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γ-Aminobutyric acid-containing basal forebrain neurons innervate inhibitory interneurons in the neocortex

(nonpyramidal cells/somatostatin/parvalbumin/disinhibition/subcortical control)

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ABSTRACT **The basal forebrain–neocortex pathway—involved in higher cognitive processing, selective attention, and arousal—is considered one of the functionally most important ascending subcortical projections. The mechanism by which this relatively sparse subcortical pathway can control neuronal activity patterns in the entire cortical mantle is still unknown. The present study in the cat provides evidence that γ-aminobutyric acid-containing basal forebrain neurons participate in the neocortical projection and establish multiple synaptic connections with γ-aminobutyric acid-releasing interneurons containing somatostatin or parvalbumin. We propose that a mechanism by which the numerically small ascending pathways can exert a powerful global effect in the neocortex is by the selective innervation of γ-aminobutyric acid-releasing interneurons, which, in turn, control the activity of large populations of pyramidal cells through their extensive axon arborizations. Finally, these results demonstrate a direct anatomical link between two cell populations implicated in Alzheimer disease pathology: basal forebrain neurons and cortical somatostatin cells.**

Efferent projections of the basal forebrain nuclei to most cortical areas (1–3) are thought important for selective attention, higher cognitive processes, cortical arousal, and plasticity (4–11). Acetylcholine has been identified as the major neurotransmitter of the basal forebrain–neocortex pathway, but noncholinergic neurons were also shown to participate in the cortical projection (12–14). In a recent study some of the cortically projecting noncholinergic neurons were shown to contain γ-aminobutyric acid (GABA) (15), and a coexistence of acetylcholine and GABA in some cortical terminals has also been reported (16). The termination pattern and postsynaptic targets of the cholinergic component of this pathway are relatively well known (16–18), but no such information is available about the GABAergic component.

The septohippocampal pathway was also shown to contain both cholinergic and GABAergic fibers (19), and the latter component was found remarkably selective in innervating GABAergic interneurons in both rat and monkey (20–22). Local GABAergic interneurons in the hippocampus, as in neocortical areas, are known to control the firing of large populations of principal cells. Thus, any pathway, even if relatively sparse, may exert a powerful effect on the electrical activity patterns of a cortical area if it modulates the activity of local inhibitory interneurons (20, 23).

In the present study we investigated the synaptic organization of the GABAergic basal forebrain–neocortex pathway to shed light on the structural basis of the mechanism by which basal forebrain neurons can effectively regulate electrical activity and higher cognitive processes in the neocortex.

METHODS

Surgery and Perfusion. Multiple injections of the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) were delivered into different parts of the basal forebrain complex in 16 adult cats of either sex (2.5–4.0 kg). PHA-L [2.5% in 10 mM phosphate buffer (PB), pH 8.0, Vector Laboratories] was iontophoresed (15 min, 5.0-μA direct current at each site, 7-sec ON–OFF cycle) (24) into the diagonal band of Broca, ventral pallidum, nucleus basalis of Meynert, and substantia innominata regions. Each hemisphere was injected at eight sites: at two anteroposterior levels (AP: +14–+17 mm), at two lateral coordinates (L: 4–6 mm), and at two dorsoventral coordinates (V: 22–18 mm from the pia of the lateral gyrus), according to the atlas of Reinoso-Suarez (25) under deep anesthesia [i.m. injection of ketamine hydrochloride at 23 mg/kg and xylazine hydrochloride at 23.32 mg/kg (Rompun, Bayer, Wüppertal, F.R.G.)]. Eight to 10 days after the operation the animals were anesthetized again with the same anesthetic and perfused through the heart, first with Tyrode's solution (2 min) and then with 1.5 liters of either fixative A (0.05% glutaraldehyde/4% paraformaldehyde/0.2% picric acid/0.1 M PB), fixative B [the same concentration of aldehydes as fixative A but in cacodylate buffer, pH 7.4 and containing 0.5% $CaCl_2$ (for parvalbumin immunocytochemistry)], or fixative C (1% glutaraldehyde/2.5% paraformaldehyde/0.2% picric acid/0.1 M PB) for 30 min. Blocks of the basal forebrain and cortical areas 4, 6, and 22; somatosensory areas 1–4; and visual areas 1 and 2 were dissected and sliced in the coronal plane, or perpendicular to the pia. The blocks were saturated with 10% and 20% sucrose, freeze-thawed in liquid nitrogen to enhance penetration of antisera, sectioned on a Vibratome at 60 μm, and washed in several changes of 0.1 M PB. Sections from animals perfused with fixative C were treated with 1% $NaBH_4$ for 30 min and washed in several changes of 0.1 M PB.

Preembedding Immunocytochemistry. A double immunostaining procedure was used to visualize the PHA-L-labeled axons and the peptide- or calcium-binding protein-containing cells simultaneously (26). (*i*) The sections were incubated in a mixture of the primary antibodies for 2 days: biotinylated goat anti-PHA-L (1:200, Vector Laboratories) was mixed with either mouse antiparvalbumin (monoclonal antibody, diluted 1:500) (27), used only in animals fixed with fixative B; or mouse antisomatostatin (monoclonal antibody, diluted 1:1000) (28), used in animals perfused with fixative A or B; or rabbit anti-neuropeptide Y (diluted 1:10,000) (29), used in animals fixed with fixative A or B; or rabbit anti-calbindin of 28 kDa (30, 31) (diluted 1:1000), used in animals fixed with

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Abbreviations: DAB, 3,3′-diaminobenzidine tetrahydrochloride; PB, phosphate buffer; PHA-L, *Phaseolus vulgaris* leucoagglutinin; TS, 0.05 M Tris-buffered saline.

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fixative A, B, or C; or rabbit anti-cholecystokinin (21) (diluted 1:10,000), used in animals fixed with fixative A or B. (*ii*) The second layer (overnight) was a mixture of avidin–biotinylated horseradish peroxidase complex (ABC, diluted 1:100, Vector Laboratories) and either goat anti-rabbit or rabbit anti-mouse IgG (according to the primary serum; diluted 1:50, ICN; Dakopatts, Glostrup, Denmark). The first immunoperoxidase reaction, for the visualization of PHA-L-labeled axons, was developed with ammonium nickel sulfate-intensified 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen (deep-blue reaction product). (*iii*) The third layer was rabbit peroxidase anti-peroxidase or mouse peroxidase anti-peroxidase complex (Dakopatts, diluted 1:100) overnight. The second immunoperoxidase reaction was developed with DAB alone, giving a brown reaction product. During the procedure 50 mM Tris-buffered saline, pH 7.4/1% normal goat or normal rabbit serum/0.5% Triton X-100 was used for washing and for diluting the antiserum. Sections immunostained for PHA-L alone for electron microscopy were incubated without Triton X-100, and DAB alone was used as a chromogen.

When the primary antisera were replaced by the respective normal sera, no specific labeling associated with neuronal structures was observed.

Postembedding Immunogold Staining. For electron microscopy, sections were treated with 1% OsO_4 for 1 hr, dehydrated, and embedded in Durcupan (ACM, Fluka). Selected areas were reembedded for further ultrathin sectioning (32). From the material fixed with fixative C alternate sections were mounted on copper and nickel grids, and the latter grids were processed for immunogold staining for GABA. The immunogold staining procedure and GABA antiserum of Somogyi and Hodgson (33) was used. The following steps were done on droplets of solutions: 1% periodic acid (H_5IO_6) for 10 min; wash by dipping in several changes of double-distilled water; 2% sodium metaperiodate ($NaIO_4$; BDH) for 10 min; wash as before; three times for 2 min in 0.05 M Tris-buffered saline (TS), pH 7.4; 30 min in 1% ovalbumin/TS; three times for 10 min in TS/1% normal goat serum; 1–2 hr in a rabbit anti-GABA antiserum (code 9, diluted 1:1000 in normal goat serum/TS); two times for 10 min in TS; 10 min in 0.05 M Tris buffer, pH 7.4/1% bovine serum albumin/0.5% Tween 20; goat anti-rabbit IgG-coated colloidal gold (15 nm, Janssen Pharmaceutica) for 2 hr (diluted 1:10 in the same buffer); 5-min wash twice in double-distilled water; saturated uranyl acetate for 30 min; wash in four changes of double-distilled water; staining with lead citrate; wash in distilled water.

When the primary antiserum was replaced by normal rabbit serum, accumulation of gold particles was not seen in any profiles either labeled or unlabeled with PHA-L. In the first experiment use of nickel-intensified DAB as a chromogen in the preembedding staining was found to lead to nonspecific immunogold staining in some of the PHA-L-labeled profiles. Therefore, in all subsequent experiments DAB alone was used as a chromogen in all sections processed for electron microscopy.

RESULTS

The multiple injection sites involved to various degrees the diagonal band of Broca, the substantia innominata, nucleus basalis, and ventral pallidal complex in all 16 cats. The density of PHA-L-labeled axons varied according to the sites of injection in the basal forebrain. The precruciate gyrus (area 6), the ectosylvian gyrus (areas 22 and 50), the primary somatosensory and visual areas, and the motor cortex (area 4) were examined in detail. The most densely innervated areas were areas 6, 22, and 50, whereas the primary sensory areas contained only a few PHA-L-labeled axons, and the motor cortex contained no PHA-L-labeled axons in any cats examined. Two types of fibers could be distinguished among the labeled axons. The most frequent type had large, mostly *en passant* varicosities (type 1), whereas the other was thin

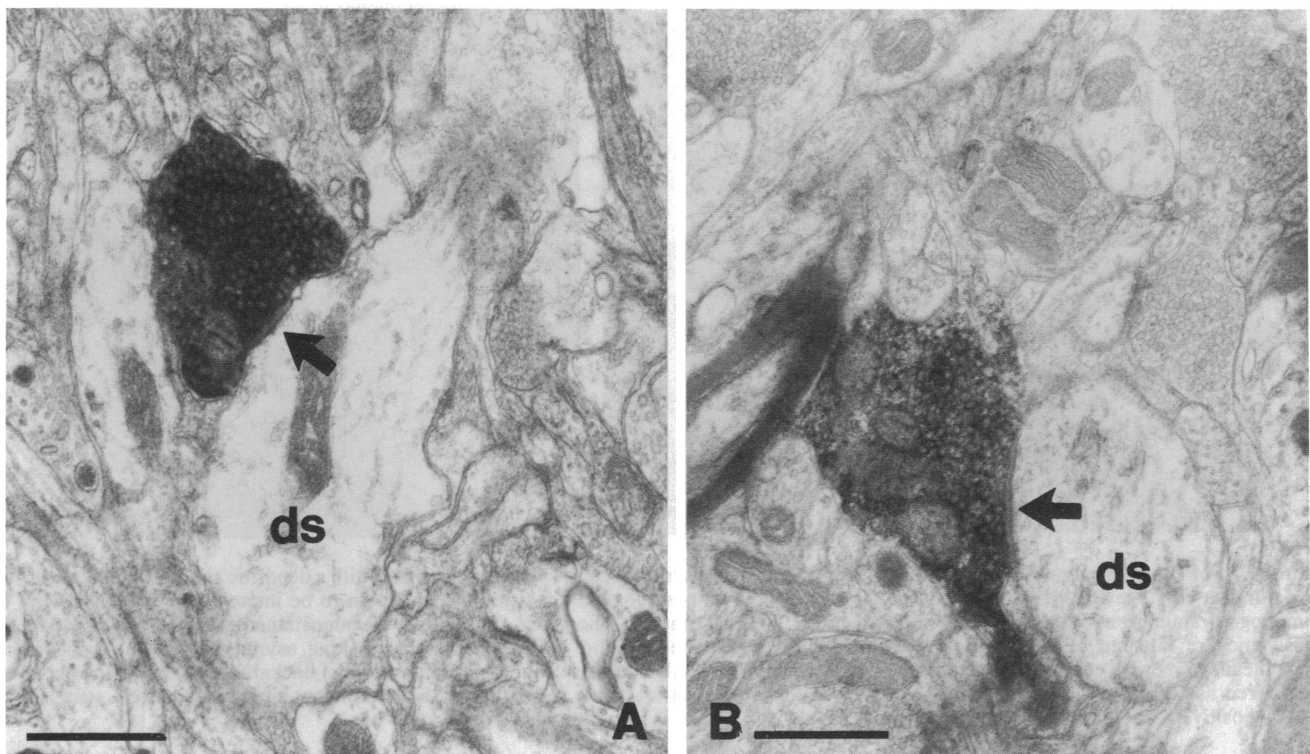

FIG. 1. Electron micrographs of PHA-L-labeled boutons originating from the basal forebrain. The boutons are shown to establish symmetrical synaptic contacts (arrows) with dendritic shafts (ds) in layer 5 (*A*) and layer 4 (*B*) of cortical area 6 in the cat. (Bars = 0.5 μm.)

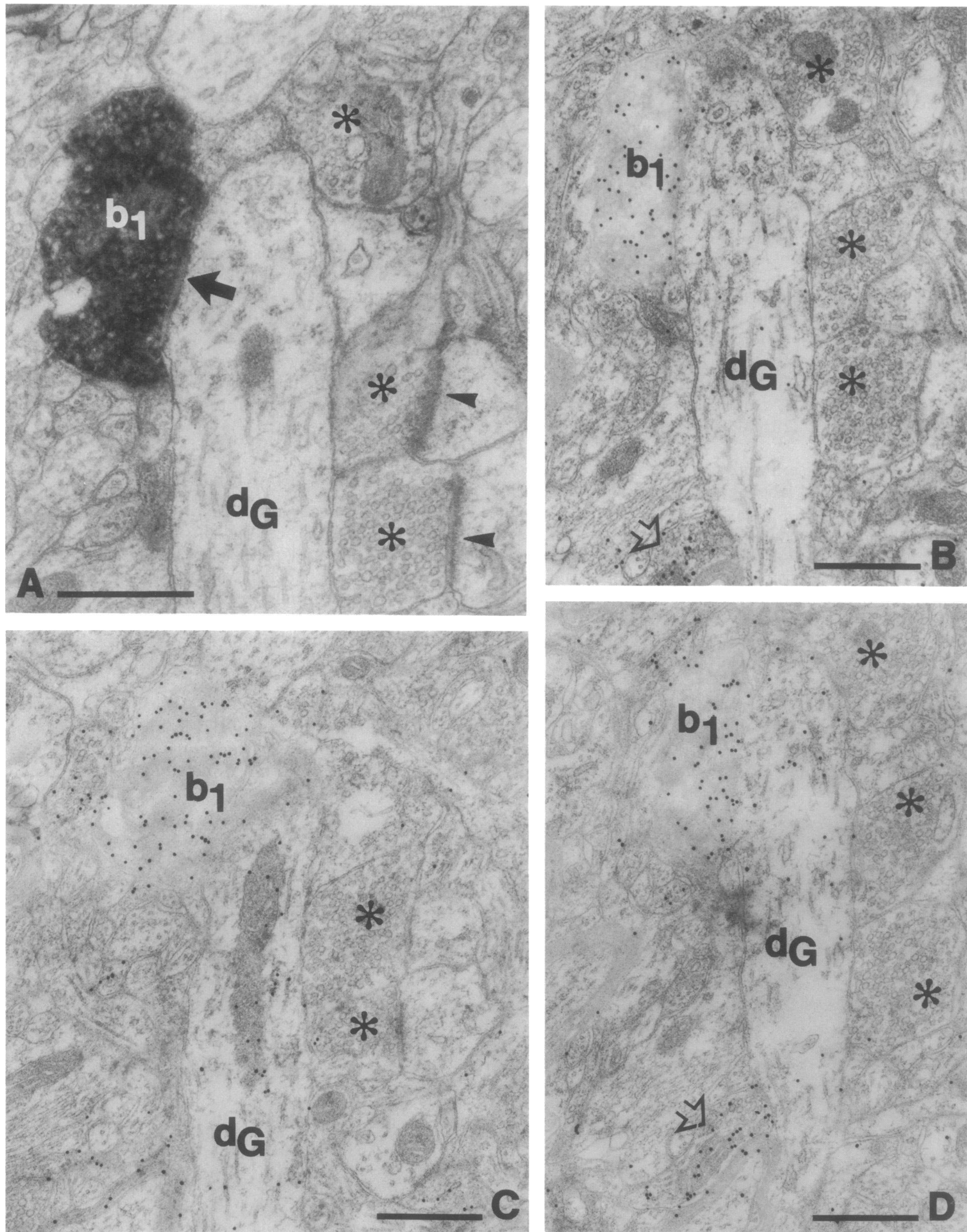

FIG. 2. A PHA-L-immunoreactive bouton (b_1) is in symmetrical synaptic contact (large arrow in *A*) with a dendritic shaft in layer 4 of cortical area 6 in the cat. The PHA-L-labeled bouton (b_1), as well as its postsynaptic dendrite (d_G), is shown to be immunoreactive for GABA by immunogold staining of consecutive ultrathin sections in *B*, *C*, and *D*. The three consecutive immunostained sections demonstrate the consistency of labeling of the different profiles. Boutons containing round vesicles (asterisks) making asymmetrical synaptic contacts (arrowheads in *A*) with unlabeled spines were always negative for GABA and served to indicate the level of background staining. Profiles were considered as GABA-positive when the gold particle density was at least five times that of the boutons making asymmetrical synapses. A GABA-positive axon terminal not labeled with PHA-L (open arrow) is also indicated. (Bars = 0.5 μm.)

and had smaller boutons, occasionally of the "drumstick" type (type 2). Type 1 axons were numerous and occurred predominantly in layers 1–3 and 5. Type 2 axons were rare and faintly stained; only one or two fibers could be seen per section, and these did not show any characteristic distribution.

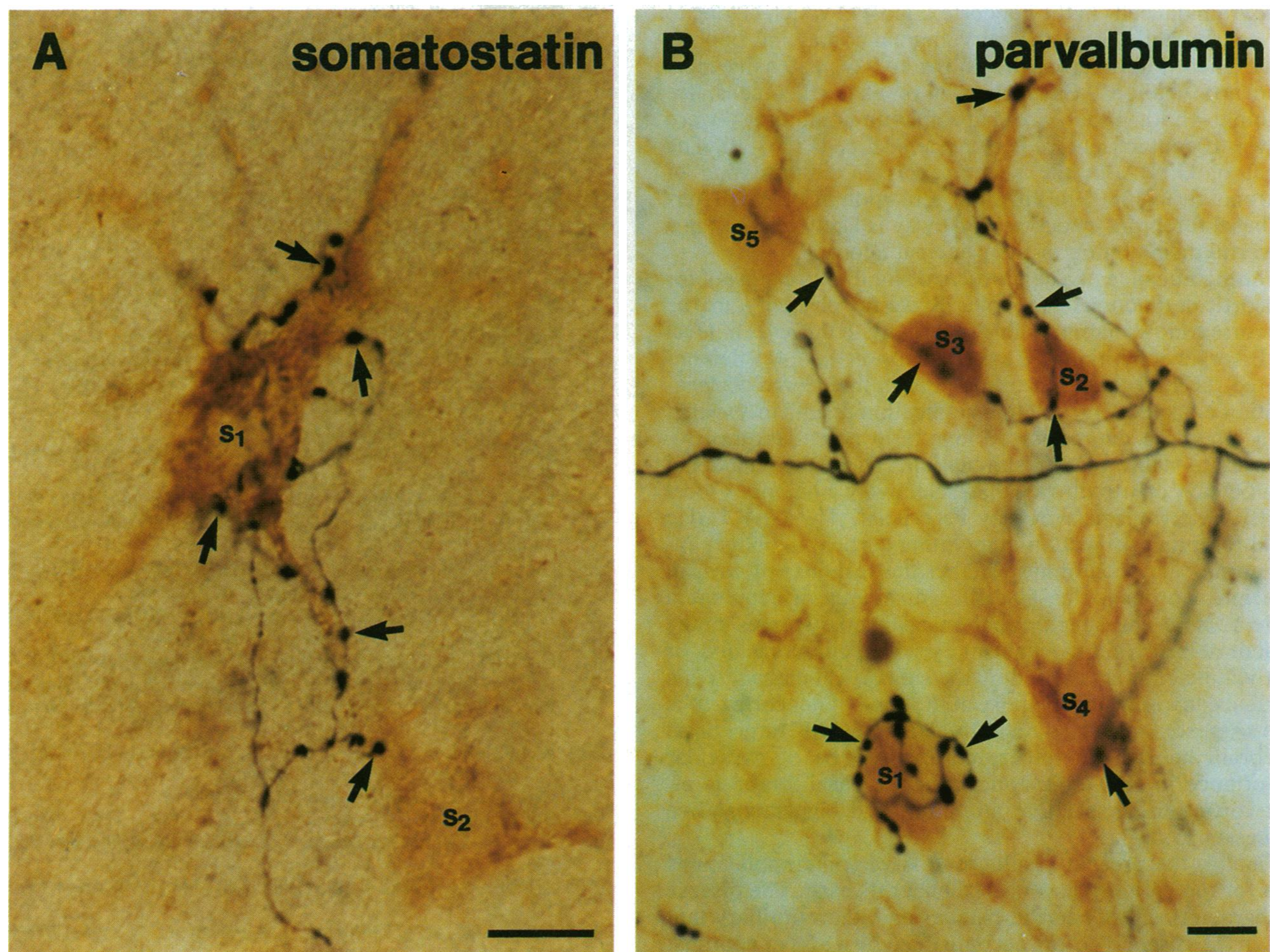

FIG. 3. Light micrographs of somatostatin-immunoreactive (*A*) and parvalbumin-immunoreactive (*B*) neurons (brown, DAB) innervated by PHA-L-labeled basal forebrain afferents (black, DAB/Ni) in area 6 of the cat neocortex. The basal forebrain axons, which belong to the thick GABAergic type, establish multiple contacts (arrows) with dendrites and/or cell bodies of the two somatostatin-containing cells (S_1 and S_2) and the five parvalbumin-immunoreactive neurons (S_{1-5}). (Bars = 10 μm.)

A total of 139 boutons of type 1 axons from three cortical areas (areas 6, 22, and 50) of three cats (fixed with fixative C) were reembedded and sectioned for electron microscopy and for postembedding immunostaining for GABA. All 139 boutons were strongly immunoreactive for GABA and established morphologically identifiable symmetrical synaptic contacts (Figs. 1 and 2*A*). Ten boutons (7.2%) terminated on cell bodies, and the remaining 129 boutons (92.8%) synapsed on dendritic shafts. All postsynaptic cell bodies and most dendrites (83.8%) were positive for GABA (Fig. 2). The majority of the target dendrites (92, 66.2% of total targets) had a thin shaft with a smooth surface, small mitochondria, and relatively sparse synaptic contacts (Figs. 1 and 2). The remaining target dendrites (37, 26.6% of total targets) were varicose in nature and had a larger diameter, large mitochondria, many free ribosomes, and numerous synaptic contacts. Spines were not seen to emerge from either of the two types of target dendrites.

Type 2 axons were very sparse and were not studied here in detail at the electron microscopic level. Boutons of type 2 axons seen within the section series were negative for GABA, similar to the findings for the septohippocampal projection (20, 21). Type 2 axons were, therefore, assumed to represent the cholinergic component of the pathway, and their relatively sparse labeling in PHA-L-tracing studies is probably due to their small diameter and/or to the preferential uptake of PHA-L by GABAergic neurons (for discussion see ref. 22).

The preferential innervation of GABAergic interneurons has been observed in a much larger sample at the light microscopic level by using double immunostaining for PHA-L and different neuropeptides or calcium-binding proteins known to be present in GABAergic neurons (27, 34, 35). In sections double-stained for somatostatin and PHA-L, a remarkably high percentage of multiple contacts—i.e., sites of putative multiple synaptic contacts—was found between basal forebrain afferents and somatostatin-positive interneurons in layers 2, 3, and 5 (Fig. 3*A*). Up to 45% of the somatostatin-positive cells present in selected, densely innervated areas were surrounded by type 1 (GABAergic) PHA-L-labeled afferents. One single somatostatin-positive cell could be contacted by as many as 28 PHA-L-labeled boutons. Nonpyramidal cells immunoreactive for parvalbumin were also among the targets (Fig. 3*B*). These cells received multiple contacts from basal forebrain axons onto their cell bodies as well as onto their proximal and distal dendritic shafts, which were—unlike those of somatostatin cells—frequently varicose.

Interneurons immunoreactive for neuropeptide Y and calbindin were rarely seen to be innervated by PHA-L-labeled axons, and even then, these contacts were limited to one to three boutons per cell. Cholecystokinin-containing interneurons were not seen to receive multiple contacts from basal forebrain axons in this material.

DISCUSSION

In the present study we demonstrated that GABAergic basal forebrain neurons terminate predominantly on GABAergic

nonpyramidal cells in the neocortex, most of which belong to the somatostatin- and the parvalbumin-containing populations. These interneurons are known to have extensive local axon arborizations. Single basket or chandelier cells, for example—both types are parvalbumin-containing—were shown to contact hundreds of pyramidal cells (36, 37). The effect of GABA in the neocortex is the suppression of neuronal firing (38, 39); thus, the activation of the GABAergic basal forebrain–neocortex pathway is likely to result in a powerful disinhibition of the principal neurons.

The significance of a subcortical disinhibitory pathway for neocortical functions is 2-fold. Synaptic plasticity in the neocortex shows a remarkable sensitivity to the level of local GABAergic inhibition (40, 41). Artola *et al.* (41) demonstrated recently that a slight increase in the degree of disinhibition evoked by bicuculline application results in a switch from long-term depression to long-term potentiation of responses to white matter stimulation. Thus, the functional significance of a pathway that selectively influences local GABAergic transmission may be crucial. The GABAergic basal forebrain projection innervating cortical inhibitory interneurons may determine whether the response of cortical principal neurons to the same afferent stimulus is potentiated or depressed. This specific anatomical link may represent the structural basis of a mechanism by which emotional and/or motivational factors carried by the subcortical pathways can influence learning and memory at the level of neocortex. It is also suggested that the direct projection of GABAergic basal forebrain neurons to the neocortex may be of particular importance in mediating noncholinergic basal forebrain effects on arousal, selective attention, and higher cognitive processes (5, 8–11).

Finally, the present results may have some implications also for the pathogenesis of Alzheimer disease. The disease is characterized by neuritic plaques and neurofibrillary tangles in the cerebral cortex accompanied by a loss of neurons, in highest proportion those that contain somatostatin (42–46), and by an extensive neuronal degeneration of both cholinergic and noncholinergic neurons in the cortically projecting areas of the basal forebrain (47, 48). Thus, the direct and specific anatomical link between basal forebrain afferents and cortical somatostatin neurons demonstrated in the present study may suggest a causal relationship between subcortical and cortical damage in the pathogenesis of Alzheimer disease.

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