conjugated rabbit antisheep IgG (1:100, Miles Biochemicals). Subsequently, sections were rinsed in PB, mounted, and coverslipped with glycerol buffered with 0.1 M lithium carbonate (3:1, pH 8.5). By using a Zeiss epifluorescent microscope with appropriate filters, the rhodamine-labeled ChAT-positive cells (RITC exciter/barrier filter set 546/590 nm) and the FITC-labeled GAD immunoreactive field (FITC filter set 450–490/520 nm) could be separately visualized in the same section.

Labeled neurons from 25 plastic-embedded sections were charted by using an X-Y plotter coupled to the microscope stage.

RESULTS

Light microscopy: cholinergic amygdalopetal cells in the ventral pallidum

A large area of the basal forebrain has been identified by Heimer and Wilson ('75) as the ventral pallidum. This area extends into the polymorph layer of the olfactory tubercle and is in continuity with the main body of the globus pallidus behind the anterior commissure (Heimer, '78; Switzer et al., '82; Haber and Nauta, '83). The ventral pallidum, like the rest of the pallidal complex, is characterized by strong GAD immunoreactivity that is located primarily in the axon terminals (Fig. 1B; see also Záborszky et al., '82). The heavily myelinated fascicles of the medial forebrain bundle (Fig. 1A) penetrate the ventral pallidum and divide it into a large subcommissural division and a thinner ventral part located primarily within the polymorph layer of the olfactory tubercle (Záborszky et al., '86). Cholinergic cells are randomly distributed in the ventral pallidum (Figs. 1C, 3).

Figure 2 shows a typical HRP injection in the basolateral amygdala and Figure 3 depicts the labeled neurons in the ventral pallidum as plotted from a thick plastic section. The number of retrogradely labeled cells was about twice as many in sections treated with the DAB-glucose oxidase reaction (Itoh et al., '79) as compared with the simple DAB procedure. About 80% of the retrogradely labeled cells were positive for ChAT immunoreactivity, whereas only 10–20% of the cholinergic cells in the ventral pallidum were also HRP-positive following injections of HRP in the basolateral amygdala (Fig. 3).

Electron microscopy

Amygdalopetal cholinergic neurons. As described (Záborszky et al., '84a; Záborszky and Leranth, '85), cholinergic amygdalopetal neurons (Fig. 4B) had an oval, fusiform, or triangular shape, with a mean size of 23 × 12 μm. Three main dendrites usually emerged from the cell body, and they could be followed as far as 150 μm in thick sections. The often eccentrically located nucleus had two to four

Fig. 1. A–C illustrate the same frontal section through the ventral pallidum with different illuminations and filter systems. A. Darkfield illumination to show the heavily myelinated fascicles of the medial forebrain bundle. B. Fluorescence photomicrograph illustrating the extent of the ventral pallidum, which is strongly GAD-positive. The outlines of the heavily myelinated fascicles of the medial forebrain bundle are superimposed on the GAD-immunopositive fields. C. Distribution of cholinergic cells. The outline of the ventral pallidum is indicated by dashed lines. VP = ventral pallidum; DB = diagonal band; ac = anterior commissure. Arrows in A points to vessels that are indicated by asterisks in B and C. Scale bar = 100 μm.

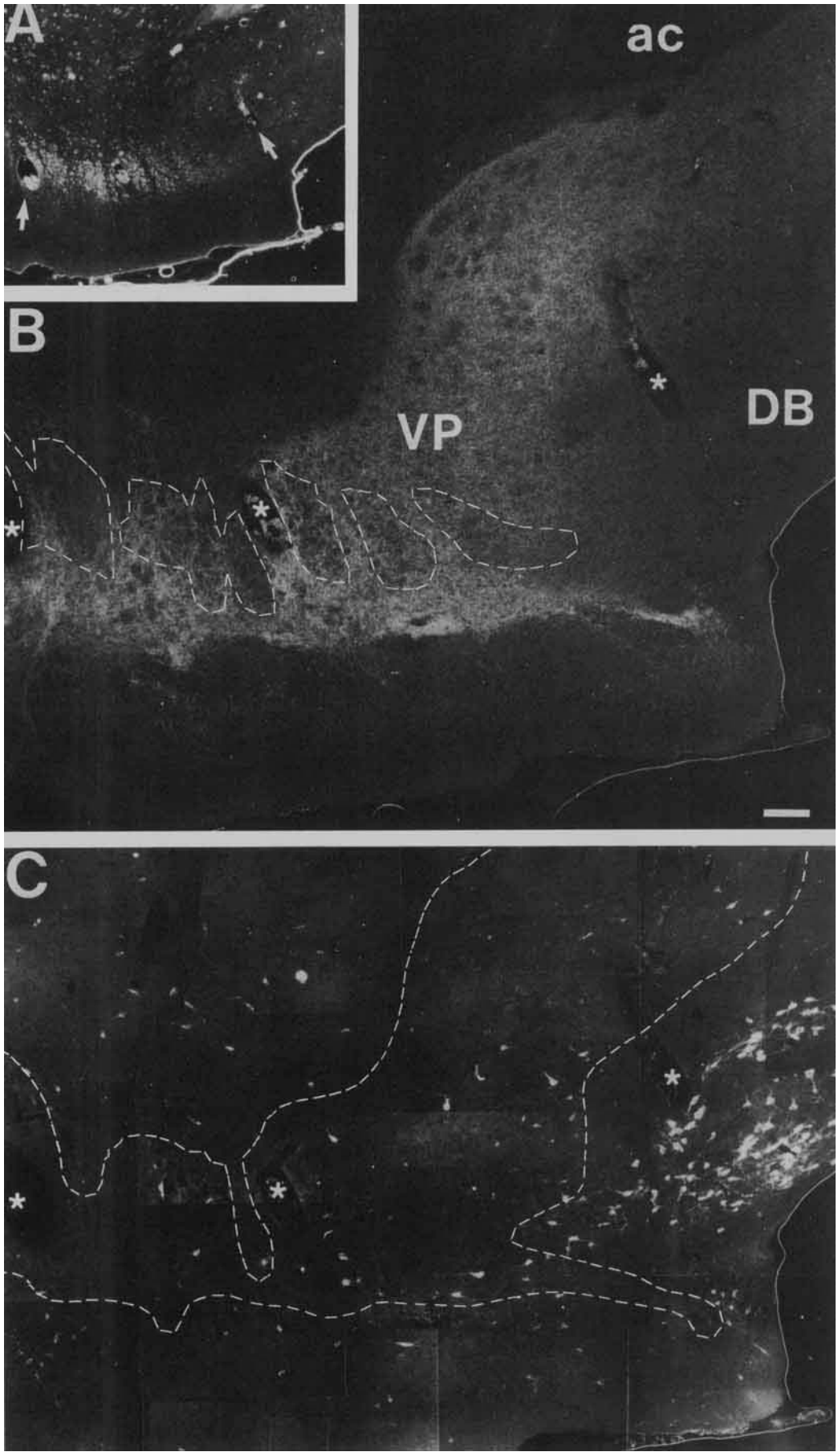


Figure 1

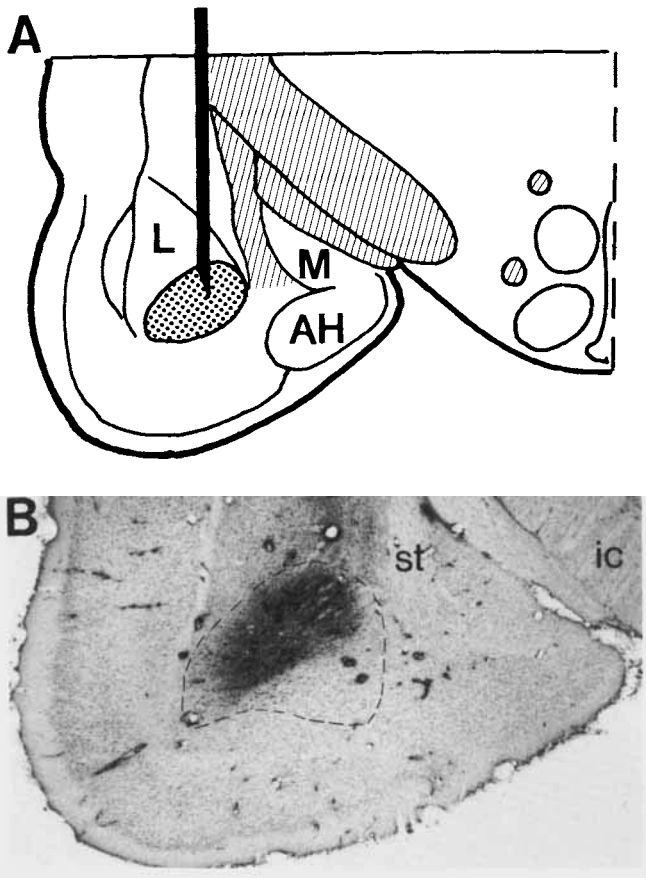


Fig. 2. A. Schematic drawing of a frontal section indicating the location of an HRP injection in the amygdala. B. Photomicrograph (×23) of the injection placement shown in A. Dashed lines indicate the border of the basolateral amygdaloid nucleus. AH = amygdalohippocampal area; L = lateral amygdaloid nucleus; M = medial amygdaloid nucleus; ic = internal capsule; st = stria terminalis.

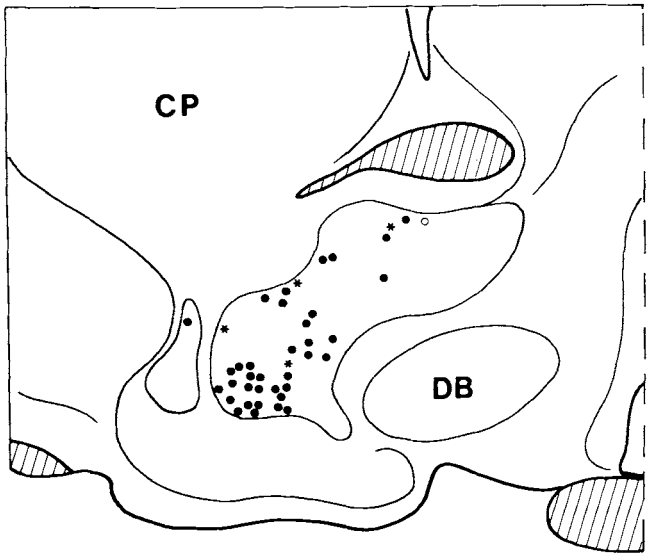


Fig. 3. Distribution of cholinergic (filled circles), retrogradely labeled (open circles), and double-labeled cells (asterisk) at the midlevel of the ventral pallidum plotted from a plastic-embedded frontal section. CP = caudate putamen; DB = diagonal band.

small invaginations and the karyoplasm contained characteristic parallel arrays of endoplasmic reticulum (ER) cisternae and several round or irregularly shaped dense bodies with a homogeneous or granular structure. These large electron-dense bodies, which reflect the accumulation of the retrogradely transported HRP, could be easily distinguished from the less dense lysosomes present normally (Fig. 4C). The immunoperoxidase reaction product was associated with ribosomes, outer membranes of the mitochondria, and microtubules. Myelinated axons often contained both the flocculent reaction product characteristic for ChAT and the large dense HRP granules. Such axons were interpreted as belonging to the amygdalopetal cholinergic projection neurons. Cholinergic cell bodies received relatively few axon terminals, none of which contained immunoprecipitate. This is demonstrated in Figure 4C, in which only one bouton is in close proximity to the cell body. Although large segments of cholinergic dendrites were often identified in our material, they were never seen to be contacted by cholinergic terminals.

GABAergic input to amygdalopetal neurons. When sections were sequentially processed for retrograde HRP visualization and GAD immunostaining, several immunonegative retrogradely labeled cells in the ventral pallidum were contacted by terminals immunopositive for GAD. The ultrastructures of these retrogradely labeled cells (Fig. 5) were similar to cholinergic retrogradely labeled neurons like the one in Figure 4. Although the relatively poor preservation of the tissue precluded a more detailed analysis of the GAD-positive terminals, it appeared that they established synapses of the symmetric type, both with cell bodies and dendrites of retrogradely labeled neurons. The boutons measured on the average of 1.13×0.45 μm and contained one to three mitochondria but never dense-core vesicles. Randomly selected sections of nine retrogradely labeled neurons were studied, and all of them received GAD-positive terminals.

Figure 6 shows details from a section that was sequentially processed for HRP retrograde labeling, for ChAT, and finally for GAD immunostaining. That the retrogradely labeled neuron in Figure 6G is cholinergic was established by comparing pictures before and after immunostaining for ChAT in wet sections. After establishing the cholinergic nature of the neuron, the section was processed for GAD immunostaining. In contrast to the material that was processed only for ChAT, several immunopositive terminals can be seen to establish synaptic contact with the perikaryon and the dendrite of this retrogradely labeled cholin-

Fig. 4. Electron micrograph of a cholinergic retrogradely labeled neuron. The section was processed sequentially for retrograde tracing of HRP and then for ChAT immunoreactivity. The boxed area in A (×21) shows the location of a retrogradely labeled neuron, which is seen with higher magnification (×225) in B (arrow) in the wet section prior to immunostaining. Arrows in C point to lysosomes, arrowheads show retrogradely transported HRP products. Asterisk indicates a nonreactive bouton. D. Immunopositive myelinated axon. Arrow points to an electron-dense body. E. Higher magnification of the rectangle in C showing the retrogradely transported HRP products. Scale bars in all electron micrographs of Figures 4–8 = 1 μm.

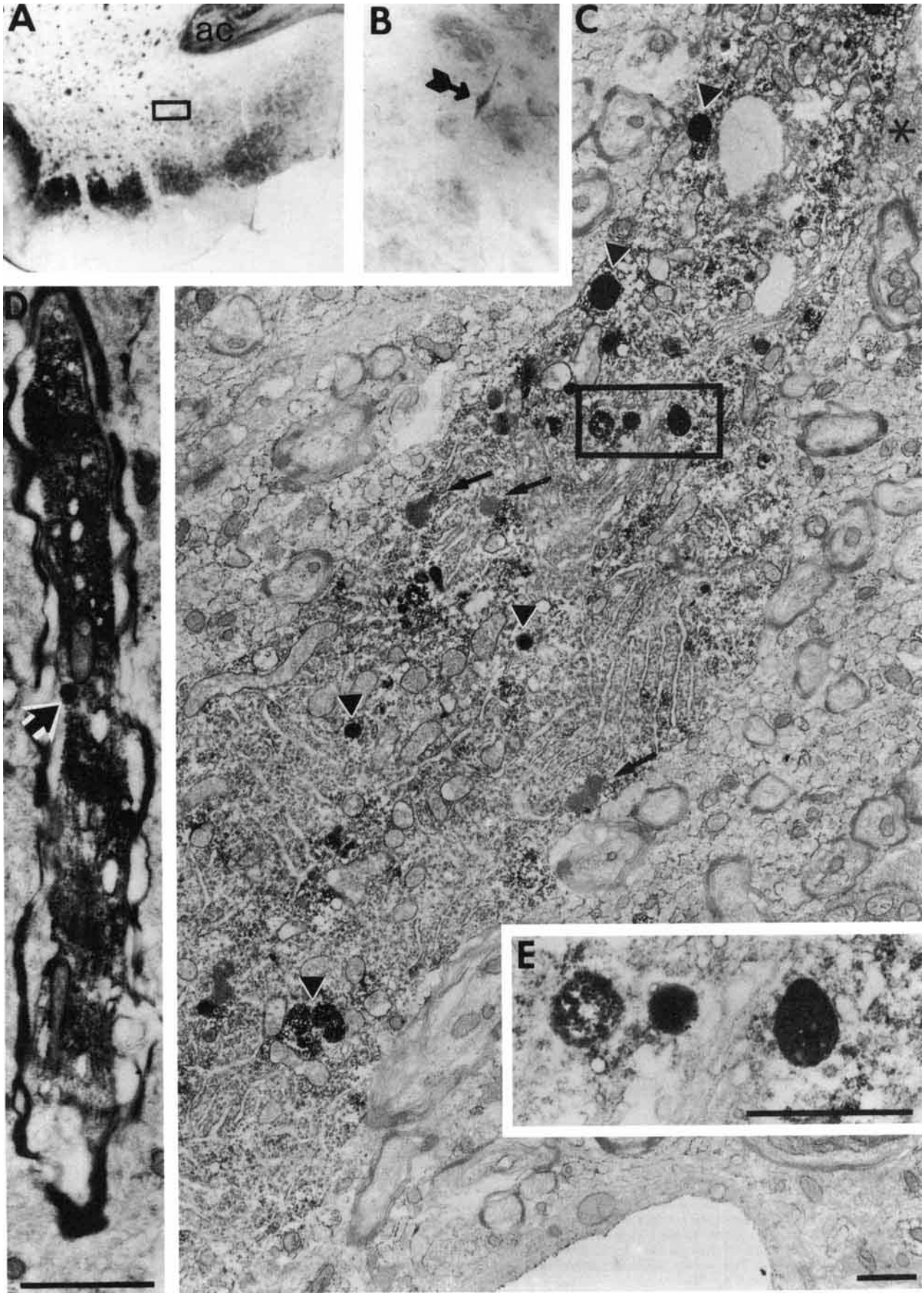


Figure 4

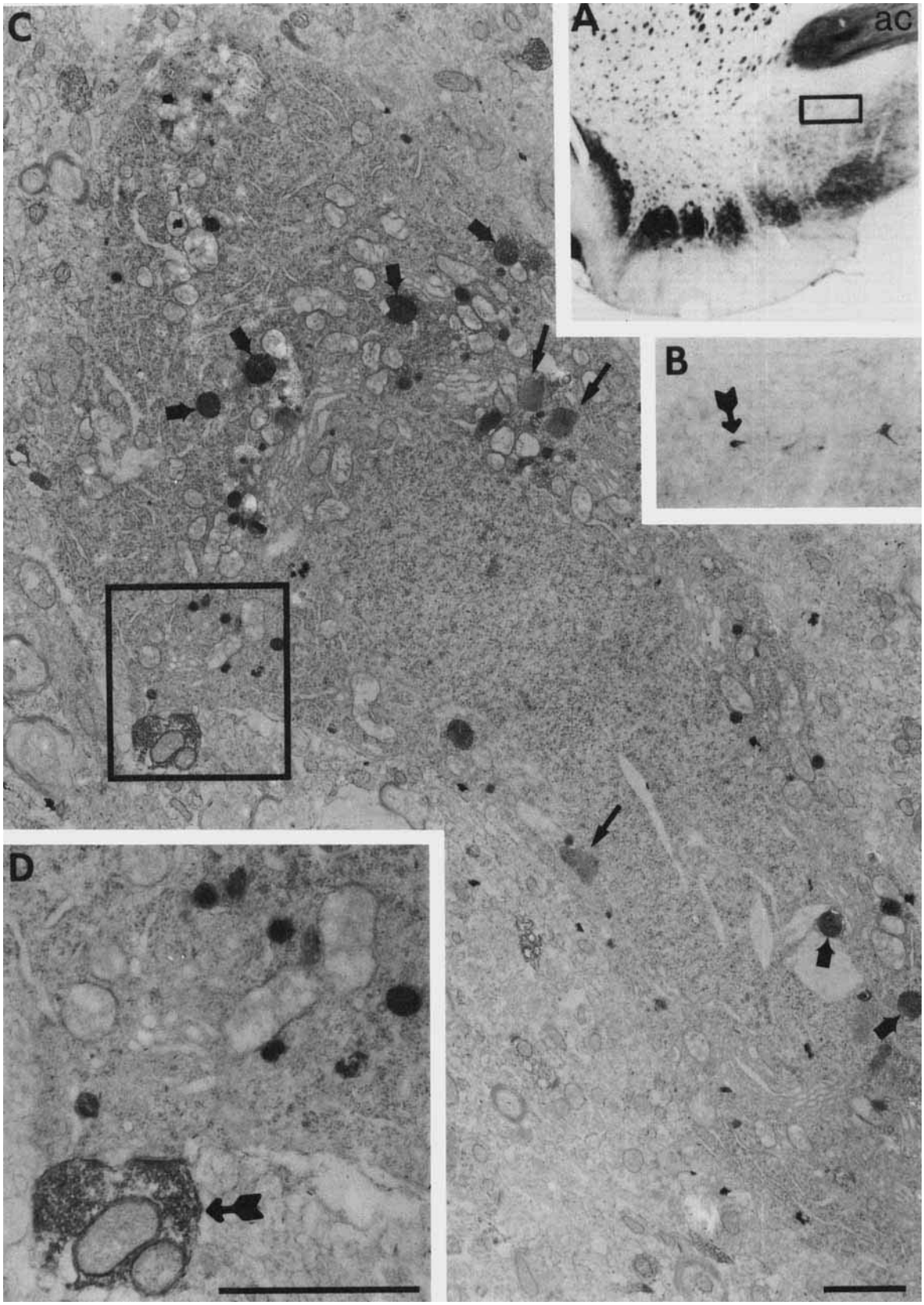


Fig. 5. Electron micrograph of a retrogradely labeled neuron that receives GAD-containing synaptic terminal. A (×20) and B (×225) show the location of retrogradely labeled neurons in the dorsomedial portion of the ventral pallidum (box in A). B arrow points to the identified neuron shown on the electron micrograph of C. This section, which is adjacent to the one shown in Figure 4, was processed for HRP retrograde tracing and GAD immunostaining. Note the similar ultrastructure of this neuron as compared to the cholinergic neuron shown in Figure 4. Thick arrows in C point to transported HRP products—thin arrows point to lysosomes. The boxed area in C is shown in higher magnification in D. The GAD-positive axon terminal contains small, clear, pleomorphic vesicles and mitochondria.

ergic neuron. Since our control experiments show (Table 2) that there is no cross-reactivity between the different immunoreagents in this experiment, the immunoreactive terminals are in all likelihood GABAergic.

When sections from intact animals were processed for ChAT immunohistochemistry by using peroxidase reaction and for GAD by using ferritin, eight randomly selected cholinergic neurons were characteristically labeled by both the large DAB immunoprecipitates and the 7-nm ferritin particles (Fig. 7). Both the perikarya and proximal dendrites of these neurons were contacted by GAD terminals containing pleomorphic synaptic vesicles, mitochondria, and single or aggregated ferritin particles. Although the immunoreactive product in the postsynaptic profile (e.g., Fig. 7C) often prevents an unequivocal classification of the synaptic contact, it seems clear from Figure 7A that the GAD-positive terminals establish symmetric contact with the cholinergic neurons.

Cholinergic terminals on GABAergic cells. In the material in which ChAT elements were labeled by the immunoperoxidase techniques and the GAD profiles were labeled with the ferritin-avidin procedure, we found ferritin-labeled cells (i.e., GAD) that were contacted by double-labeled (immunoperoxidase + ferritin, i.e., cholinergic) terminals. Although a systematic effort was made to search for ChAT-GABAergic synaptic interactions in several randomly selected areas of the ventral pallidum, only a few examples were found. These cholinergic synapses were located only on cell bodies of GAD-containing neurons. Whether cholinergic terminals also contact GAD-containing dendrites is not clear, since in our material we were not able to identify GAD-positive dendrites. The cholinergic terminals appear to establish symmetric contacts (Fig. 8).

DISCUSSION

Technical considerations

It is generally accepted that fluorescent retrograde tracers are more sensitive than the HRP retrograde technique (Sawchenko and Swanson, '81). Accordingly, we found a lower percentage (11–14%) of amygdalopetal cholinergic cells in the ventral pallidum in this HRP study than in our fluorescent retrograde experiments (Carlsen et al., '85), in which about 25% of the cholinergic cells in the ventral pallidum were double labeled. Since it is possible that double labeling techniques may result in less actual labeling due to the competition of the two markers (Alheid et al., '84), the actual percentage of cholinergic amygdalopetal neurons in the ventral pallidum may be somewhat higher than indicated in our studies.

In order to understand the function of neuronal systems it is necessary to determine both the precise wiring of the circuits involved and the transmitters of the pre- and postsynaptic neurons. In some cases, the same marker can be used to define two different antigens, although the evidence becomes rather circumstantial and extensive controls have to be used. In the double-label protocol used for the experiment illustrated in Figure 6, the DAB endproduct marked both the cholinergic cell and the presumptive GABAergic terminals. The two primary antibodies were raised in different species (Oertel et al., '81; Eckenstein and Thoenen, '82), and according to our control experiments cross-reactivity between the different immunoreagents can be ruled out. Furthermore, and in agreement with several reports (Armstrong et al., '83; Ingham et al., '85a; Armstrong, '86), cholinergic forebrain projection neurons do not seem to receive cholinergic afferents. These data support the notion that the labeled terminals on the identified cholinergic neurons are in all likelihood GABAergic.

In the second double label experiments (Fig. 7), we used two distinctly different electron-dense markers for the two antigens (Leranth et al., '85). The antigen that was identified in the first sequence, i.e., ChAT, is labeled with both DAB and ferritin. The presence of the two labels in immunoreactive ChAT cells is due to the fact that the tissue bound biotinylated antirat IgG is not completely saturated with the avidin-biotin-peroxidase in the first sequence and the residual biotin is therefore still able to recognize some ferritin-avidin. However, the second antigen, GAD, is labeled only with the ferritin particles. In control experiments, when the anti-GAD antibody or the biotinylated antisheep IgG was omitted (Table 2), no terminals were labeled with ferritin. The problem of nonspecific binding of the avidin-ferritin complex has been discussed by Leranth et al. ('85). Although the ferritin-avidin technique in combination with the PAP technique would avoid the double labeling of the antigen identified in the first sequence (i.e., ChAT in our case), we choose the ABC technique due to convenience of the ABC kit, the higher sensitivity of the technique, and lower costs of the immunoreagents.

Figures 7 and 8 show the advantage of using ferritin for immunolabeling, in the sense that the ultrastructural details of the stained profile can be easily studied. The DAB technique, in contrast, does not allow such detailed analysis, since large DAB particles mask many of the details. The ferritin-labeling technique, however, also has its disadvantages. For instance, high-power electron microscopy is necessary for screening, and the penetration of the compound is rather limited.

Afferents to cholinergic forebrain neurons

In the past few years, the cholinergic forebrain projection system has received special attention due primarily to its implication in memory and other cognitive functions (Aigner et al., '84; Forbes and Macrides, '84; Hepler et al., '85; Miyamoto et al., '85) and to its disruption in some cases of Alzheimer's disease (Price et al., '83). Although recent light microscopic studies have provided detailed information about the topography of the forebrain cholinergic projection neurons (Rye et al., '84; Carlsen et al., '85; Záborszky et al., '86), little is known about the organization and the transmitters of the afferents to these neurons. Since removal of the synaptic input often leads to disruption of cellular metabolism and sometimes to death of the deafferented target neuron (Heimer and Kalil, '68; Steward and Rubel, '85), it is possible that a more detailed analysis of the afferents to the cholinergic projection neurons might provide some clue for the understanding of the pathophysiological mechanisms of Alzheimer's disease.

Light microscopic tracing techniques are generally unable to discriminate between terminals and fibers of passage. The afferents to the cholinergic neurons, therefore, have to be studied at the EM level. To date only few EM studies (Juraniec, '79; Walker et al., '83; Lemann and Saper, '85) have addressed the problem of synapses on magnocellular forebrain neurons, and little effort has been made to determine the chemical nature of the receiving neuron. Since cholinergic projection neurons in the basal forebrain are intermingled with other types of neurons, including GABAergic projection neurons (Köhler et al., '84; Záborszky et al., '86), this issue can be studied only if the

TABLE 2. Summary of Immunocytochemical Procedures in the Controls

Immunostaining sequence	Results
1. PB + antirat IgG +ABC +DAB	No immunoreactivity
2. NSS or PB + antisheep IgG + ABC + DAB	No immunoreactivity
3. PB + antirat IgG + ABC + DAB + anti-GAD + antisheep IgG + ABC + DAB	Immunopositive terminals, characteristic for GAD staining, establish contacts with both positive and negative postsynaptic profiles (dendrites, cell bodies)
4. anti-ChAT + antirat IgG + ABC + DAB + NSS[1] or PB + antisheep IgG + ABC + DAB	Immunoreactive cell bodies, terminals, and myelinated axons characteristic for ChAT-staining; immunopositive terminals contact only immunonegative postsynaptic profiles
5. PB + antirat IgG + ABC + DAB + NSS or PB + antisheep IgG + ABC + DAB	No immunoreactivity
6. anti-ChAT + antirat IgG + ABC + DAB	Same as No. 4
7. anti-GAD + antisheep IgG + ferritin-avidin	Similar to No. 3, except profiles are labeled by ferritin instead of DAB particles
8. anti-ChAT + antirat IgG + ABC + DAB + PB + antisheep IgG + ferritin-avidin	Profiles, double labeled by DAB + ferritin, are morphologically similar to labeled structures seen in No. 4; no single ferritin labeled profiles
9. anti-ChAT + antirat IgG + ABC + DAB + anti-GAD + PB + ferritin-avidin	Same as No. 8

[1]NSS, preimmune or normal sheep serum.

chemical identity of both the afferent fiber system and the postsynaptic target is being established in the same ultrathin section with appropriate combinations of tracer and immunocytochemical methods. In addition, since cholinergic neurons projecting to different regions are intermingled in most forebrain regions (Carlsen et al., '85; Záborszky et al., '86) the cholinergic projection neuron has to be identified also in regard to its target.

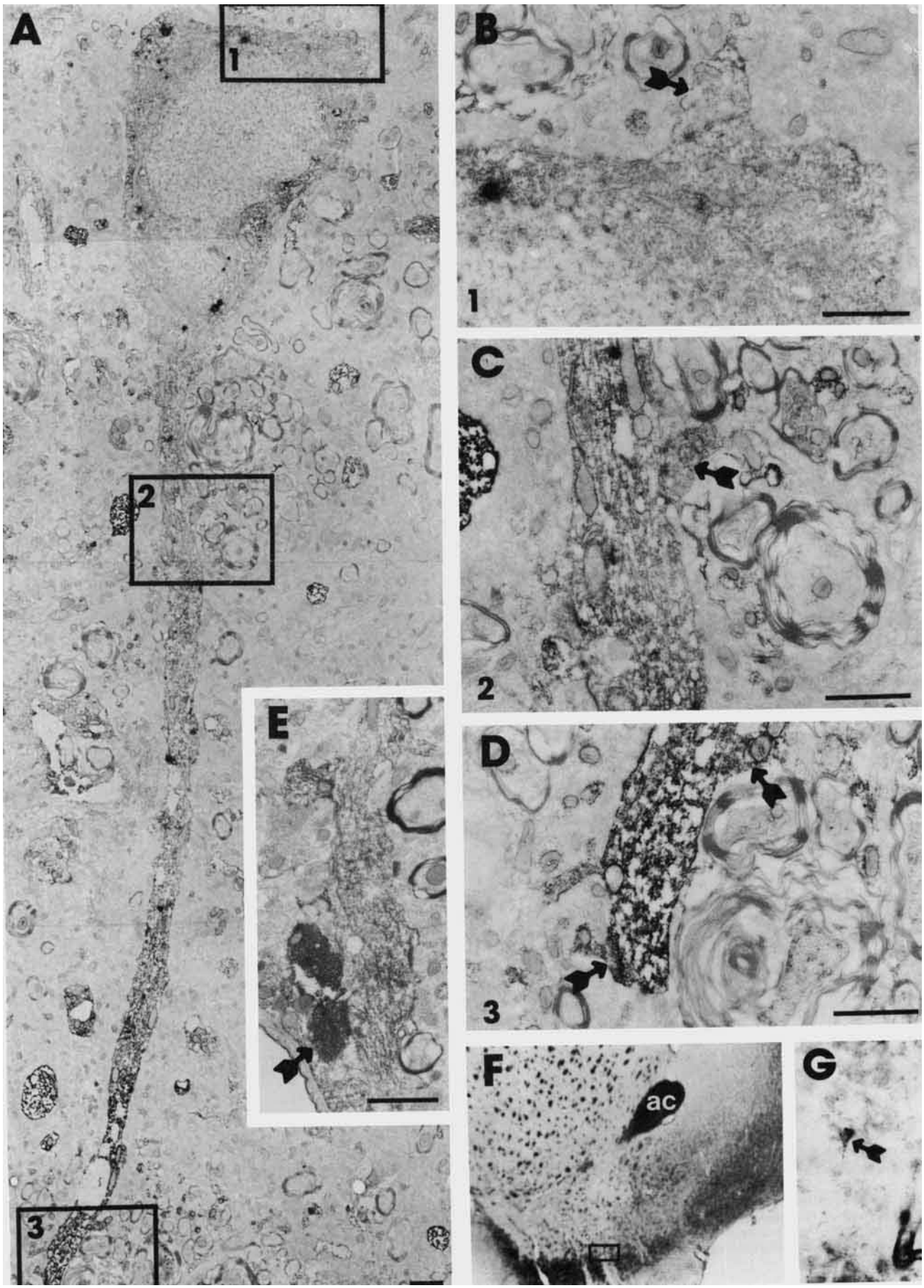
Cholinergic projection neurons are located along several fiber systems originating in different cortical areas, the hippocampus, amygdaloid body, bed nucleus of the stria terminalis, preoptic area, and hypothalamus as well as various brainstem nuclei (for references see Mesulam and Mufson, '84; and Russchen et al., '85). Future EM studies have to determine whether these fibers only pass through the area of cholinergic cells, or indeed establish synaptic contacts. To date, only one of the afferents to the cholinergic neurons in the ventral pallidum seems to have been identified on the electron microscopic level (Záborszky et al., '84a). In this study, we showed that amygdalofugal fibers terminate on cholinergic cells of the ventral pallidum. However, neither the targets of these cholinergic neurons, nor the transmitters of the amygdalofugal fibers were determined in this case. According to pharmacological experiments (Wenk, '84; Davies et al., '84) and a study using ^{3}H-D-Asp as a transmitter-specific tracer for putative glutamatergic and aspartergic neurons (Fuller et al., '85), at least part of the amygdalofugal projection to the basal forebrain utilizes excitatory amino acids as transmitter. To what extent cholinergic neurons receive cholinergic input is not known. It is interesting to note, however, that cholinergic terminals were seen to contact cholinergic neurons in the striatum and amygdala (Phelps et al., '85; Carlsen and Heimer, '86), but in these cases the cholinergic neurons are believed to be short-axon interneurons. Cholinergic terminals have so far not been found to contact cholinergic projection neurons (Armstrong et al., '83; Ingham et al., '85a; Armstrong, '86). Due to the limited penetration of immunoreagents and the fact that in these studies as well as in our own experiments no attempts were made for a complete reconstruction of the cholinergic projection neurons, these observations should be cautiously interpreted.

A number of other putative neurotransmitters have been localized (see for references Palkovits, '84) in various forebrain regions containing cholinergic projection neurons and it is likely that several of these will be identified in afferent pathways to the cholinergic neurons. Indeed, it has recently been shown in a preliminary report (Ingham et al., '85b) that substance-P-containing terminals contact cholinergic neurons in the basal forebrain.

GABAergic—cholinergic, cholinergic—GABAergic interactions

Our results with the ferritin-avidin technique leave little doubt that cholinergic cells in the ventral pallidum are being contacted by a significant number of GAD-positive terminals. It is well known that the ventral pallidum receives afferents from ventral striatal regions (Heimer and Wilson, '75; Heimer, '78; Záborszky et al., '82; Haber and Nauta, '83; Grove and Nauta, '84; Heimer et al., '85), which contain a large number of GABAergic neurons (Mugnaini and Oertel, '85). It has furthermore been shown that lesions of the nucleus accumbens result in a depletion of GAD

Fig. 6. Electron micrographs of a retrogradely labeled cholinergic neuron that receives GAD-containing axon terminals (A). This section was sequentially processed for HRP retrograde labeling, ChAT, and finally for GAD immunostaining. The box in F (×20) shows the location of the retrogradely labeled neuron, which is displayed in G (×144) prior to immunostaining. In A, large electron-dense granula in the perikaryon and dendrite represent transported HRP. Diffuse flocculent material in the cytoplasm indicates that the neuron is positive for ChAT. Rectangles labeled 1, 2, and 3 are shown in B, C, and D with higher power. Note the presence of immunopositive terminals (arrows) establishing synapses with the cholinergic projection neuron. E shows another detail from a dendrite of the same neuron. The synapses seem to be of the symmetric type.


A
1
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G

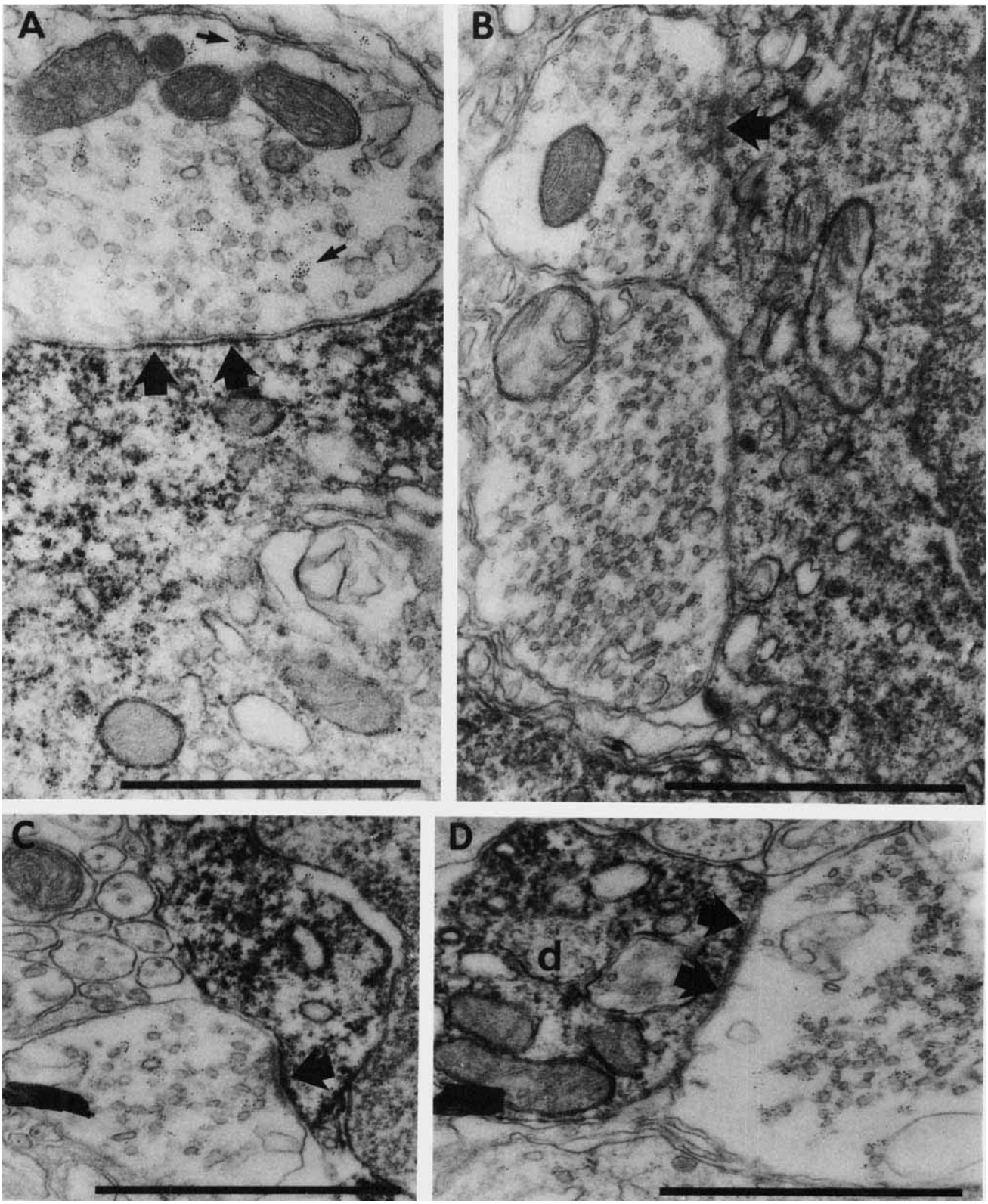

Fig. 7. Electron micrographs showing GAD-containing axon terminals synapsing with cholinergic cell bodies (A–C) and dendrites (d in D) in the ventral pallidum. Double antigen label technque. Cholinergic profiles are labeled by the presence of immunoperoxidase + ferritin; GAD-containing terminals are labeled by ferritin. Large arrows point to synaptic thickenings; small arrows point to accumulation of ferritin particles. The synapse in A is clearly of the symmetric type, while that in C shows a slightly increased postsynaptic density.

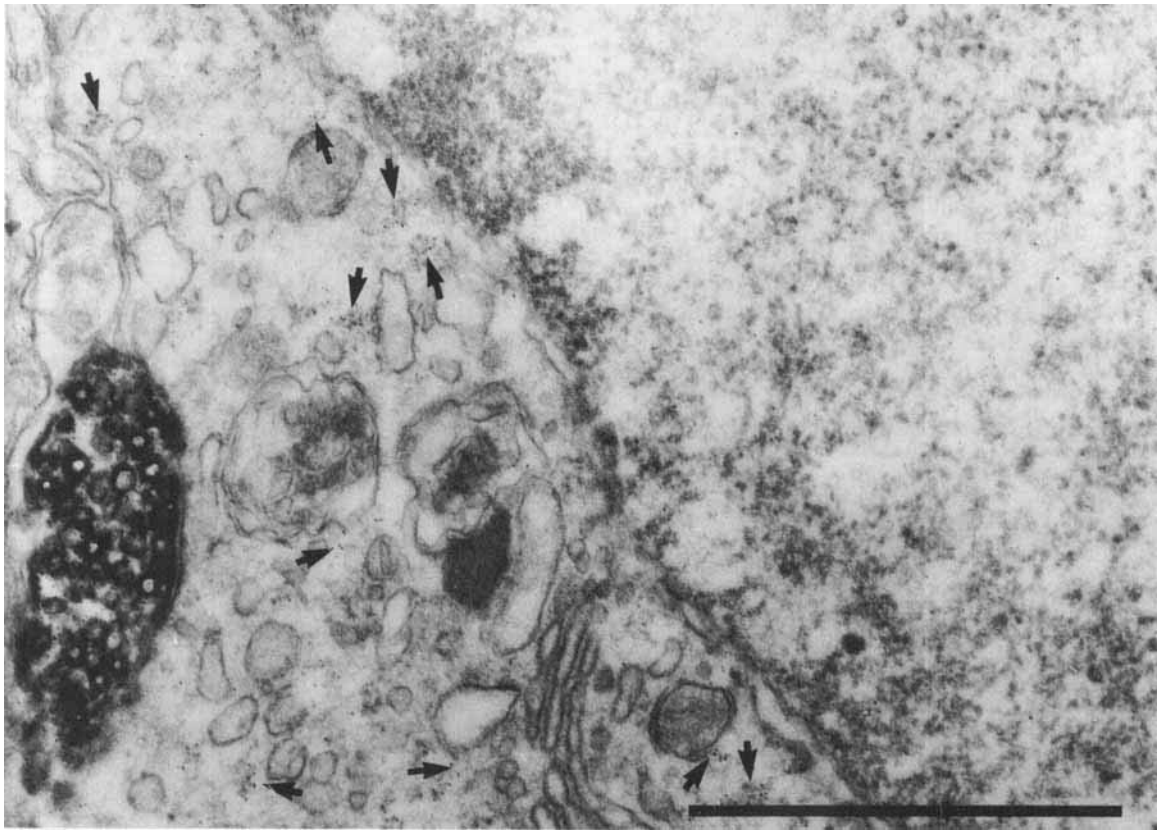

Fig. 8. Electron micrograph of a GAD-containing cell body indicated by the presence of ferritin (arrows), which is contacted by a ChAT-positive axon terminal (containing immunoperoxidase + ferritin). The synapse is symmetric.

immunoreactivity of the ventral pallidum (Záborszky et al., '85). Whether the GABAergic input to the cholinergic neurons in the ventral pallidum originates in ventral striatal GABA-containing neurons or whether it consists of axon collaterals of locally arborizing or projective GABAergic neurons of the ventral pallidum cannot be decided at present. Although the ferritin-avidin technique was not combined with retrograde labeling for the identification of projection target of the cholinergic neurons, the experiments in group D (see Table 1), in which the target of the cholinergic neurons was identified, do suggest that amygdalopetal neurons are part of the cholinergic projection system receiving GABAergic input.

That GABAergic-cholinergic interaction may be a more general phenomenon is suggested by pharmacological experiments that indicate GABAergic inhibition of cholinergic neurons in the substantia innominata (Wood and Richard, '82; McGeer et al., '83; Wenk, '84, Blaker, '85), septum (Costa et al., '83; Allen and Crawford, '84), or in cultured retina (Agardh et al., '85). It is interesting to note in this context that Wenk ('84) could also show that enkephalin, like GABA, inhibits cholinergic transmission in the substantia innominata. This fits well with our suggestion (Zahm et al., '85) that enkephalin and GABA may coexist at least in a population of forebrain terminals. It remains to be determined in future combined EM studies whether GABAergic-cholinergic interaction is characteristic also for those cholinergic projection neurons that are located in such areas as the sublenticular substantia innominata and the nucleus of the horizontal limb of the diagonal band regions, which seem to be less heavily supplied by GABAergic terminals.

Our studies also revealed that ChAT-containing axon terminals establish synapses with GAD-containing cell bodies. Since so little is known about the intrinsic organization of the ventral pallidum, and we did not use colchicine treatment to enhance the detection of GAD-containing neurons, the significance of this phenomenon cannot be fully appreciated at present. However, a possible cholinergic-GABAergic synaptic arrangement has already been suggested in different parts of the CNS by ChAT immunohistochemistry (Houser et al., '85; de Lima et al., '85; Phelps et al., '85) or pharmacological experiments (Krnjević and Ropert, '81; Sillito and Kemp, '83). The predominant effect of acetylcoline (Ach) in the CNS seems to be excitatory (Bird and Aghajanian, '76; Dodd et al., '81; Benardo and Prince, '82; Lamour et al., '82). According to some reports, however, Ach can produce an inhibitory effect on inhibitory interneurons (Ben-Ari et al., '81; Sillito et al., '83; Francesconi et al., '84). Since we found that cholinergic terminals establish symmetric contacts with GABAergic neurons, and symmetric synaptic contacts are supposed to be inhibitory (Colonnier, '81), one can speculate that cholinergic excitation in the ventral pallidum could lead to disinhibitory effects lo-

cally. More definite answers to these questions can only be obtained in future studies, in which sophisticated intracellular electrophysiologic techniques are combined with morphological and chemical identification of the participating units.

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