

CORTICAL INPUT TO THE BASAL FOREBRAIN

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Abstract—The arborization pattern and postsynaptic targets of corticofugal axons in basal forebrain areas have been studied by the combination of anatomical tract-tracing and pre- and postembedding immunocytochemistry. The anterograde neuronal tracer *Phaseolus vulgaris* leucoagglutinin was iontophoretically delivered into different neocortical (frontal, parietal, occipital), allocortical (piriform) and mesocortical (insular, prefrontal) areas in rats. To identify the transmitter phenotype in pre- or postsynaptic elements, the tracer staining was combined with immunolabeling for either glutamate or GABA, or with immunolabeling for choline acetyltransferase or parvalbumin.

Tracer injections into medial and ventral prefrontal areas gave rise to dense terminal arborizations in extended basal forebrain areas, particularly in the horizontal limb of the diagonal band and the region ventral to it. Terminals were also found to a lesser extent in the ventral part of the substantia innominata and in ventral pallidal areas adjoining ventral striatal territories. Similarly, labeled fibers from the piriform and insular cortices were found to reach lateral and ventral parts of the substantia innominata, where terminal varicosities were evident. In contrast, descending fibers from neocortical areas were smooth, devoid of terminal varicosities, and restricted to the myelinated fascicles of the internal capsule *en route* to more caudal targets. Ultrastructural studies obtained indicated that corticofugal axon terminals in the basal forebrain areas form synaptic contact primarily with dendritic spines or small dendritic branches (89%); the remaining axon terminals established synapses with dendritic shafts. All tracer labeled axon terminals were immunonegative for GABA, and in the cases investigated, were found to contain glutamate immunoreactivity. In material stained for the anterograde tracer and choline acetyltransferase, a total of 63 *Phaseolus vulgaris* leucoagglutinin varicosities closely associated with cholinergic profiles were selected for electron microscopic analysis. From this material, 37 varicosities were identified as establishing asymmetric synaptic contacts with neurons that were immunonegative for choline acetyltransferase, including spines and small dendrites (87%) or dendritic shafts (13%). Unequivocal evidence for synaptic interactions between tracer labeled terminals and cholinergic profiles could not be obtained in the remaining cases. From material stained for the anterograde tracer and parvalbumin, 40% of the labeled terminals investigated were found to establish synapses with parvalbumin-positive elements; these contacts were on dendritic shafts and were of the asymmetrical type.

The present data suggest that corticofugal axons innervate forebrain neurons that are primarily inhibitory and non-cholinergic; local forebrain axonal arborizations of these cells may represent a mechanism by which prefrontal cortical areas control basal forebrain cholinergic neurons outside the traditional boundaries of pallidal areas. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: cholinergic neurons, GABAergic neurons, glutamate, electron microscopy, double immunolabeling, rat.

The term basal forebrain refers to a heterogeneous collection of structures, located close to the medial and ventral surfaces of the cerebral hemispheres. This highly complex brain region has been implicated in

attention, motivation, memory^{5,22,61} as well as in a number of neuropsychiatric disorders such as Alzheimer's disease, Parkinson's disease, and schizophrenia.^{6,30,45,57,64} Part of the difficulty in understanding the functions of the basal forebrain, as well as the aberrant information processing characteristic of these disease states lies in the anatomical complexity of the region. Basal forebrain areas, including the ventral pallidum, horizontal limb of the diagonal band (HDB), substantia innominata (SI) and peripallidal regions contain cell types different in transmitter-content, morphology and projection pattern.^{8,9,19,32,87} Among these different neuronal populations, the cholinergic corticopetal projection neurons have received particular attention in

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Abbreviations: ABC, avidin–biotin–peroxidase complex; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; BFC, basal forebrain cholinergic projection neurons; ChAT, choline acetyltransferase; DAB, 3,3'-diaminobenzidine; HDB, horizontal limb of the diagonal band; MCP, magnocellular preoptic nucleus; NGS, normal goat serum; PAP, peroxidase–antiperoxidase; PB, sodium phosphate buffer; PFC, prefrontal cortex; PHA-L, *Phaseolus vulgaris* leucoagglutinin; RT, room temperature; SI, substantia innominata; TBS, Tris-buffered saline.

numerous functional and pathological studies.^{11,78,96} However, cholinergic projection neurons represent only a fraction of the total cell population in these forebrain areas, which also contain GABAergic and peptidergic neurons.^{9,32,86,98}

In studies conducted in behaving monkeys, DeLong and co-workers⁶⁷ and Rolls and colleagues^{68,90,91} found that basal forebrain neurons respond to a range of visual and auditory stimuli that have been reinforced. It has been proposed⁶⁸ that cholinergic neurons in the basal forebrain receive information on the expected availability of reinforcement through afferent inputs from the orbitofrontal cortex. Through their widespread corticopetal projections the cholinergic neurons may then be able to modulate sensory, motor and higher order cognitive processes in the cerebral cortex.

A number of tracing studies have suggested that the cerebral cortex is a major source of input to the basal forebrain.^{1,36,41,54,60,70,73,83,89} In the primate brain, the origin of this innervation appears to be largely restricted to the so called limbic and paralimbic cortical areas.^{60,70} On the other hand, most of the sensorimotor areas and parasensory association cortices do not seem to project to the basal forebrain. A light microscopic study²⁷ using a combination of *Phaseolus vulgaris* leucoagglutinin (PHA-L) tracing and choline acetyltransferase (ChAT) immunocytochemistry in rat described that axons from the prefrontal cortex arborize in the vicinity of basal forebrain cholinergic projection neurons (BFC). Furthermore, an electron microscopic study in rat suggested that corticopetal neurons in the ventromedial globus pallidus receive synaptic input from the insular cortex.⁵³ The verification of actual synaptic contact between these afferent fiber systems and cholinergic projection neurons requires appropriate combinations of double labeling methods at the ultrastructural level, in which the afferent system and the cholinergic nature of the postsynaptic target can be unequivocally determined. Since such studies have not been performed, it has remained unclear if cortical regions influence the BFC system directly. The present study sought to identify the postsynaptic target of corticofugal terminals in the basal forebrain.

To determine whether cytoarchitecturally distinct cortical regions, including neocortical (frontal, parietal, occipital), allocortical (piriform) and mesocortical (prefrontal, insular) areas project to basal forebrain areas, depositions of the anterograde tracer PHA-L were made in these regions. Since prefrontal axons, in contrast to neocortical axons, showed rich terminal arborizations in basal forebrain areas, PHA-L tracing following injection of the tracer in prefrontal cortex was combined with ChAT immunostaining to determine whether prefrontal axons terminate on cholinergic basal forebrain neurons. In selected cases, PHA-L labeling was combined with parvalbumin immunostaining to examine another

potential basal forebrain target of corticofugal axons. In addition, PHA-L immunostaining was combined with GABA and glutamate immunostaining in a postembedding protocol to identify the transmitter of the presynaptic axons.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

Twelve male Sprague-Dawley rats (Zivic-Miller Laboratories, Inc., Zelienople, PA, U.S.A.; 275 ± 25 g) were used in this study. All animals received Purina Mills Lab Chow No. 5001 and tap water *ad libitum*. The Rutgers University Research Animal Facility where the animals were housed, is maintained in accordance to NIH guidelines and is USDA registered and AAALAC accredited. Prior to surgery, the rats were anesthetized with sodium pentobarbital (50 mg/kg body weight). A 2.5% PHA-L (Vector Labs, Burlingame, CA) solution in 0.1 M sodium phosphate buffer, (PB; pH 8.0) was backfilled into glass micropipettes with tip diameter of 10–20 μ m and placed into various cortical regions as depicted in Fig. 1 and Fig. 5. The stereotaxic coordinates were chosen according to the atlas of Paxinos and Watson.⁶³ PHA-L was iontophoretically delivered for 15–20 min at 6 μ A with a 7 s on/7 s off pulse.²⁹ In several cases, multiple injections were made to maximize labeling. In one case (C028), multiple injections of a 2.5% PHA-L solution (100 nl) were delivered by pressure using a Hamilton microsyringe.

Tissue preparation

After 5–10 days of survival, the animals were given an overdose of pentobarbital. They were then briefly perfused transcardially with normal saline (0.9% NaCl, 1 min), followed by 500 ml of cold fixative. The first 350 ml of the fixative was composed of 4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid in PB (pH 7.4). Glutaraldehyde was omitted in the subsequent 150 ml of fixative. After the perfusion, the brains were removed and further fixed overnight in the same fixative at 4°C.⁸⁰

One animal (C001) was perfused with a different fixative solution containing 2.5% paraformaldehyde, 1% glutaraldehyde and 0.2% picric acid in 0.1 M PB (first 350 ml) followed by the same fixative without glutaraldehyde (150 ml). This brain was postfixed for 2 h and processed for combined pre-embedding PHA-L and postembedding GABA/glutamate immunolabeling (see below).

After fixation, transverse sections (50 μ m thick) were Vibratome-cut from the forebrain and were collected into six series in ice-cold 0.1 M PB. The sections that were fixed with 1% glutaraldehyde were incubated in a solution of 1% sodium borohydride in 0.1 M PB for 30 min, and then thoroughly rinsed in PB.

Tissue processing for light microscopy

Prior to all incubation steps, the sections were rinsed several times in ice-cold PB. Normal sera and antibodies were diluted in a PB solution to which 0.5% Triton X-100 had been added.

Phaseolus vulgaris leucoagglutinin immunolabeling. One series of forebrain sections was processed for the tracer PHA-L only, using the avidin-biotin-peroxidase (ABC) method. Sections were incubated overnight at 4°C in goat anti-PHA(E+L) (Vector Labs) at a dilution of 1:1000. This was followed by incubation in biotinylated donkey anti-goat IgG (Jackson Immunoresearch Labs, West Grove, PA) at 1:100 for 2 h, and the ABC complex (Vector Labs) at 1:500 for 2 h, both steps at room temperature (RT). Subsequently sections were treated with the coupled glucose oxidation reaction of Itoh *et al.*⁴² with 3,3'-

diaminobenzidine (DAB) as a chromogen intensified with nickel ammonium sulfate which yielded a black reaction product. The solution was made up in PB and contained 50 mg DAB, 40 mg ammonium chloride, 0.4 mg glucose oxidase (Sigma, type VII), 200 mg β -D-glucose/100 ml, and 1 mM nickel ammonium sulfate. The reaction medium was filtered before use. The sections were rinsed three times after the staining, mounted on slides, dehydrated and coverslipped in DPX (BDH Laboratory Supplies, Poole, U.K.). The forebrain sections were counterstained with Cresyl Violet to determine the exact location of the PHA-L injection sites.

Simultaneous localization of Phaseolus vulgaris leucoagglutinin and choline acetyltransferase. The second series of forebrain sections was processed to visualize PHA-L and ChAT with a sequential double-labeling method. The first part (PHA-L immunolabeling) was similar to that described for the first series of sections (above). PHA-L was visualized with nickel-intensified DAB. The sections were then treated with the second immunocytochemical protocol. This involved incubation in a rat monoclonal anti-ChAT antibody²³ at 1:10 for two overnights at 4°C, followed by donkey anti-rat IgG (Jackson Immunoresearch Labs) at 1:100 for 2 h, rat monoclonal peroxidase-antiperoxidase (PAP) (Sternberger Monoclonals Inc) at 1:100 for 2 h (RT), and finally development using DAB alone as a substrate, again by the coupled glucose oxidation reaction. Sections were rinsed, dehydrated, and coverslipped.

Simultaneous localization of Phaseolus vulgaris leucoagglutinin and parvalbumin. A third series of forebrain sections were incubated in a mixture of primary antibodies containing goat anti-PHA-L (1:200) and rabbit anti-parvalbumin (1:1000) for two days at 4°C, followed by incubation in a mixture of biotinylated donkey anti-goat IgG (1:200) and donkey anti-rabbit IgG (Jackson Immunoresearch Labs, 1:100) for 2 h. PHA-L was further processed with the ABC complex and visualized with nickel-intensified DAB. Parvalbumin staining was accomplished using donkey anti-rabbit PAP (Sternberger Monoclonals Inc., 1:100, 2 h) and regular DAB.

The possibility of cross-reactivity between immunoreagents in double-labeling experiments was controlled by omitting of one of the primary antibodies, or by replacing with normal serum. One series of sections were run for this purpose. Evidence of cross-reactivity, however, was never encountered.

Tissue processing for electron microscopy

Sections selected for electron microscopy were immersed in 15% and then 30% sucrose, exposed to two freeze-thaw cycles within liquid nitrogen to improve antibody penetration, and extensively washed in PB.

Phaseolus vulgaris leucoagglutinin/choline acetyltransferase double labeling. The fourth series of forebrain sections was used for electron microscopy and was processed for PHA-L and ChAT double immunolabeling. The sequence and the antibody solutions were the same as described previously, except that biotinylated anti-PHA(L+E) (Vector Labs, dilution 1:200) was used and that the incubation times of the secondary antibodies, ABC and PAP, were 3–4 h. The entire procedure was carried out at low temperature (4°C) and only 0.04% Triton X-100 was added to the primary antibody solution.

Phaseolus vulgaris leucoagglutinin/parvalbumin double labeling. The fifth series of sections was used for PHA-L/parvalbumin staining for electron microscopic analysis. The sequence and the antibody solutions were the same as for light microscopy, except that only 0.04% Triton X-100 was added to the antibody solution.

Embedding. Following the immunostaining procedure, the sections were postfixed in 1% osmium tetroxide for 30–45 min, dehydrated in an ascending ethanol series (1% uranyl acetate was included in the 70% ethanol step for 40 min) and propylene oxide, and flat-embedded in Durcupan ACM (Fluka) between glass slides and coverslips coated with liquid releasing agent (Electron Microscopy Sciences, Ft Washington, PA). The embedded sections were examined under the light microscope (Zeiss Axioplan). Selected areas containing PHA-L-labeled varicosities apparently contacting ChAT or parvalbumin-immunolabeled cell bodies or dendrites in the basal forebrain were photographed, dissected and mounted with cyanoacrylate on the flat surface of cylindrical resin blocks, trimmed and ultrathin-sectioned on a Reichert Ultracut E ultramicrotome with a Diatome diamond knife. Serial ultrathin sections were collected on single-slot Formvar-coated gilded grids.

Postembedding staining for GABA and glutamate. In the brain fixed with 1% glutaraldehyde and 2.5% paraformaldehyde (C001), PHA-L was visualized with DAB. Ultrathin sections containing PHA-L-labeled structures were processed for postembedding GABA and/or glutamate immunostaining. Serial sections (3–4/grid) were mounted on Formvar-coated single-slot gilded grids. Every second grid was subjected to treatment with periodic acid and sodium metaperiodate. The steps of the immunogold staining procedure followed those described by Somogyi and Hodgson⁷⁹ using a well-characterized antiserum against GABA³⁸ and glutamate.³⁷ The following steps were carried out on drop-lies of Millipore-filtered solutions in humid Petri dishes: i) 2% periodic acid (H5IO6, BDH) for 10 min; ii) wash by dipping in several changes of double-distilled water; iii) 2% sodium metaperiodate (NaIO4, BDH) for 10 min; iv) wash as before; v) three times 2 min in Tris-buffered saline (TBS; 0.05 M Tris buffer, pH 7.4); vi) 30 min in 1% ovalbumin dissolved in TBS; vii) three times 10 min in TBS containing 1% normal goat serum (NGS); viii) rabbit anti-glutamate (Arnel Products, New York, NY; 1:10000) or rabbit anti-GABA (1:1000), 1–2 h diluted in NGS/TBS; ix) two times 10 min in TBS; x) two times 5 min in TBS containing 1% bovine serum albumin and 0.5% Tween 20; xi) goat anti-rabbit IgG-coated colloidal gold (15 nm, BioCell Research Labs, Cardiff, U.K., 1:20 for glutamate or 1:10 for GABA) for 2 h; xii) three times 5 min wash in double-distilled water; xiii) staining with lead citrate (1 min); xiv) wash in distilled water.

Data analysis

Light microscopy. The PHA-L injection sites were evaluated by plotting PHA-L filled cell bodies from coronal sections at magnifications between 4× and 20× with the aid of the Neurolucida image analysis system (MicroBright-Field Inc., Colchester, VT) connected to a Zeiss Axioplan microscope. These Neurolucida images were then aligned and overlaid on the corresponding brain maps using the graphic software provided with the atlas of Swanson.⁸² From cases (C010, C020, C026) where PHA-L-labeled fibers approached cholinergic projection neurons, drawings of sections were made at 10× with the aid of a camera lucida drawing tube at various basal forebrain levels. Both PHA-L-labeled fibers and ChAT-positive cell bodies and proximal dendritic segments were drawn (25×). In addition, sections from case C026 were screened at high magnification (63×) with the aid of an ocular reticule for the presence of appositions between PHA-L and ChAT-labeled elements. The same criteria for selecting cases for analysis at the electron microscopic level, were used, i.e. a clearly identified PHA-L-labeled terminal swelling (including associated axon) directly abutting a ChAT-labeled profile, with both structures appearing in the same focal plane.

Electron microscopy. A total of 90 PHA-L-labeled varicosities were reconstructed in scans of serial sections from case C001. From this material 69 boutons were processed for co-detection of GABA, and 8 boutons were stained in alternate sections for detection of GABA and glutamate. Since the same fields always contained heavily-labeled axon terminals and the sections showed low background staining, no quantification of gold particle density was performed. PHA-L varicosities associated with cholinergic profiles ($n=63$) and PHA-L varicosities in close contact with parvalbumin neuronal elements ($n=10$) selected from five brains were scanned in serial sections for the presence of synapses.

RESULTS

Light microscopy of Phaseolus vulgaris leucoagglutinin-labeled cortical axons in the basal forebrain

Nomenclature. For the localization of PHA-L injection sites in Fig. 1 and Fig. 5 cortical divisions were labeled according to the atlas of Swanson.⁸² The prefrontal cortex (PFC) is generally defined as that part of the frontal cortex that has reciprocal connection with the mediodorsal thalamic nucleus^{35,50} and receives dense dopaminergic input from the ventral tegmental area.²⁰ The parcellation of the PFC used in this paper is largely adapted from Krettek and Price.⁵⁰ Accordingly, the PFC can be partitioned into medial, lateral or sulcal and ventral or orbital subdivisions. The medial parts comprise the medial precentral area (labeled secondary motor area in Fig. 1 and Fig. 5), the anterior cingulate, the prelimbic and infralimbic areas.* The ventral areas encompass the medial, ventral, ventrolateral and lateral orbital areas, whereas the lateral subdivisions include the ventral and dorsal agranular insular areas. More posteriorly, the rhinal sulcus becomes less deeply invaginated, and the agranular insular area and the overlying granular insular cortex receive a massive viscerosensory input from the thalamic ventroposterior (parvicellular) and the brainstem parabrachial nuclei.^{4,72} A PHA-L injection involving this more caudal portion of the insular cortex (C020) is separately discussed, since this area is not considered as part of the PFC. The PFC and insular cortex because of its transitional architectonic organization between neocortex and allocortex (piriform cortex) are often termed as periallo-¹⁰¹ or mesocortex.⁵⁹ The substantia innominata (SI) was defined according to the atlas of Paxinos and Watson.⁶³

Phaseolus vulgaris leucoagglutinin injections into the frontal and parietal cortices. The forebrain projections from the frontal and parietal cortices in cases C018, C019, and C028 were similar and are therefore described together. The locations of the tracer deposits are seen in Fig. 1. Case C018 involved a dual injection in the frontal cortex, the more medial of which was located primarily in layers V and VI of the

lateralmost part of the precentral cortex, while the more lateral injection was within the lateral frontal cortex (primary motor area), mainly within layer V and upper layer VIa (Fig. 1A). The PHA-L injection case C019 was located in the lateral part of the primary somatic sensory cortex, with the majority of labeled neurons in the deeper layers (V and VI), although some labeled cells were detected in layers II and IV (Fig. 1C). In case C028, pressure injections of PHA-L were made in three portions of the frontoparietal cortex. The first injection site was located at the border between the lateral frontal cortex (primary motor area) and the primary somatosensory cortex, and involved mostly layer V (Fig. 1B). The second (Fig. 1C) and third injections (Fig. 1D) were located more caudally, within the medial part of the primary somatosensory cortex. It should be noted that pressure injections of PHA-L tend to produce very large injection sites, and indeed, labeled neurons were found to some extent within the cortical areas separating the injection sites described, spanning a rostro-caudal distance of 2.5–3.0 mm. As a consequence, the forebrain labeling (described below) was heavier in case C028 than in cases C018 and C019.

From all cases a dense innervation of the striatum was seen, primarily within its dorsal and lateral parts, although in case C028 this terminal network also extended to more central portions of the striatum. Descending fibers were also seen in the striatum within the myelinated bundles of the internal capsule. These fibers were thick, smooth, and devoid of varicosities, and many could be followed to the thalamus, where they established dense terminal networks in several regions, including the ventrolateral, ventromedial, ventroposterior medial, gelatinosus, and reticular nuclei. Other fibers remained within the internal capsule, and descended further caudally. Labeled fibers and terminals were not detected in other forebrain regions.

The dark-field photomicrograph of Fig. 2 illustrates the pattern of labeling at one level of the basal forebrain (approximately 1.3 mm caudal to bregma) from case C028. Although labeled fibers were present in the striatum (including within the myelinated fascicles of the internal capsule at this level), no labeling was seen within more ventral parts of the forebrain.

Phaseolus vulgaris leucoagglutinin injection in the occipital cortex. Three PHA-L injections were made in case C025 within the occipital cortex, involving several subsectors of this region, although the majority of labeled neurons were within the medial and lateral subfields of the primary visual cortex (Fig. 1F). The most lateral injection slightly encroached upon the posterior parietal association area.

Labeling within the striatum was found primarily within its dorsomedial portion. Fibers could be followed in the internal capsule as they coursed beneath

*The infralimbic area sends projections to the mediodorsal nucleus, but appears not to receive input from it.³⁴

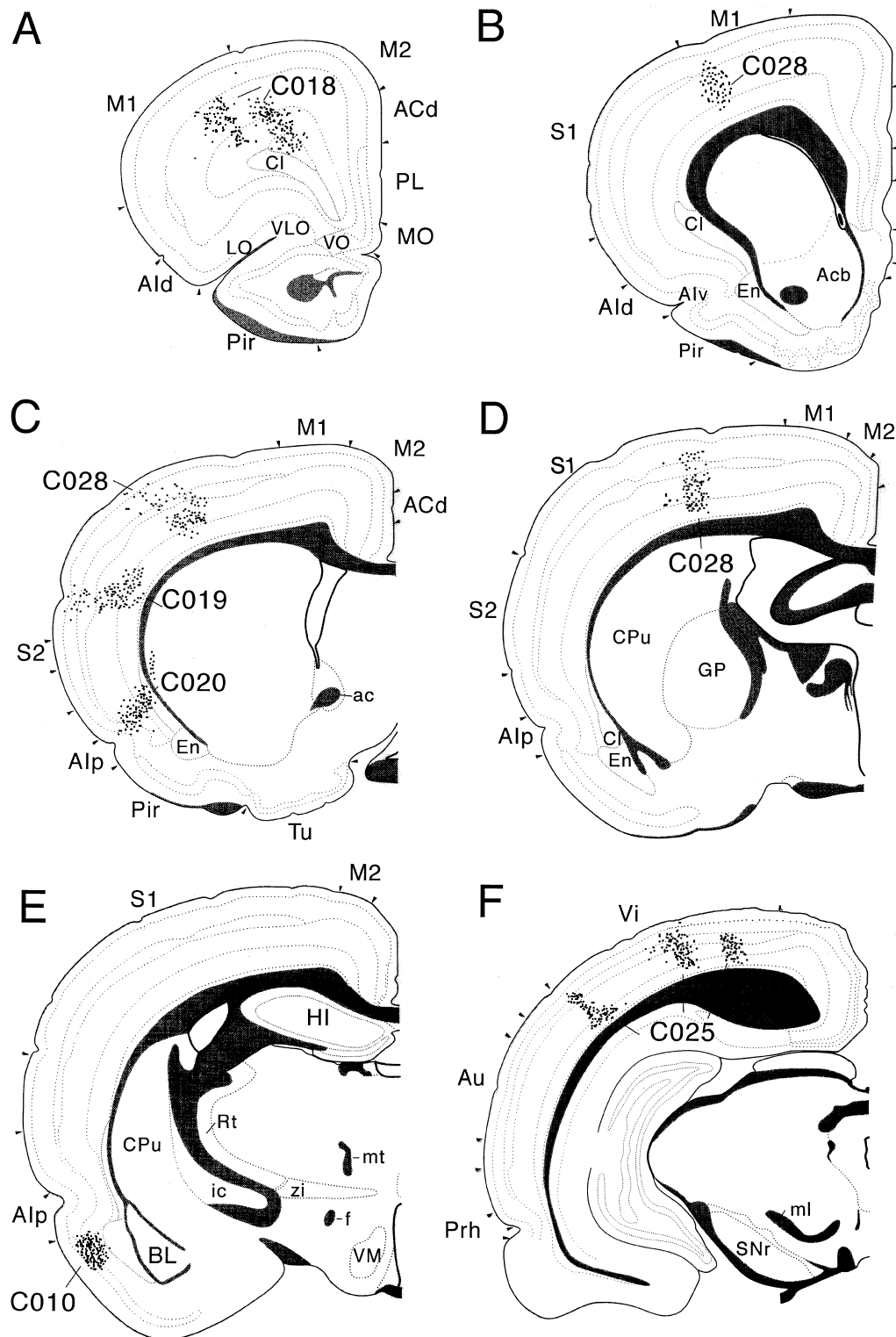


Fig. 1. Composite diagram illustrating the locations of cortical PHA-L injection sites for the light microscopic study. Camera lucida plots of sections containing PHA-L-labeled neurons at the level of their maximum extent were aligned and superimposed of the corresponding maps from the atlas of Swanson.⁸² ac, anterior commissure; Acb, nucleus accumbens; Alp, agranular insular area, posterior part; ACd, anterior cingulate area, dorsal part; Ald, agranular insular area, dorsal part; Alv, agranular insular area, ventral part; Au, auditory cortex; BL, basolateral amygdaloid nucleus; Cl, claustrum; CPu, caudate-putamen; En, endopiriform nucleus; f, fornix; GP, globus pallidus; HI, hippocampus; ic, internal capsule; LO, lateral orbital area; M1, primary motor cortex; M2, secondary motor cortex; MO, medial orbital area; ml, medial lemniscus; mt, mammillothalamic tract; Pir, piriform cortex; PL, prelimbic cortex; Prh, perirhinal cortex; Rt, reticular thalamic nucleus; S1, primary somatosensory cortex; S2, supplemental somatosensory area; SNr, substantia nigra pars reticulata; Tu, olfactory tubercle; Vi, visual cortex; VLO, ventrolateral orbital area; VM, ventromedial hypothalamic nucleus; VO, ventral orbital area; zi, zona incerta.



Fig. 2. Dark-field photomicrograph of frontal section (from level approximately 1.3 mm caudal to the bregma) that has been labeled for PHA-L following delivery of the tracer to the frontoparietal cortex (case C028). PHA-L-labeled fibers and terminals were detected in the dorsolateral portions of the caudate-putamen (arrows). Boxed area is shown with higher magnification in Fig. 7A. The heavily myelinated fiber bundles in this and subsequent photomicrographs show white reflection. CPu, caudate-putamen; GP, globus pallidus; HDB, horizontal limb of the diagonal band; ic, internal capsule; ox, optic chiasm; SI, substantia innominata; 3V, third ventricle; sm, stria medullaris. Scale bar=1 mm.

the floor of the lateral ventricle to the thalamus. Within the thalamus labeled fibers and terminals were seen in the lateral geniculate nucleus, and to some extent within the lateroposterior nucleus. A few scattered fibers were also seen within the internal capsule at caudal forebrain levels, however these had few or no terminal varicosities. Similar to the frontoparietal injections, striatal labeling was observed in this case, but no labeled fibers/terminals were detected elsewhere in the forebrain.

Phaseolus vulgaris leucoagglutinin injection in the piriform cortex. The injection site in case C010 involved the piriform cortex just lateral to the dorsal endopiriform nucleus (Fig. 1E). From the injection site, labeled fibers were seen to course medially through the piriform cortex. From this point fibers

passed ventromedially through the anterior amygdaloid area and caudolateral SI, to the area just ventral to the magnocellular preoptic nucleus (MCP) and HDB. Terminal networks were seen along the course of these projections. Other fibers were collected more ventrally, and ran medially along the ventral surface of the brain to the olfactory tubercle. A small contingent of fibers continued rostrally before turning dorsally and running through the lateral portion of the medial septum.

Phaseolus vulgaris leucoagglutinin injection in the insular cortex. The PHA-L injection in case C020 was located just lateral to the claustrum in the posterior agranular insular area and in overlying deep layers of the gustatory insular cortex (Fig. 1C). A few neurons labeled along the pipette track were seen in the lateral

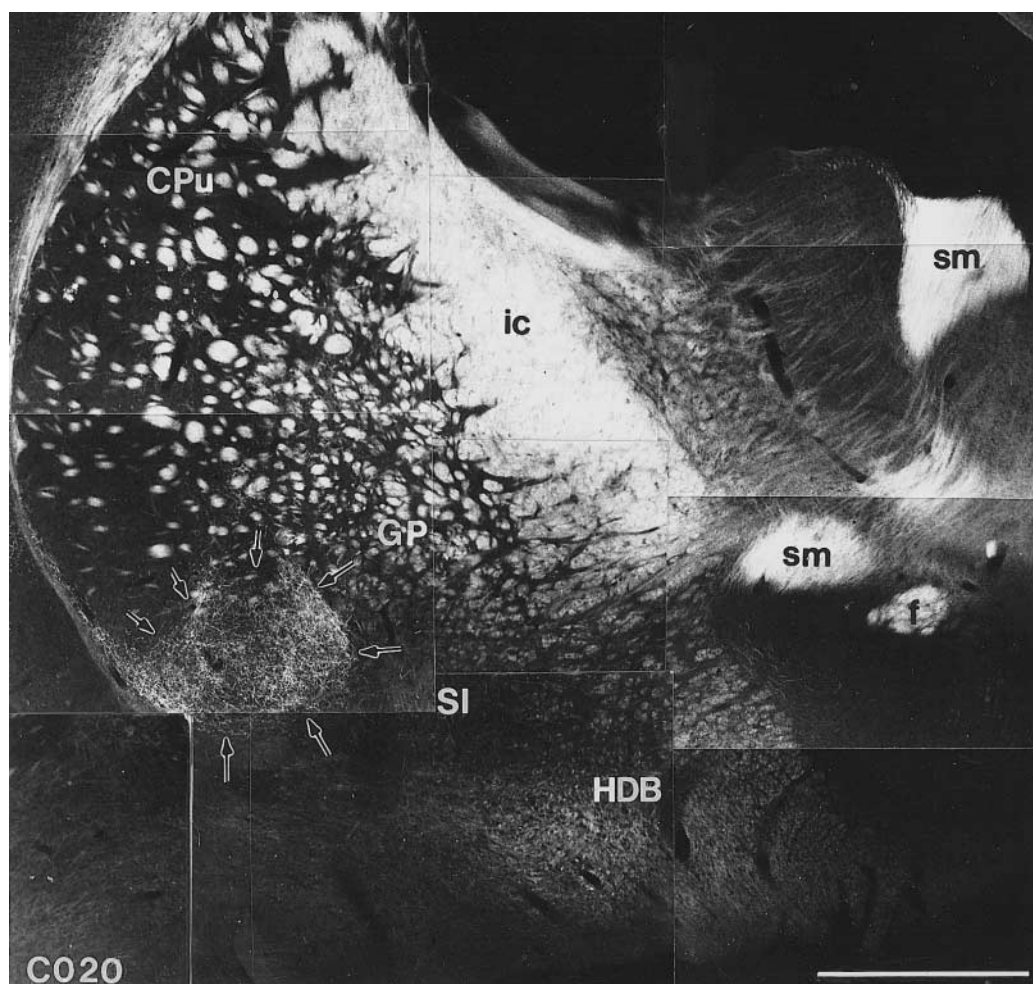


Fig. 3. Dark-field photomontage of frontal section to show the distribution of PHA-L-labeled terminals in the ventral part of the caudate-putamen (arrows) following tracer delivery in the agranular insular cortex (case C020). Scale bar=1 mm.

portion of the striatum. Fibers were seen to course into the striatum, where dense networks of fibers and terminals were seen in the ventrolateral portions (Fig. 3). Some fibers continued medially through the striatum, and at more rostral levels reached the lateral portion of the bed nucleus of the stria terminalis. Labeled fibers were also seen in the globus pallidus and lateral portion of the internal capsule. These axons were primarily fibers of passage, most of which were collected within the myelinated fascicles of the internal capsule, although a few fibers bearing *en passant* varicosities coursed medially within the globus pallidus. Some fibers were seen as detached and coursed ventrolaterally through the caudolateral SI to the central amygdaloid nucleus.

At more caudal levels, fibers were seen within the internal capsule *en route* to the thalamus, where dense terminal networks were distributed within the reticular nucleus, and in particular, the parvocellular ventroposterior thalamic (viscerosensory) nucleus. Lighter labeling was also seen within the mediodorsal thalamic nucleus.

Figure 4 shows the relationship of PHA-L-labeled fibers and cholinergic neurons in cases in which the tracer was injected into the piriform cortex (Fig. 1E: case C010) or the insular cortex (Fig. 1C: case C020), respectively. In these cases, PHA-L-labeled fibers had *en passant* varicosities as well as short terminal branches, and labeled terminals occasionally were seen in close proximity to cholinergic neuronal elements in the ventral MCP/HDB (Fig. 4A) and caudolateral SI (Fig. 4B), respectively.

Phaseolus vulgaris leucoagglutinin injections in the prefrontal cortex. Six animals received multiple PHA-L injections in different parts of the prefrontal cortex. In two cases (C088, C104) the majority of labeled cells were located in the prelimbic and infralimbic cortices, in the other cases, PHA-L-labeled cells filled various portions of the ventral and lateral parts of the prefrontal cortex. Figure 5 shows the location of labeled cells at the injection sites from these cases.

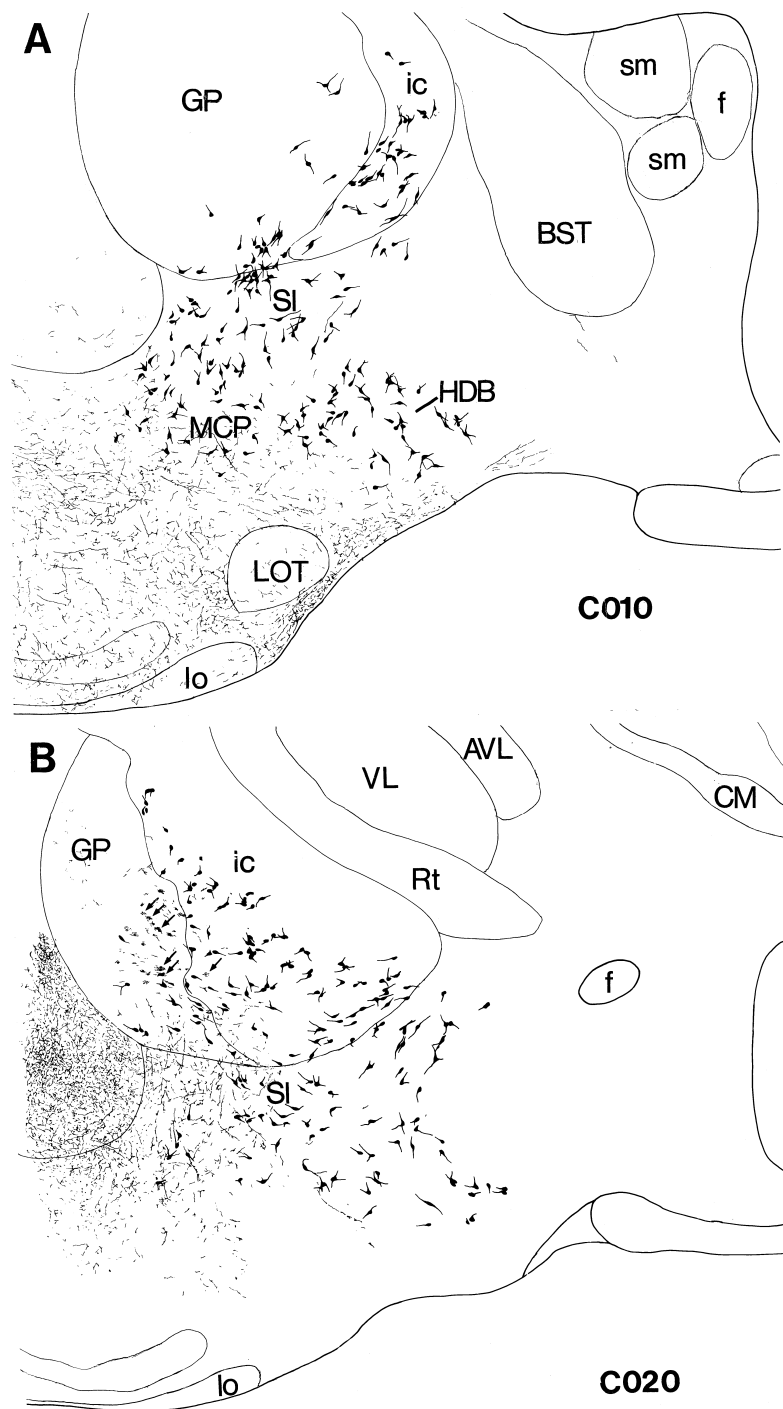


Fig. 4. Camera lucida drawings ($10\times$) from double-labeled sections illustrating the distribution of PHA-L fibers and terminals in relation to cholinergic cells following PHA-L injections in the piriform (A, case C010) and insular (B, case C020) cortices. Levels of drawings are approximately 1.0 mm (A) and 1.6 mm (B) caudal to the bregma, respectively. Small arrows in (B) denote fibers confined to the internal capsule bundles within the globus pallidus. AVL, anterior ventral thalamic nucleus; BST, bed nucleus of the stria terminalis; CM, central medial thalamic nucleus; lo, lateral olfactory tract; LOT, nucleus of the lateral olfactory tract; MCP, magnocellular preoptic nucleus; Rt, reticular thalamic nucleus; VL, ventrolateral thalamic nucleus.

In general, PHA-L-labeled axons were distributed in the basal forebrain according to a mediolateral

topography.^{27,39,76} PHA-L-labeled axons were profusely arborizing in ventral striatal areas, including

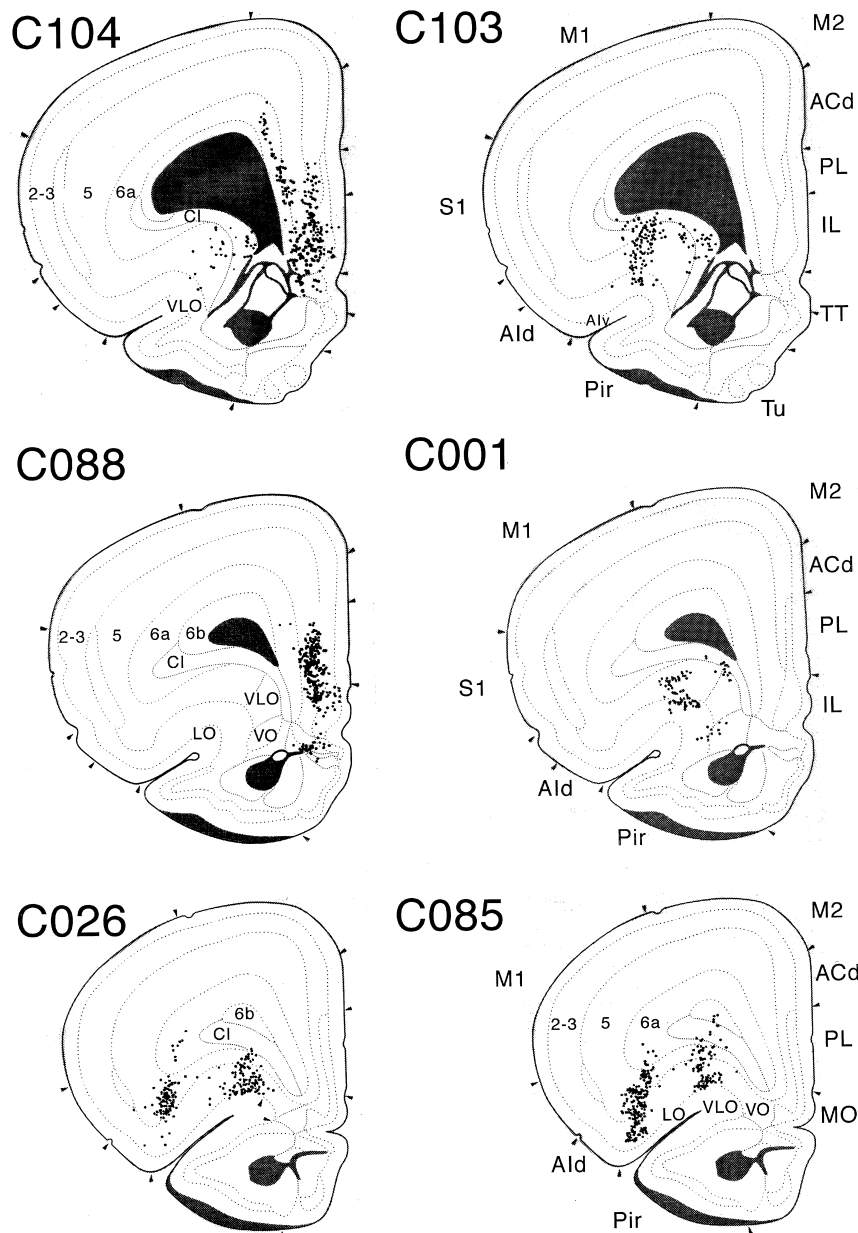


Fig. 5. Composite diagram illustrating the location of PHA-L-labeled cells at their maximum extent in the prefrontal cases for light and electron microscopic analysis. Injection sites are arranged according to their caudorostral (upper-middle-lower rows) location. Each dot represents one neuron. Drawings adapted from Swanson.⁸² IL, infralimbic cortex; TT, tenia tecta. Other abbreviations, see Fig. 1.

the ventral part of the nucleus accumbens, olfactory tubercle and the interconnected striatal cell bridges. The terminals often formed patches in striatal regions. On the other hand, adjacent and interdigitating ventral pallidal territories remained mostly free of terminals.

In case C026, dual PHA-L injections were made within the orbital (orbitofrontal cortex), and lateral (insular or sulcal) PFC (Fig. 5). From the injection site many fibers coursed into the striatum, where they i) terminated massively in its mid-portion at rostral

levels, ii) ran within the bundles of the internal capsule (Fig. 6, boxed region), or iii) coursed ventrally through the striatum to more ventral fore-brain territories. Some labeled fibers entered the external capsule in which they coursed caudally to distribute to the deep piriform cortex. Some of these fibers proceed underneath the striatum towards the HDB and SI. Of the fibers that coursed ventrally through the striatum, some could be followed to the olfactory tubercle. A small contingent turned medially and then ran dorsally, through the vertical limb

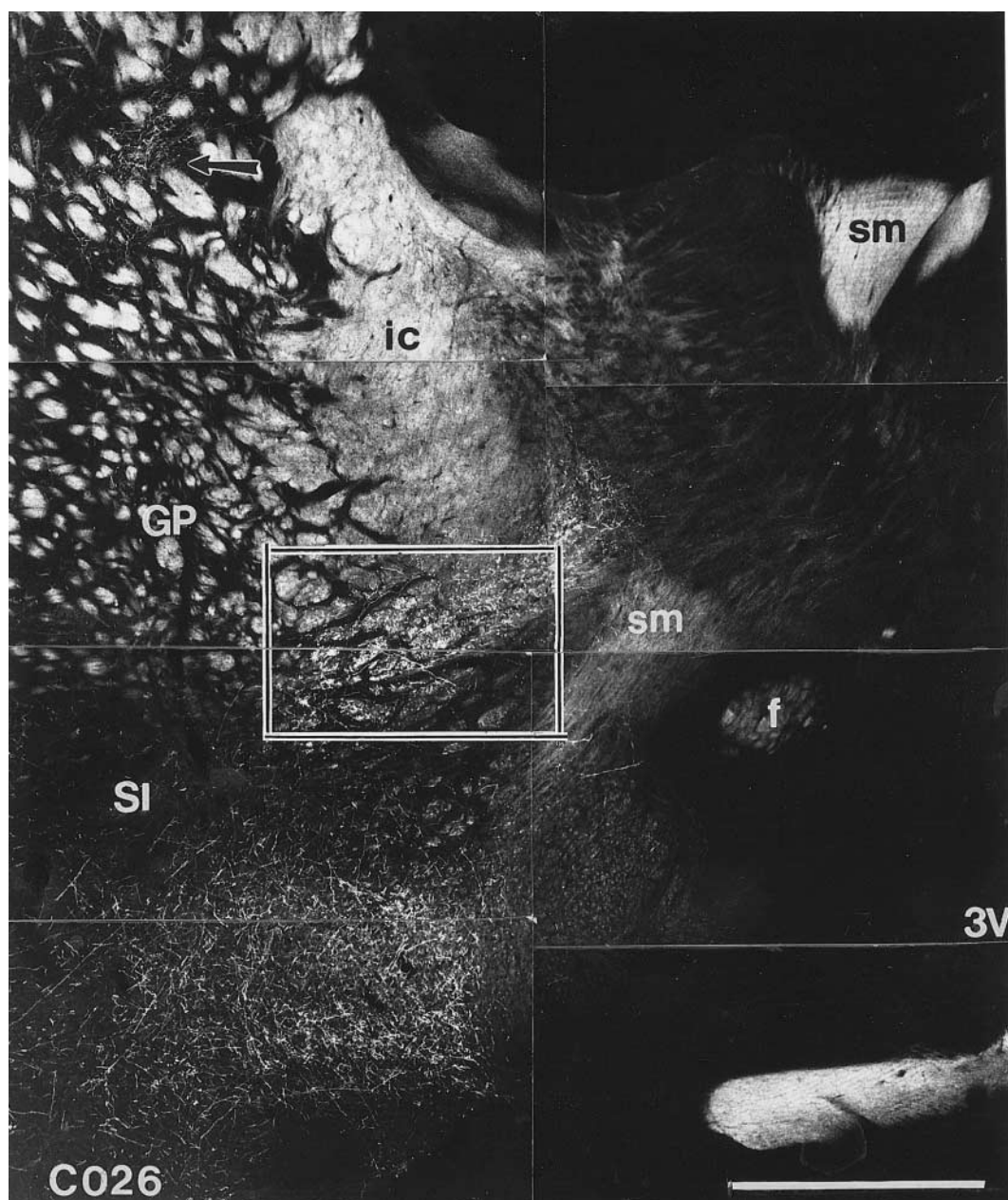


Fig. 6. Dark-field photomicrograph of a frontal section illustrating the distribution of PHA-L fibers and terminals in the basal forebrain (case C026). Heavy labeling is evident in the horizontal limb of the diagonal band, ventral SI, and internal capsule. Labeling is also evident within the caudate-putamen. Boxed area is shown in Fig. 7B. f, fornix. Scale bar=1 mm.

of the diagonal band nucleus and medial septum, before coursing over the genu of the corpus callosum to the cortex. Other fibers that coursed ventrally through the striatum left this structure at more caudal levels, establishing networks of fibers/terminals in the amygdala, SI, MCP, and in particular, the HDB and regions ventral to the HDB, as shown in the dark-field photomicrograph of Fig. 6. Fibers also arrived at these regions after detaching from the internal capsule (Fig. 7B). In comparison,

Fig. 7A shows that descending fibers from neocortical areas (e.g. case C028) remained in the internal capsule *en route* to caudal areas.

In all prefrontal injections, PHA-L fibers showed profuse varicosities in basal forebrain areas rich in cholinergic neurons. A high magnification light microscopic analysis of case C026 shows that some of the PHA-L-labeled terminals are apparently abutting cholinergic elements in the HDB and MCP area (Fig. 8).

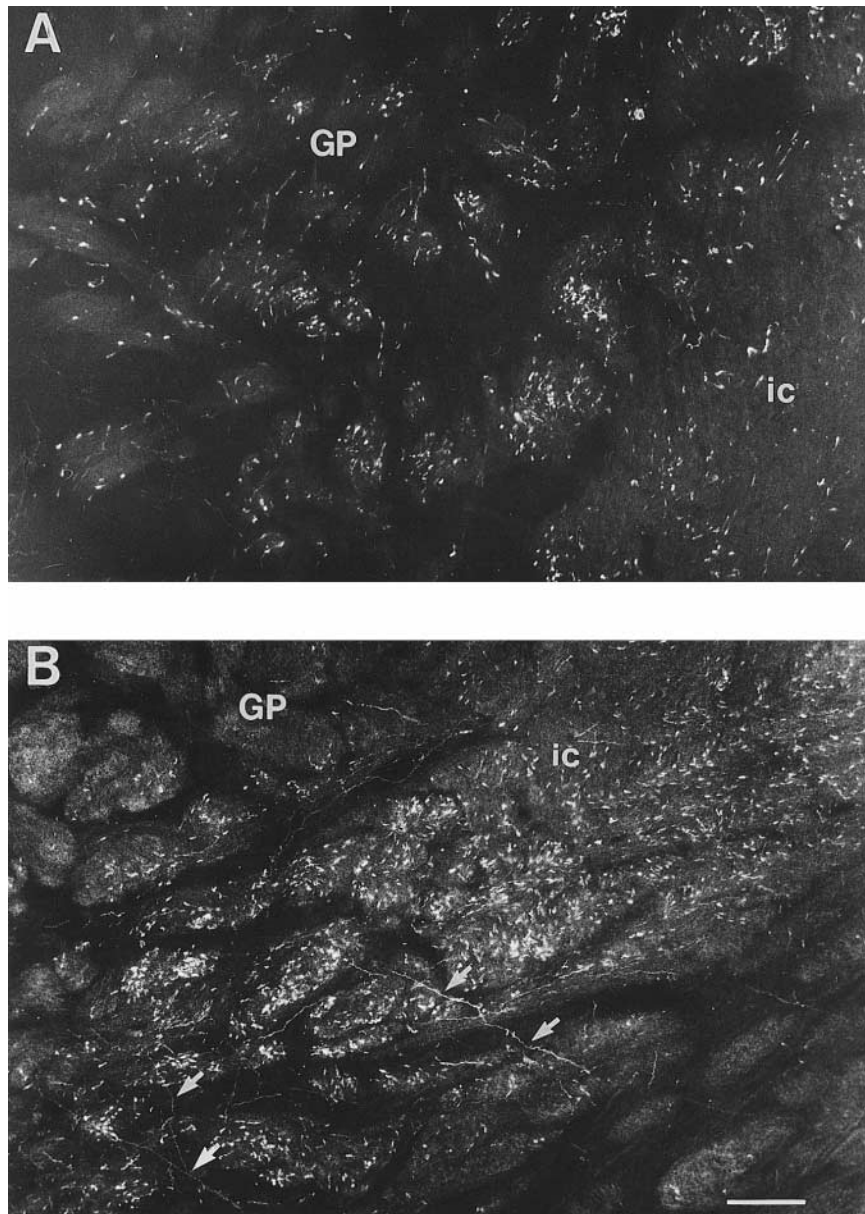
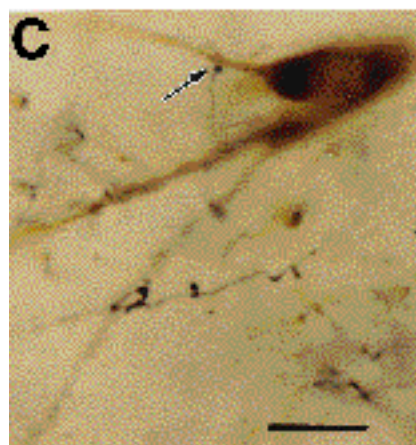
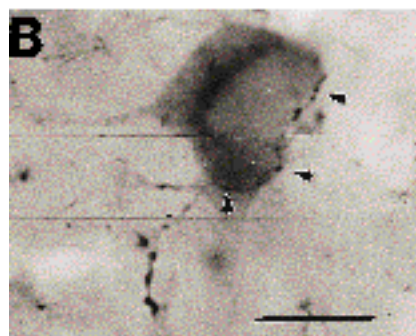
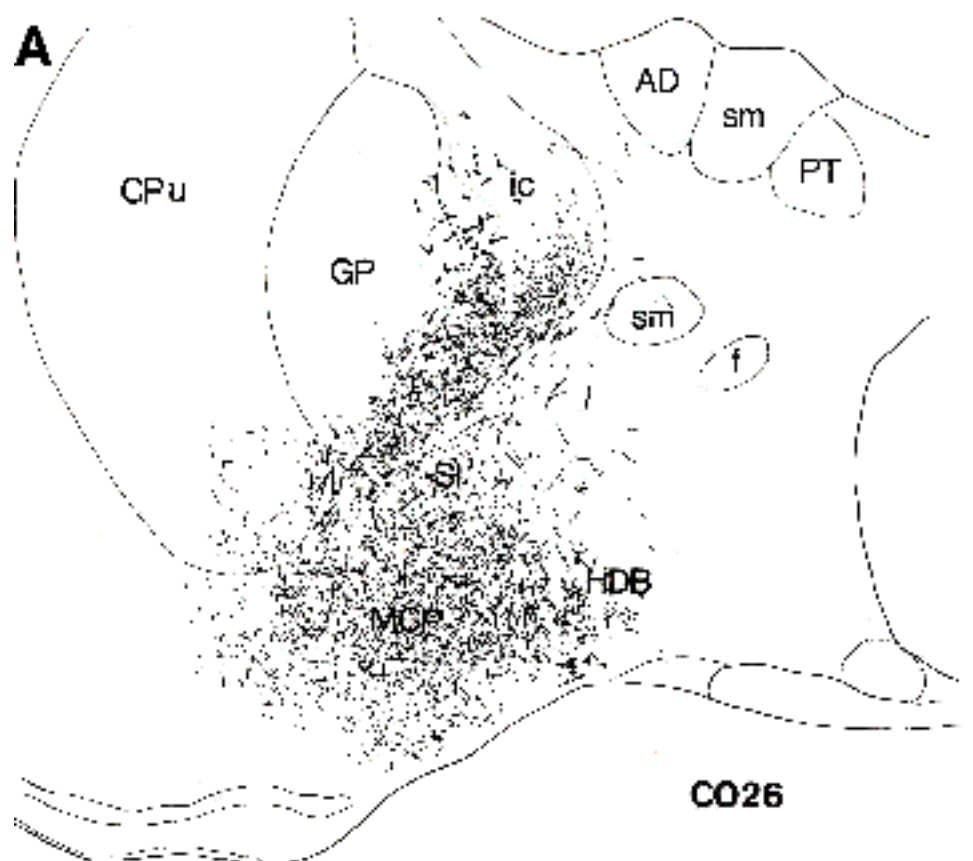


Fig. 7. Dark-field photomicrographs illustrating the relationship of descending PHA-L-labeled axons in the internal capsule. Numerous PHA-L-labeled axons are seen in A (case C028) that are cut in cross section, and confined to the myelinated bundles of the internal capsule. A similar situation is seen in B (case C026), although a few fibers are seen to detach (arrows) and course ventrally and ventromedially. Scale bar=10 μ m.

Electron microscopy of prefrontal axon terminals in the basal forebrain

In general, two types of labeled axons can be observed in the basal forebrain: axons with very small varicosities with thin, long intervaricose segments, which proved to be axons of passage under the electron microscope; the other type had larger varicosities, with smaller intervaricose segments; these varicosities always contained a large number of synaptic vesicles and many of these boutons entered into synaptic contacts with various profiles. A total

of 90 randomly collected PHA-L-labeled boutons were serially reconstructed from case C001 (i.e. that case perfused with 1% glutaraldehyde, thereby allowing GABA or glutamate postembedding staining of these profiles). From this pool 80 boutons were identified to establish synaptic specializations while the remaining 10 varicosities did not display synaptic contacts, or represented axons of passage. From the 80 PHA-L-labeled synaptic boutons, in 89% of the cases the synapse was found with a spine or with a small dendritic branch and in only 11% of the cases was the synapse with a proximal dendritic shaft.



Examples from this material are shown in Figs 9, 10 and 11. From a single myelinated prefrontal axon, depicted in Fig. 9C, altogether 14 varicosities were identified as synaptic boutons and two are displayed in Fig. 9F and G (arrow). Both synapses are with dendritic spines. Labeled varicosities were occasionally found adjacent to cell bodies such as shown in Fig. 10, but these did not make synapse with the perikaryon. Indeed, the large labeled varicosity adjacent to the cell body shown in Fig. 10 entered into synaptic contacts with a small dendritic branch and a spine (Fig. 10C, D).

Phaseolus vulgaris leucoagglutinin/GABA/glutamate. From the 90 reconstructed boutons, 69 were stained for GABA in at least three consecutive sections. In all cases, the PHA-L-labeled boutons contained only background immunogold labeling, while axon terminals non-labeled for PHA-L showed clear accumulation of gold particles indicating the presence of GABA in these boutons. Even after longer treatment with sodium metaperiodate, in which case the PHA-L-labeled boutons lost most of their electron density, we could not identify selective accumulation of gold particles indicative of GABA in the corticofugal presynaptic axons. In eight cases synaptic varicosities were stained in alternate grids for GABA and glutamate. Figure 11 shows a representative example from this material. The same large axon terminal is shown in three adjacent sections selectively accumulating gold particles, indicating glutamate immunoreactivity, but no GABA immunoreactivity could be established when sections were processed for GABA postembedding staining (fourth section). This axon terminal is in synaptic contact with a dendritic spine.

Transmitter phenotype of the postsynaptic target neurons

Phaseolus vulgaris leucoagglutinin/choline acetyltransferase. A total of 63 PHA-L-labeled varicosities abutting ChAT-immunopositive elements were selected for ultrastructural analysis from five brains (cases C026, C085, C088, C103, C104).

Labeled terminals were encountered on cholinergic cell bodies ($n=33$), proximal dendrites within 50 μm distance from the parent cell body ($n=19$), and distal cholinergic dendritic processes ($n=11$). Sixteen out of the 63 selected terminals appeared to make no direct contact with ChAT-positive neurons, but were separated by layers of membranes of interposed profile. The remaining 47 boutons were found indeed to abut directly against the apposed ChAT-immunolabeled structure. Figure 12 shows the location of these identified PHA-L-labeled varicosities. It can be seen that a large number of synaptic terminals were identified from areas near the ventral striatum/ventral pallidal border (Fig. 12A), where the density of the prefrontal axons was high. Interestingly, a relatively large number of terminal boutons were localized in close apposition to cholinergic profiles in the HDB/SI area (Fig. 12E) where the density of prefrontal terminals was low, but as the descending fibers to more caudal areas were passing through this area, they gave rise to terminal varicosities. Under the electron microscope, in several cases a slight increase in the density of the cholinergic postsynaptic membrane was discernible and no other synapse of the selected bouton was found (Fig. 13). However, due to the lack of other signs of synaptic specialization (straightening of the apposed membranes, widening of the intercellular space, etc.), no definitive conclusion could be drawn about the presence of synaptic contact with cholinergic profiles. In contrast to this, from the 63 boutons, 37 PHA-L varicosities clearly established synaptic contact with non-cholinergic elements. The majority of postsynaptic targets were dendritic spines (74%), often characterized by the presence of spine apparatus (Fig. 14). The synapses occurred both on the head (Fig. 14) and the neck of the dendritic spines (Fig. 15C, lower right inset). Additional contacts were with small dendritic processes and dendritic shafts (Fig. 15C, upper left inset), each with 13%. In all cases the synapses with the non-cholinergic profile were unequivocally of the asymmetric type.

Phaseolus vulgaris leucoagglutinin/parvalbumin. From 10 PHA-L-labeled varicosities adjacent to

Fig. 8. A) Camera lucida drawing (10 $\times$) from a double-labeled section (the same as shown in the dark-field image of Fig. 6) illustrating the distribution of PHA-L-labeled fibers in relation to cholinergic neurons following delivery of the tracer to the orbitofrontal cortex (C026). B) High magnification light micrograph showing PHA-L-labeled *en passant* boutons (arrowheads) in apposition to a cholinergic cell body. C) Colour micrograph showing close association of a PHA-L-labeled bouton with cholinergic dendrites (arrow). Note the colour difference between the brown DAB-labeled cholinergic profiles and the NiDAB-labeled black PHA-L varicosities. D) Schematic drawing illustrating the distribution pattern of PHA-L-labeled varicosities in apposition to cholinergic neurons from section (A) that was analysed using high magnification light microscopy. Cholinergic cell bodies are represented by dots. Zones containing PHA-L-positive varicosities adjacent to cholinergic profiles are depicted as red squares. Sections were screened using an ocular reticule (80 $\times$ 80 μm) at 63 $\times$, and putative contact sites were marked on camera lucida drawing using a proportional grid. A comparison of (A) and (D) suggests that globus pallidus and internal capsule contain mostly PHA-L-labeled fibers of passage devoid of varicosities adjacent to cholinergic neurons. Scale bar in B and C=10 μm . AD, anterodorsal thalamic nucleus; PT, paratenial thalamic nucleus.

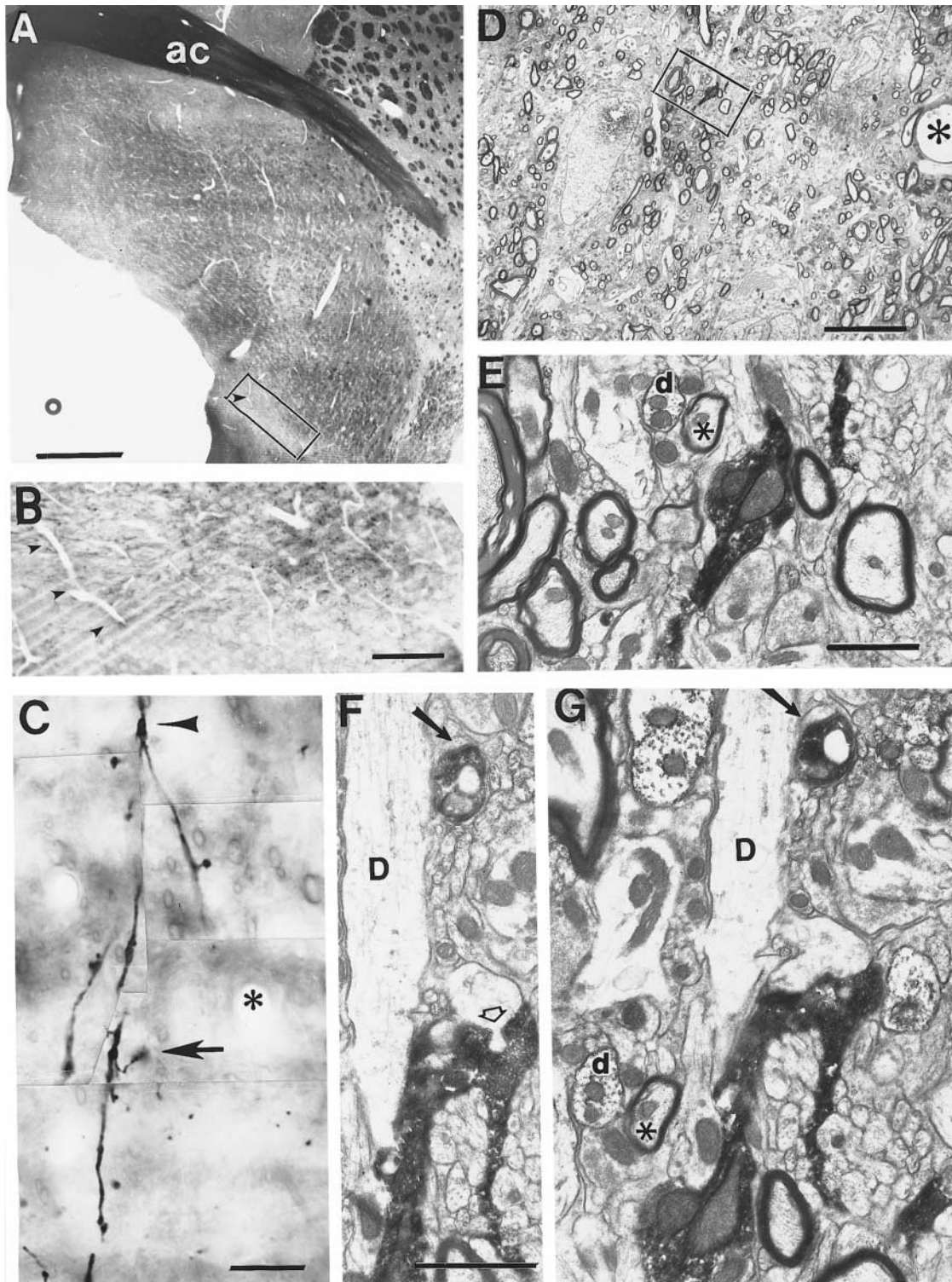


Fig. 9. Arborization pattern of prefrontal axons in the basal forebrain (case C001). A) Low magnification photomicrograph of a forebrain section at the level of the crossing of the anterior commissure (ac) processed for electron microscopy. B) Enlargement of the boxed area from (A). Capillaries serve as fiducial markers (arrowheads). Note the dense distribution of PHA-L-labeled terminals. C) A corticofugal axon emanating from a myelinated sheath labeled with arrowhead gives rise to several terminal varicosities in the ventral part of the HDB (boxed area in A), seen under the electron microscope in (D–G). The varicosity marked with arrow in (C) is shown with progressively higher magnification under the electron microscope in (D–G). D) Low magnification electron micrograph, the boxed area, shown at higher magnification in (E), contains a PHA-L-labeled axon enlargement. Asterisk marks identical capillaries in (D) and (C). F, G) Adjacent thin sections to (E) showing that the labeled varicosity from (E) is synapsing with a small dendritic evagination in (F) (open arrow). Another axon terminal (arrow) in (F) and (G) is in synaptic contact with a spine in the upper part of the pictures. d, D marks dendritic shafts and stars denote the same myelinated fibre serving as fiducial marker to correlate sections in panels (E–G). Scale bar A=500 μ m, B=100 μ m, C, D=10 μ m, E=1 μ m, F=1 μ m (also valid for G).

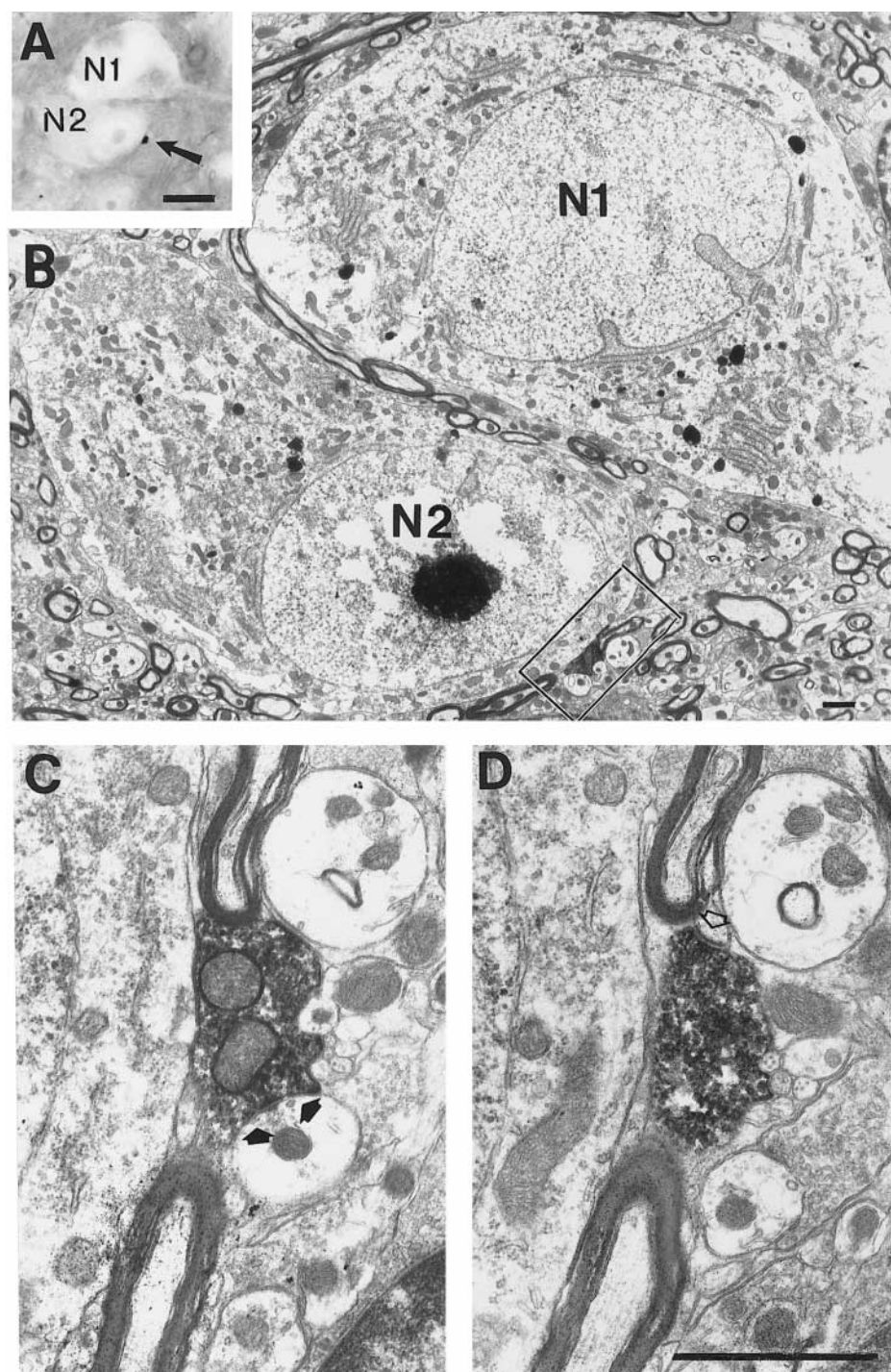


Fig. 10. Illustrates the typical pattern of termination of a prefrontal axon in the ventral part of the HDB (from case C001). A) Adjacent to the cell body of N2 a PHA-L-labeled varicosity (arrow). B) Electron micrograph showing the two neurons (N1 and N2) depicted in (A). Note the richness of the endoplasmic reticulum in both perikaryon. The region of boxed area in B is enlarged in two adjacent sections in (C) and (D). C) The large axon terminal adjacent to cell N2 is synapsing with a small dendritic profile. Arrows point to the postsynaptic thickening. D) The same bouton in an adjacent thin section is in synaptic contact with a small dendritic spine (open arrow). Scale bar in A=10 μ m, B=1 μ m, D=1 μ m (also valid for C).

parvalbumin-immunoreactive profiles selected for ultrastructural analysis, four showed unequivocal sign of synapse with dendritic shafts immunoreactive

for parvalbumin (Fig. 16, 17). The other six PHA-L-labeled boutons established synapses with unlabeled dendritic shafts or spines. Figure 16A shows the

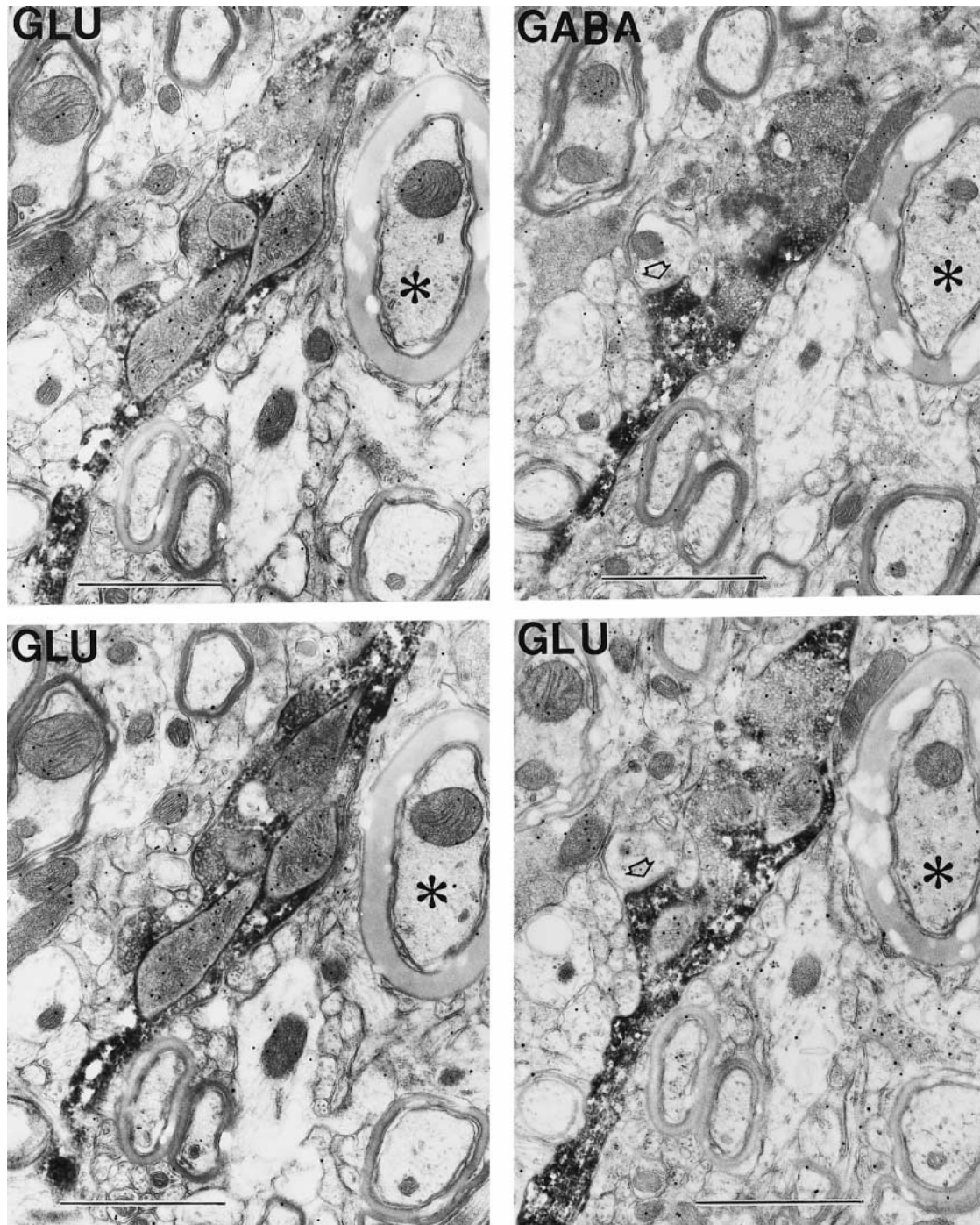


Fig. 11. Pre-embedding PHA-L immunostaining combined with alternate GABA/glutamate postembedding staining (case C001). Four adjacent ultrathin sections immunogold-labeled for GABA or glutamate (GLU). The obliquely running PHA-L-labeled axon varicosity is easily appreciated in all sections. Note that this axon is rich in gold particles indicating glutamate-immunostaining but lacks GABA immunolabeling. Open arrow points to a synapse with a small dendritic process. Asterisk marks the same myelinated axon in all sections. Scale bar=1 μ m.

distribution of parvalbumin-positive neurons in the ventral pallidum and Fig. 16B and C depict a parvalbumin-containing dendritic shaft in this region receiving a large PHA-L-positive vesicle containing varicosity. Figure 17A shows a parvalbumin-

positive cell body in the ventral pallidum. Under the light microscope many light-brown parvalbumin-containing varicosities surrounded this cell body, however one varicosity was particularly apparent because of its density and black colour. This

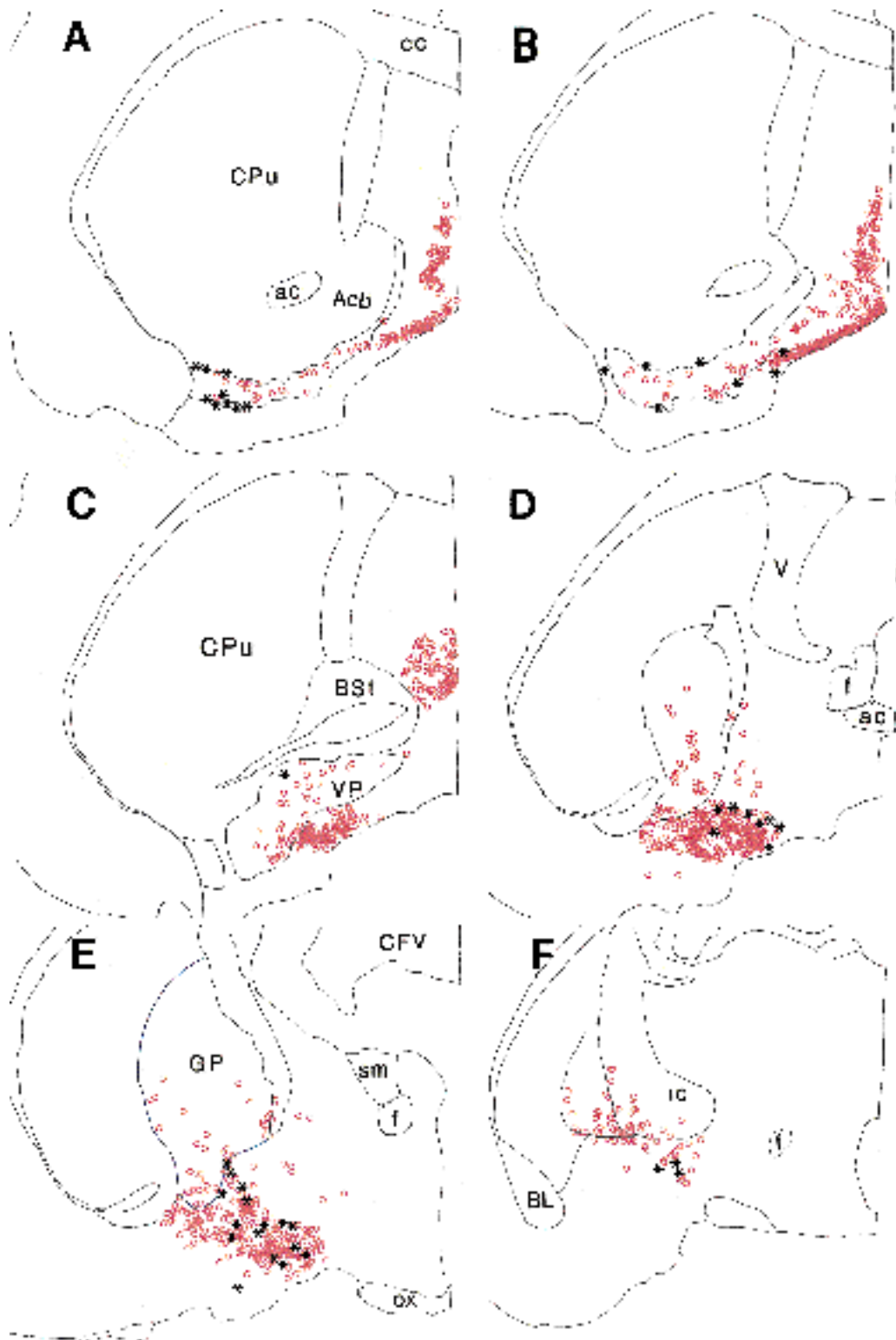


Fig. 12. Composite maps showing the approximate location of PHA-L varicosities (asterisks) abutting directly to cholinergic profiles selected for serial electron microscopic reconstruction. The location of identified synapses were entered into a reference database, plotted from the original embedded sections using fiducial markers for orientation. Several cases from within a 500 μ m distance were pooled into one figure. Open circles represent cholinergic cell bodies. cc, corpus callosum; CFV, ventral hippocampal commissure; VP, ventral pallidum.

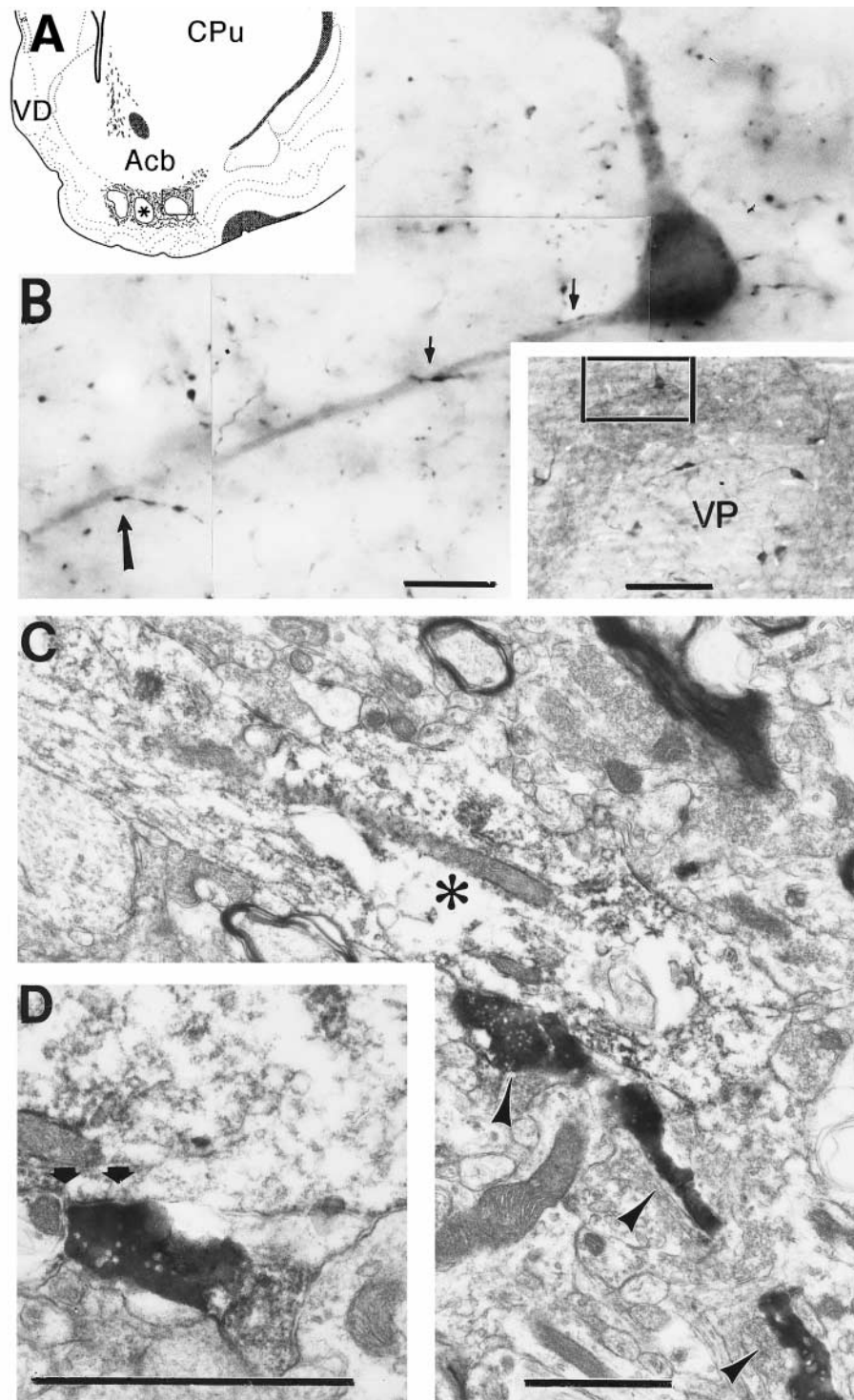


Fig. 13. PHA-L-labeled axon terminals in close association with a cholinergic dendrite. A) Schematic drawing (adapted from Swanson's atlas) to show the location of the cholinergic neuron at the border between the ventral shell of the nucleus accumbens (Acb) and ventral pallidal pockets (asterisk). Note that the ventral pallidal pockets are surrounded by dense PHA-L-labeled terminals. Boxed area in (A) is shown at higher magnification in the inset. Boxed area from the inset is partially depicted in (B) at higher magnification. The cholinergic neuron is approached by an axon (large arrow) which has a varicosity next to the dendrite. Several other axons are crossing over this dendrite (small arrows), but none of them were identified as synaptic. C) Low magnification electron micrograph showing a portion of the cholinergic dendrite (asterisk) with several fragments of the PHA-L-labeled axon (arrowheads) that are labeled with the large arrow in (B). D) High magnification view of the contact site from a section adjacent to (C). Note the small thickening of the postsynaptic membrane (arrows). Synaptic vesicles are visible in the presynaptic bouton. Scale bar in B=10 μ m, inset: 100 μ m, C and D=1 μ m. VD, vertical limb of the diagonal band; VP, ventral pallidum.

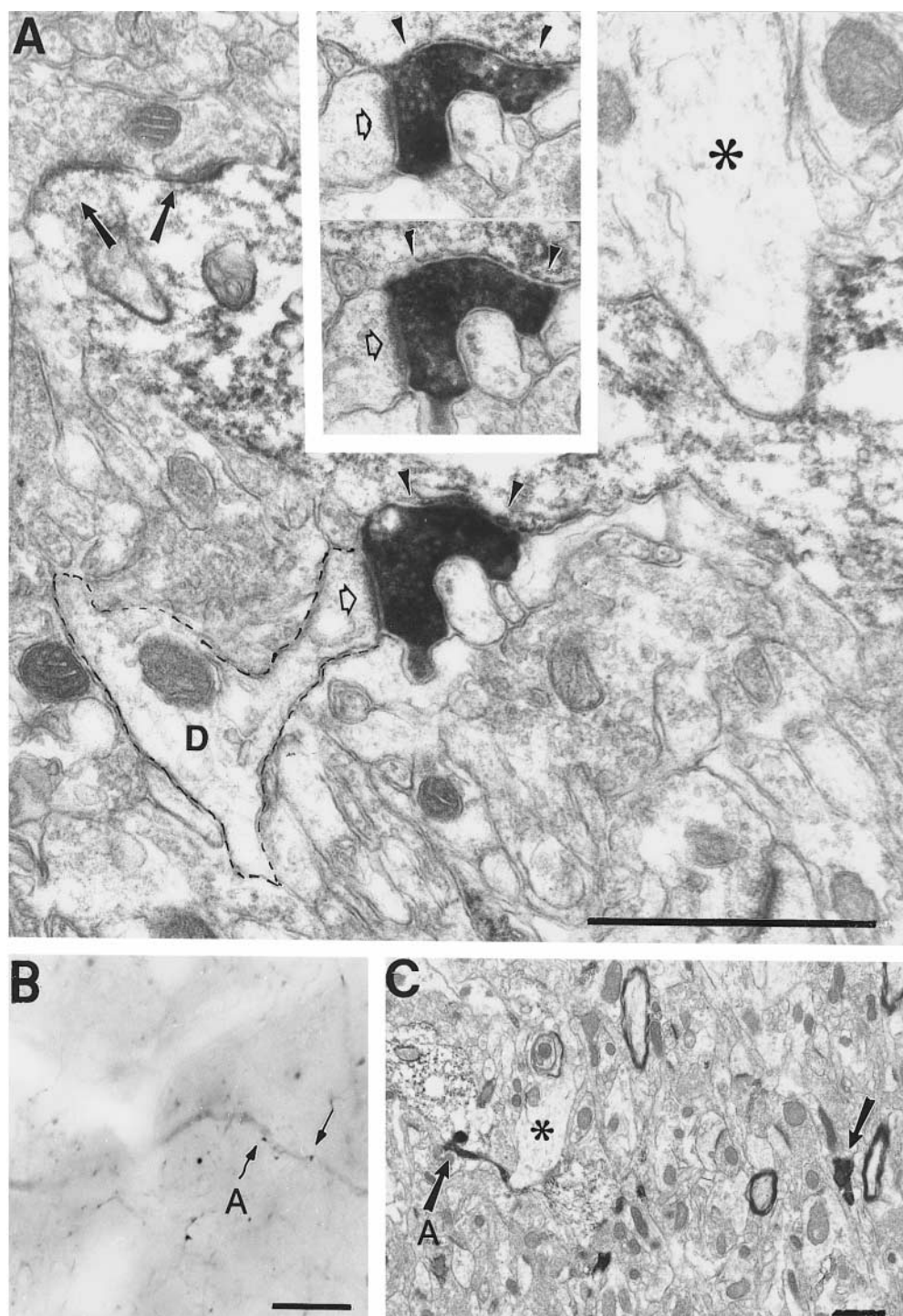


Fig. 14. Corticofugal axon terminal synapses with an unlabeled dendritic spine-head in the close vicinity of a distal cholinergic dendrite. A) High magnification view of the PHA-L-labeled bouton marked with an arrow connected to an "A" in (B) and (C). The labeled varicosity in the close vicinity of the cholinergic dendrite enters into an asymmetric synapse with a dendritic spine. Open arrow points to the postsynaptic membrane. D marks the parent dendritic shaft which is connected through a narrow neck with the spine head. The inset shows images of the same synapse from two adjacent sections. In all three sections the synapse with the non-cholinergic profile is unambiguous, but the cholinergic membrane (between arrowheads) facing the labeled bouton shows no clear indication of a synapse. An unlabeled bouton in the upper left corner shows a slightly asymmetric synapse with the same cholinergic dendrite (arrows). B) Light micrograph of the same distal cholinergic dendrite is surrounded by several PHA-L-labeled varicosities. C) Low magnification electron micrograph showing approximately the area between the arrows in (B). Asterisk in (C) and (A) marks the same non-labeled dendrite. Arrows in (B) and (C) point to identical PHA-L-labeled boutons. Scale bar in B=10 μ m, A, C=1 μ m.

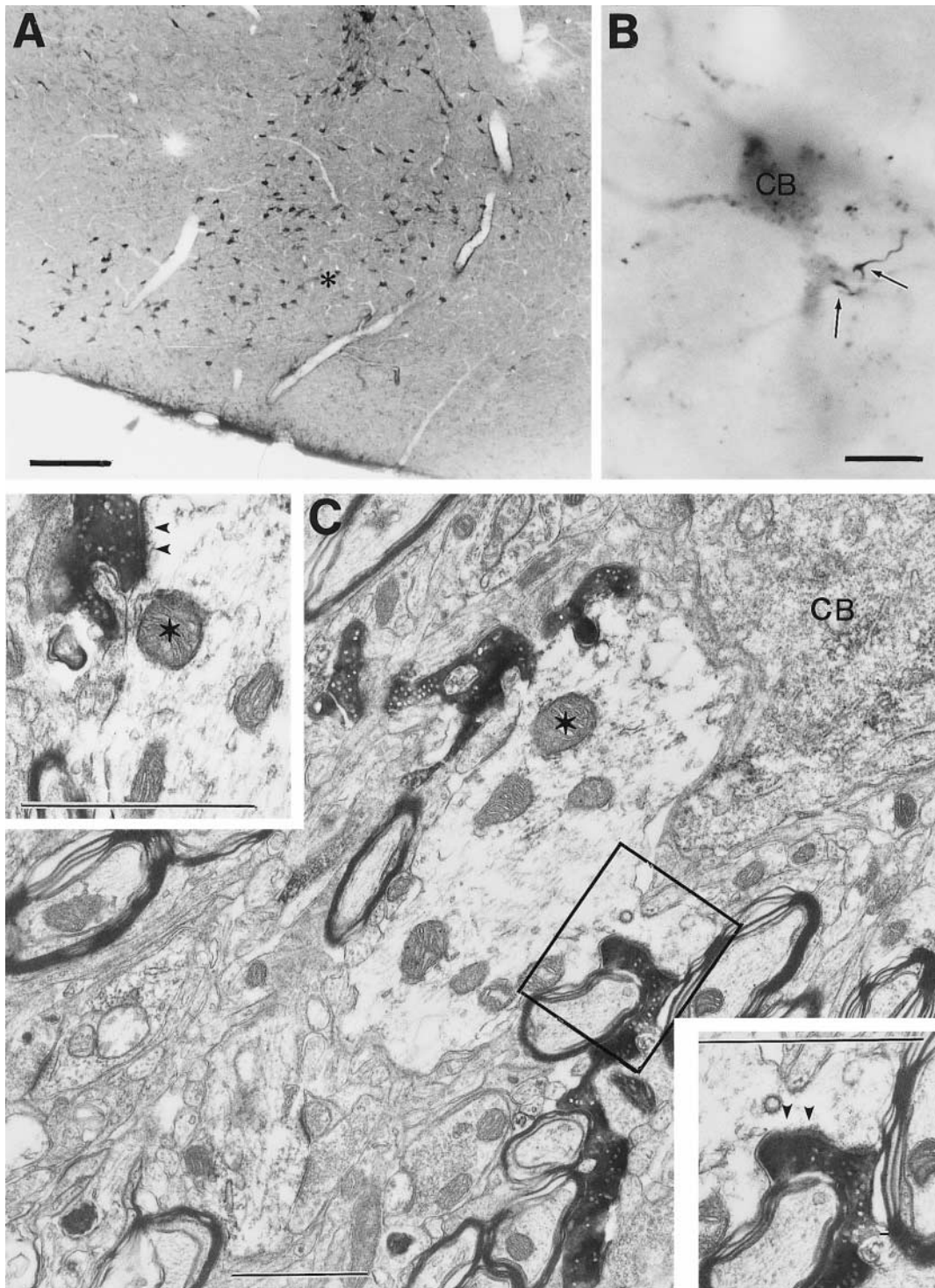


Fig. 15. Corticofugal terminals in synaptic contacts with an unlabeled dendritic shaft and spine in the vicinity of a cholinergic neuron in the basal forebrain. A) Low magnification photomicrograph showing the area for electron microscopic analysis. Asterisk labels the location of micrograph (B). B) A horseshoe-like labeled axonal segment bearing several varicosities in close proximity to a weakly-labeled cholinergic dendrite. C) Low magnification electron micrograph to show this cholinergic neuron and PHA-L-labeled axon varicosities. The PHA-L-labeled varicosities synapse an unlabeled dendrite. One of the synaptic contact with the dendritic shaft is shown in the upper left inset from an adjacent thin section corresponding to the left arrow in (B). The synapse with the spine-neck (boxed area) is enlarged in the inset in the lower right hand corner. This PHA-L bouton corresponds to the one indicated by the right arrow in (B). Note that (C) is rotated about 70 degree clockwise relative to (B). Arrowheads point to postsynaptic thickenings. CB marks the same cholinergic cell body in (B) and (C). Stars indicate the same mitochondrion in (C) and in the upper left inset. Scale bar in A=100 μ m, C and insets=1 μ m.

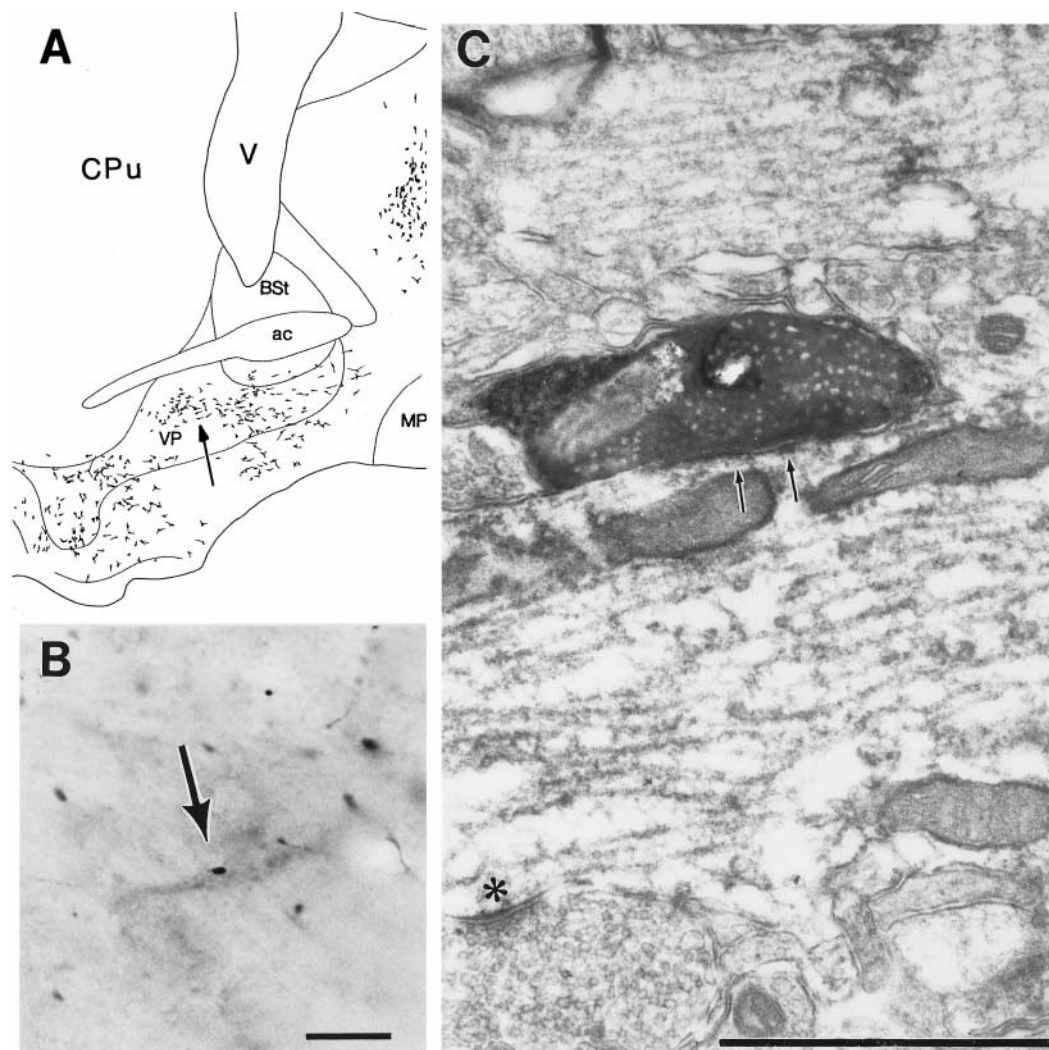


Fig. 16. PHA-L-labeled terminal in contact with a distal parvalbumin-containing dendritic shaft. A) Distribution of parvalbumin-containing neurons in the ventral pallidum. Approximate location of the identified dendritic shaft is indicated by an arrow. B) High magnification light micrograph of the identified bouton (arrow) in close association with a parvalbumin-containing dendritic profile. C) High magnification electron micrograph showing the synaptic region. Arrows delineate the postsynaptic density. Asterisk marks another synapse with an unlabeled bouton. Scale bar in B=10 μ m, in C=1 μ m. MP, medial preoptic nucleus, V, lateral ventricle.

varicosity was a PHA-L bouton and entered into synaptic contact with a parvalbumin-immunoreactive dendritic shaft adjacent to the cell body. The synapses in both cases are of the asymmetric type.

DISCUSSION

The present study provides evidence that i) among the corticofugal projections to the basal forebrain examined, only prefrontal, piriform and insular axons terminate in extended basal forebrain areas that are associated with cholinergic projection neurons; ii) PHA-L-labeled prefrontal boutons were found to be GABA-negative, and were confirmed to contain glutamate immunoreactivity; iii) the majority of prefrontal axons form asymmetric synapses with

small dendritic shafts and spines that are ChAT-immunonegative; iv) despite light microscopic indications of prefrontal terminals abutting cholinergic profiles, no clear electron microscopic evidence could be obtained for synaptic contact with cholinergic neurons; v) postsynaptic targets so far identified are parvalbumin-positive, furthermore small dendrites and spines of unidentified, but most likely non-cholinergic neurons.

Cortical afferents to the basal forebrain

The findings of the present light microscopic study suggest several conclusions. Firstly, the prefrontal cortex is a major source of input to basal forebrain areas that contain cholinergic projection neurons,

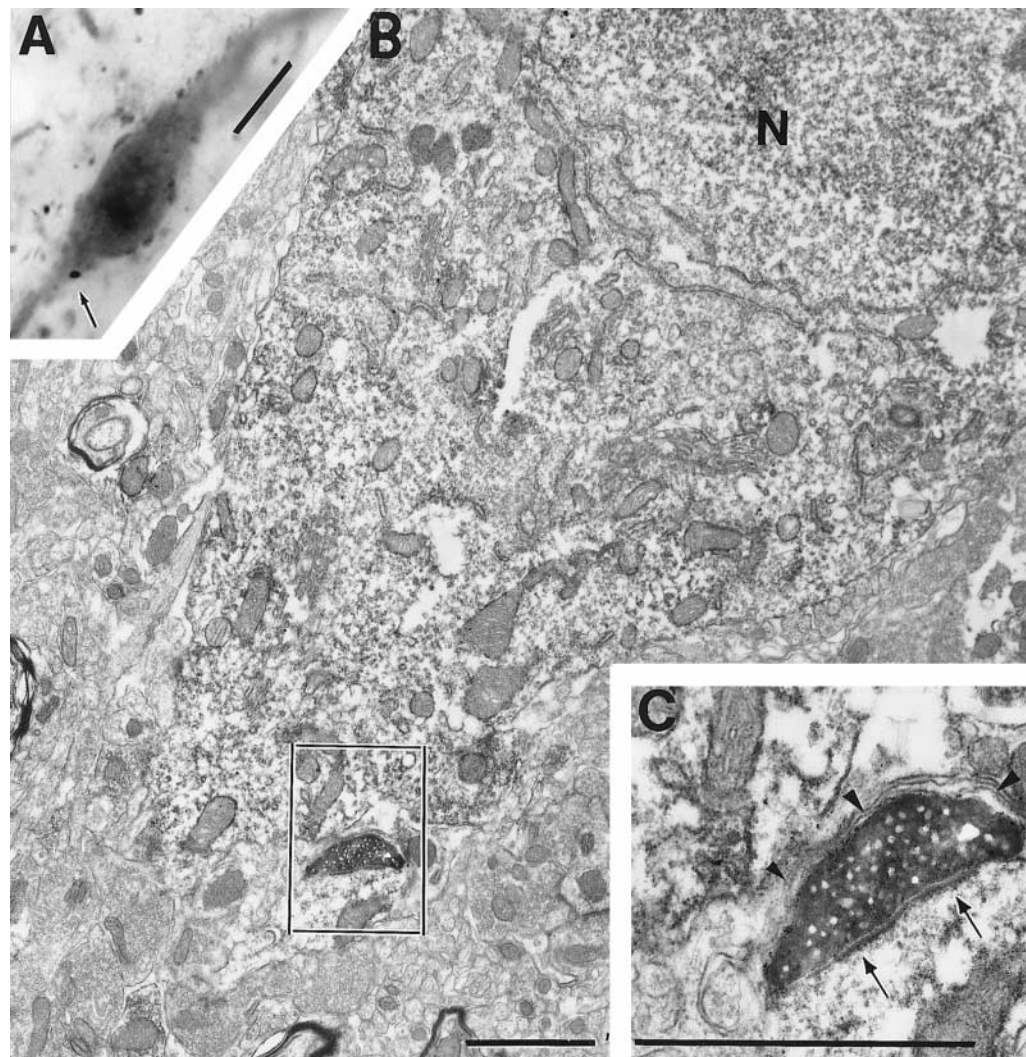


Fig. 17. PHA-L-labeled axon varicosity in synaptic contact with a parvalbumin-positive dendritic shaft. A) A heavily-stained PHA-L profile (arrow) contacts a parvalbumin-containing cell body in the ventral pallidum. Note several lightly-stained parvalbumin-positive boutons surround the cell body. B) Medium power electron micrograph depicting this neuron. The PHA-L-labeled varicosity with the postsynaptic thickening is facing away from the perikaryon (boxed area). C) High magnification view of the area of the synaptic contact. The PHA-L bouton enters into asymmetric synaptic contact (arrows denote the postsynaptic density) with a parvalbumin-positive dendritic profile. The presynaptic axon terminal is separated from the parvalbumin-positive perikaryon by several membrane foldings (arrowheads). N, nucleus. Scale bar in A=10 μ m, B, C=1 μ m.

including the horizontal limb of the diagonal band, ventral pallidum and substantia innominata, supporting previous observations.²⁷ Labeled terminals from the insular and piriform cortices also appeared to be distributed near cholinergic neurons, although less frequently in comparison to those from the prefrontal cortex. Such differences might be partially explained by the fact that the prefrontal cases involved a dual injection, and thus, a stronger projection was seen to forebrain regions containing BFC neurons.

Secondly, in contrast to allo- and mesocortical regions, the neocortical areas examined apparently do not send projections to the cholinergic basal

forebrain area. This is at variance with a previous study in the rat which suggested that neocortical areas are reciprocally interconnected with the BFC system.⁷³ In that study, corticopetal neurons retrogradely labeled with wheat germ agglutinin-horseradish peroxidase from the neocortex, which are found largely within the internal capsule, globus pallidus, and peripallidal regions, were always seen in close proximity to anterogradely-labeled descending fibers and were deemed likely to receive this cortical input. Insight into the discrepancy with the current findings appears to lie in the morphology of these corticofugal axons. Although in the present study the positions of fibers descending from neocortical

regions were remarkably consistent with previous results,⁷³ the PHA-L method revealed that these axons were smooth, devoid of varicosities, and largely restricted to the myelinated fascicles of the internal capsule. These fibers apparently remain within the internal capsule *en route* to more caudal targets. In contrast, fibers from the orbitofrontal or insular cortices which reached the basal forebrain through the internal capsule could sometimes be seen to detach from it, where they appeared as axons bearing *en passant* terminals, usually coursing to more ventral forebrain regions. These contrasting patterns are illustrated in the dark-field micrographs of Fig. 7, in which descending fibers from the frontoparietal (case C028) and orbitofrontal cortices (C026) are shown in relation to the internal capsule.

Transmitter identity of prefrontal axon terminals

The observation that prefrontal axons contain glutamate immunoreactivity and most of the synapses with non-cholinergic neurons were of the asymmetric type, traditionally associated with excitation, suggest that prefrontal axons use excitatory amino acid as their transmitter. A similar conclusion was reached in a tracing study using high-affinity uptake of radiolabeled aspartate.¹² These data together with the fact that all of the synaptic specializations formed by cortical boutons in the dorsal and ventral striatum are exclusively asymmetrical^{21,52,75} suggest that corticofugal terminals in the entire forebrain may originate from cortical pyramidal neurons.

Additional evidence that afferents to the basal forebrain utilize excitatory amino acids glutamate or aspartate as their transmitter has also been advanced in studies using electrophysiology, pharmacology, *in vivo* dialysis or receptor localization. First, depolarizing effects of glutamate have been reported in dissociated basal forebrain cultures⁶² and identified basalcortical neurons were readily excited by iontophoretically-applied glutamate.⁵¹ Second, injections of glutamate in the SI significantly increased cortical high-affinity choline uptake⁸⁸ and cerebroventricular injections of glutamate receptor antagonists increased cortical acetylcholine efflux.³¹ Third, a calcium-dependent, potassium-evoked release of endogenous glutamate has been demonstrated in basal forebrain slices,¹⁶ and presynaptic high-affinity glutamate uptake occurs in the basal forebrain.^{16,24} Fourth, postsynaptic excitatory amino acid receptors have been localized in the region⁵⁸ and excitatory postsynaptic currents in basal forebrain slices were activated through glutamate-gated ion channels^{2,77} indicating that neurons in the basal forebrain are regulated by glutamate synapses.

Postsynaptic target of prefrontal axons in the basal forebrain

Surprisingly, none of the selected anterogradely-labeled boutons located adjacent to cholinergic

neurons could be clearly shown to form synaptic contacts with cholinergic profiles. In contrast, unequivocal evidence of synapse formation with adjacent ChAT-immunonegative elements was obtained in 59% of the selected boutons. In our previous electron microscopic studies with PHA-L,^{15,28,94,95} the frequency of putative contacts between PHA-L-labeled terminals and cholinergic neurons was usually proportional to the density of terminals present in a given area, and approximately 40–50% of the boutons selected with high magnification light microscopy could be confirmed to be synapses under the electron microscope. In this study, however, unequivocal synapses on cholinergic neurons could not be ascertained, despite the large number of selected profiles. One reason for this negative finding could be that corticofugal axons terminate exclusively on far distal dendrites, which due to the low ChAT-immunoreactivity in these processes were not identified as cholinergic and many synapses remained undetected. Indeed, according to our high magnification light microscopic screening of material treated with high detergent concentration to increase antibody penetration, appositions between labeled prefrontal axons and cholinergic dendrites were often located along distal dendritic segments whose cell bodies were not identifiable in the same sections, indicating that these contacts may be at least 200 μ m away from the cell body, a distance calculated based upon the average length of dendrites which remained connected to their parent cell body in a 50- μ m-thick section.⁸¹ Despite the fact that our selection was slightly biased towards PHA-L-labeled varicosities adjacent to proximal cholinergic profiles with well identifiable cell bodies, nine boutons were found abutting far distal cholinergic dendrites, but none showed unequivocal signs of synapse. However, since the distal dendritic staining was light in the material processed for electron microscopy, it is possible that some of the small spine-like processes identified as non-cholinergic postsynaptic targets of prefrontal axons could belong to cholinergic neurons. As cholinergic dendrites are known to have a few of such protrusions, this possibility cannot be ruled out. Nevertheless, since such spines are rather infrequent, the likelihood that a major input to cholinergic neurons was missed appears rather small. Interestingly, a lack of direct cortical inputs to basal forebrain cholinergic neurons is consistent with the connectional studies of Lapper and Bolam,⁵² who examined cortical input to striatal cholinergic neurons and found very little evidence for such a projection, although in the same material an abundance of thalamic input to striatal cholinergic neurons was found.

Although a much smaller pool ($n=10$) of putative varicosities near parvalbumin-containing perikarya or dendrites was reconstructed, unequivocal signs of asymmetric synapses, occurring on dendritic shafts, were found in 40% of the cases. A recent study by

Toth *et al.*⁸⁵ provided evidence that the majority of hippocamposeptal fibers terminating in the medial septum/vertical limb of the diagonal band complex form synaptic contact with parvalbumin-positive, septohippocampal neurons. Parvalbumin is contained in subpopulations of GABAergic interneurons^{7,14,49} and projection neurons^{25,47,66} in the basal forebrain. In our material stained for PHA-L/GABA, due to technical difficulties, we did not trace the postsynaptic spines or small dendritic segments back to their parent dendritic shafts or the cell body, where postembedding GABA staining is more reliable in case of GABAergic interneurons. On the other hand, the somatic level of GABA in the GABAergic neurons with distant projections is below the sensitivity of immunocytochemistry.⁸⁵ Since parvalbumin neurons in our study were not further characterized, it is unclear whether parvalbumin-containing neurons that were targets of prefrontal axon terminals are interneurons or projecting neurons.

The present study disclosed that a large number of corticofugal axons in the basal forebrain terminate on non-cholinergic neurons, particularly on dendritic spines. Such spiny neurons were distributed throughout broad forebrain areas containing cholinergic projection neurons. Some of these neurons at the border between striatum/pallidum may be striatal spiny neurons. Spiny neurons in the ventral striatum have been shown to receive prefrontal input.⁷⁵ In Golgi studies of the basal forebrain,^{8,19} several classes of spiny neurons were described, the transmitter character of them, however, was not identified. Since parvalbumin-containing neurons in the striatum⁷ or globus pallidus⁴⁸ have been shown to be aspiny neurons and most of the synapses in our material were associated with unlabeled spines, another type of neuron may be major recipient of cortical input. Basal forebrain areas rich in cholinergic neurons contain in addition to GABAergic neurons,^{13,25,44,47,98} several classes of peptidergic neurons.^{86,93,97} Somatostatin interneurons are rich in basal forebrain areas; they often can be found in the close vicinity of cholinergic cell bodies. These neurons have short dendrites, with few spines and their axons richly terminate with symmetric synapses on the cell bodies and proximal dendrites of BFC neurons.⁹³ The proximity of those small dendritic profiles to cholinergic neurons, which received labeled prefrontal terminals make the somatostatin interneurons another possible candidate for the target of prefrontal axons.

Comparison with primate studies

Previous data from primate studies have indicated that cortical projections to forebrain regions containing BFC neurons originate from the orbitofrontal, insular, temporo-polar regions, the entorhinal and prepiriform cortices.^{41,60,70} Results from recent retrograde tracer studies in rats^{36,46,74} taken

together with the present findings suggest that cortical input to the basal forebrain territories containing cholinergic cells in rodent are likely to be restricted to the same type of cortical areas. Mesulam and co-workers⁶⁰ suggested that fibers emanating from these aforementioned cortical regions terminate on cholinergic neurons in the basal forebrain of primates. A similar conclusion was drawn from the electrophysiological studies of Rolls and colleagues in behaving monkeys. Namely, it has been known for some time,^{10,17} that basal forebrain neurons respond to a range of visual and auditory stimuli that have been reinforced. Since similar units were also found in the orbitofrontal cortex and amygdala,^{26,84} Rolls and colleagues proposed^{68,90,91} that cholinergic neurons in the basal forebrain receive information on the expected availability of reinforcement through afferent inputs from the orbitofrontal cortex and amygdala and relay this information to widespread cortical areas to facilitate sensory, motor or associative functions. Indeed, neuronal responsiveness in sensory cortex appears to be related to similar motivational changes in basal forebrain neurons.⁴³ In these behavioural studies, however, basal forebrain neurons were only occasionally identified antidromically from the cortex and none of the studies attempted to identify the transmitter of the recorded neurons. While amygdaloid projections to the basal forebrain have been described in primates and rodents^{65,69,70} and have been shown to terminate, at least in part, directly on cholinergic neurons,¹⁰⁰ the termination of cortical input on cholinergic neurons remains to be addressed directly in primates. Interestingly, the major α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subtype GluR1 has been shown to be co-localized in monkey cholinergic neurons but not in rat,⁵⁸ suggesting that cholinergic neurons in rat either do not receive glutamatergic synapses or utilize alternative glutamate receptor subtypes. On the other hand the expression of AMPA receptors in cholinergic neurons in the basal forebrain in primates may reflect a direct, excitatory, glutamatergic feedback from allo- and mesocortical regions.

CONCLUSION

A comparison of the present results with data regarding the cortical targets of cholinergic neurons,^{56,71,73,92} suggests that cholinergic neurons in specific forebrain subterritories may ultimately be interconnected with cortical regions sending projections to the same basal forebrain regions. For example, inputs from the piriform cortex were limited mainly to the ventral MCP-HDB regions where cholinergic projection neurons to the same cortical area have been described. Similarly, afferents from the insular cortex were restricted to the caudolateral SI, an area which is rich in projection neurons to the

same cortical area. Finally, fibers from prelimbic/infralimbic areas were distributed in the SI, medial HDB, medial part of the internal capsule/globus pallidus border, regions where many of the projection neurons to the same cortical areas can be found. On the basis of the present results, it appears that any reciprocity in such projections requires intervening connections with local forebrain interneuronal populations, which may be the principle recipients of corticofugal axonal projections (see below). Such interneuronal relays may be comprised of GABAergic neurons, as BFC neurons are known to receive a substantial GABAergic input.^{40,55,99}

Interestingly, BFC neurons located in the internal capsule, globus pallidus, and peripallidal regions, areas not reached by projections from the prefrontal cortex, may receive indirect cortical influence through GABAergic striatal efferents,³³ similar to afferent connections of ventral pallidal cholinergic neurons, which receive input from the nucleus accumbens.⁹⁵ The nucleus accumbens, in turn, receives topographically organized projections from function-

ally different parts of the prefrontal cortex.³⁵ Thus, an essentially similar indirect cortical control mechanism can be envisaged for most BFC neurons, either through striatal GABAergic or local inhibitory neurons. Such cholinergic circuits may subserve similar functions as to the parallel forebrain circuits that involve the frontal cortex, the basal ganglia and the thalamus.^{3,18,35} Ultimately, an understanding of the impact of corticofugal input to the basal forebrain may come from the determination of the transmitter and output relationships of the neurons targeted by cortical afferents in multiple tracing/immunocytochemical studies, as well as from physiological and pharmacological characterizations of these connections.

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