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Short communication

Parvalbumin-containing neurons in the basal forebrain receive direct input from the substantia nigra-ventral tegmental area

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Abstract

By means of anterograde tracing of *Phaseolus vulgaris*-leucoagglutinin (PHA-L) it was determined if parvalbumin-immunoreactive neurons in the basal forebrain receive a direct synaptic input from the A9–A10 dopaminergic nuclei of the substantia nigra and ventral tegmental area. Forebrain sections were processed for immunocytochemical detection of PHA-L and parvalbumin (PV) at light and electron microscopic levels. At the ultrastructural level, PHA-L-labeled terminals were found to establish synaptic contacts with PV-immunoreactive neuronal somata in the ventromedial globus pallidus, the ventral pallidum, the internal capsule, and the substantia innominata. PV-containing neurons in pallidal and adjacent basal forebrain territories are thus directly targeted by presumably A9–A10 dopaminergic neurons and represent a novel aspect of midbrain dopaminergic control of basal forebrain neuronal activity.

Keywords: Ventral pallidum; Substantia innominata; GABA; Dopamine; Anterograde tracing; *Phaseolus vulgaris*-leucoagglutinin

Parvalbumin (PV), a member of a family of calcium-binding proteins, has been found to be widely distributed in the central nervous system. It is present in distinct subpopulations of GABAergic neurons [2,4,14] and is thought to be associated with neurons that have high firing rates and a highly active metabolism. Almost the entire basal forebrain region contains neurons showing PV immunoreactivity [3]. Strongly PV-immunoreactive cells are distributed across the globus pallidus and ventral pallidum. Neurons with variably intense PV immunoreactivity are furthermore present in the substantia innominata, diagonal band nuclei and medial septum. Those in the medial septum-diagonal band have been shown to contain GABA and to innervate inhibitory interneurons in the hippocampus [9]. PV-positive cells in the other territories of the basal forebrain are most likely GABAergic as well.

Previous studies have shown that, besides the striatum, dopaminergic neurons in the substantia nigra (SN) and the ventral tegmental area (VTA) innervate basal forebrain areas, including ventral pallidum, substantia innominata, diagonal band nuclei and septum [1,6,7,18,22]. Since little is known about the nature of target neurons of the A9–A10 dopaminergic nuclei, the present study sought to determine if the PV-containing neurons are among the recipients of the SN-VTA afferents. Such a connection would imply that presumably dopaminergic fibers monosynaptically influence the PV-containing subclass of GABAergic neurons in the basal forebrain.

Twenty-five male Sprague–Dawley rats (b.wt. 250–300 g) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and received iontophoretic deposits of the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PHA-L, 2.5% in 10 mM phosphate buffer, pH 8.0, Vector Labs, Burlingame, CA), as described previously [25], into discrete parts of the SN-VTA. After a 5–8 day survival period, the animals were deeply anesthetized and perfused transcardially with saline (50 ml), followed by 500 ml of either fixative A (4% paraformaldehyde (PF), 0.1% glutaraldehyde (GA) and 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4) or fixative B (2.5% PF and 1% GA in 0.1 M PB). The brains were removed and postfixed overnight in fixative without GA at 4°C and sectioned at a thickness of 50 μm with a Vibratome. Sections from animals perfused with fixative B were treated with 1% $NaBH_4$ for 30 min and washed in 0.1 M PB. Sections selected for electron microscopy were saturated in successively 15% and 30% sucrose, freeze-thawed in liquid nitrogen twice to enhance penetration of antisera, and

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rinsed in 0.1 M PB. Prior to each incubation step described below, sections were rinsed three times in PB. Antibodies were diluted in a PB solution containing 2% normal blocking donkey serum (Jackson Immunoresearch Labs, West Grove, PA), to which 0.5% Triton X-100 was added when processed for light microscopy, and only 0.04% when processed for the electron microscope. For single PHA-L staining, sections were incubated in goat anti-PHA(E + L) (Vector Labs) at a dilution of 1:1000 overnight at 4°C, followed by incubation in biotinylated donkey anti-goat IgG (Jackson Immunoresearch Labs) at 1:100 for 2 h, and then in the ABC complex (Vector Labs) at 1:500 for 2 h. Peroxidase staining was performed using 3,3′-diaminobenzidine (DAB) as a chromogen intensified with ammonium nickel sulfate (yielding a black reaction product), as described previously. For dual immunolabeling of PHA-L and PV, sections were incubated in a mixture of primary antibodies containing goat anti-PHA-E + L (1:200) and rabbit anti-PV (provided by Dr. Baimbridge, 1:1000) for 2 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. PV staining was accomplished using rabbit peroxidase anti-peroxidase (PAP) (Sternberger Monoclonals, Baltimore, MD, 1:100, 2 h) and visualization with DAB. Reacted sections were prepared for light and electron microscopic analyses as described previously [25]. For electron microscopy, osmium-postfixed and dehydrated sections were flat-embedded in Durcupan ACM (Fluka). Basal forebrain areas containing PHA-L-labeled varicosities contacting PV-immunoreactive cell bodies or dendrites were selected for ultrathin sectioning. Serial ultrathin sections were collected on single-slot Formvar-coated gilded grids and examined with a Philips 201 electron microscope.

Ultrathin sections from animals perfused with fixative B on every second grid were subjected to a postembedding GABA immunostaining protocol, as described previously [23]. The following steps were done on droplets of solutions: 0.1% periodic acid (H_5IO_6) for 10 min; 3 × distilled water (wash); 0.1% sodium metaperiodate ($NaIO_4$) for 10 min; wash; 3 × 2 min in Tris-buffered saline (TBS); 15 min in TBS containing 1% ovalbumin; 3 × 5 min in TBS with 1% normal goat serum (NGS); 1.5 h in rabbit anti-GABA (Incstar, 1:1000 dilution in TBS/NGS), 2 × 10 min in TBS; 2 × 10 min in TBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20; 1.5 h in goat anti-rabbit IgG-coated colloidal gold (15 nm, Biocell, 1:15) in TBS/BSA/Tween 20; 5 washes in distilled water; contrasting in lead citrate; wash in distilled water. When the primary antibody was replaced by normal rabbit serum, accumulation of gold particles was not seen on these sections.

PHA-L depositions into the dopamine-rich cell regions of the SN and the VTA (Fig. 1) resulted in a dense

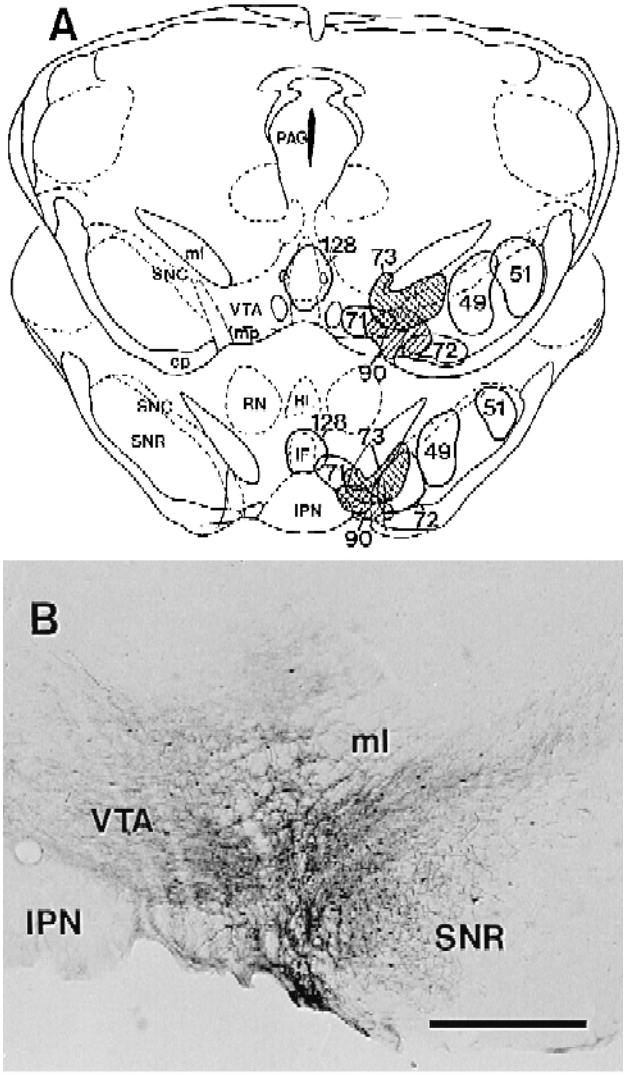

Fig. 1. A: chartings of representative PHA-L injection sites giving rise to siginificant innervation of striatal and basal forebrain regions. Hatched injection sites represent cases (73 and 90) in which connections with PV-ir neurons are illustrated at the EM level. B: micrograph of PHA-L-labeled neurons after injection in the SN-VTA border region. Scale bar = 500 μm. Abbreviations: cp, cerebral peduncle; IF, interfascicular nucleus raphe; IPN, interpeduncular nucleus; ml, medial lemniscus; mp, mammillary peduncle; PAG, periaqueductal gray; RL, rostral linear nucleus raphe; RN, red nucleus; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; VTA, ventral tegmental area.

innervation by labeled fibers of the striatum in a topographic distribution as described previously [1,7]. In addition, profusely branching PHA-L-labeled fibers traversed through the ventral part of the internal capsule and the ventromedial corner of the globus pallidus (GP) and radiated into the substantia innominata (SI), the ventral pallidum (VP), and adjacent dorsal part of the diagonal band nuclei. The topographic distribution of SN-VTA afferents in the basal forebrain has been described in detail with reference to cholinergic neurons in a separate report [11].

Light microscopic observations of PHA-L-PV double-immunostained sections revealed black PHA-L-labeled

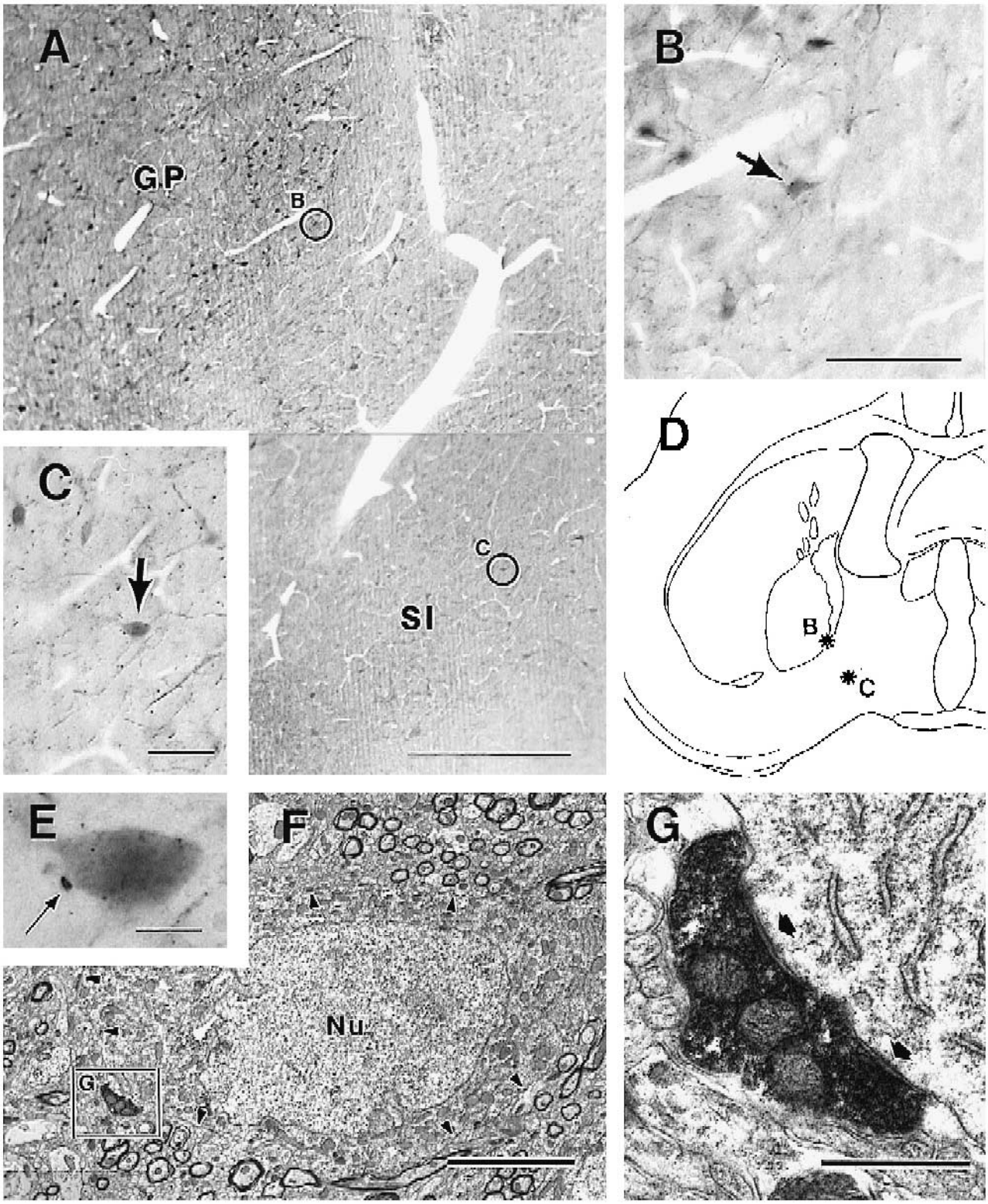

Fig. 2. Correlated light and electron microscopy of PHA-L-labeled SN-VTA afferents terminating on PV-containing neurons. A: low power light micrograph of ventral forebrain. Two recipient cells are indicated by circles (B and C indicate panels where these cells are shown in more detail). B: PV-ir cell receiving nigral input (arrow) in the ventromedial GP (see further details in Fig. 3). C: recipient PV-ir cell in ventral part of SI (arrow, also shown in E–G). D: drawing of section indicating the location of the identified PV-ir neurons (asterisks). E–G: light and electron micrographs of PV-ir neuron in SI establishing a synaptic contact with a single PHA-L-labeled bouton (arrow in E). F: electron micrograph of PV-ir soma containing rich cytoplasm and indented nucleus. Box indicates area enlarged in G. G: symmetric synapse with the PV-ir soma (solid arrows). Scale bars in A: 500 μm, B: 100 μm, C: 50 μm, E: 10 μm, F: 5 μm, G: 0.5 μm.

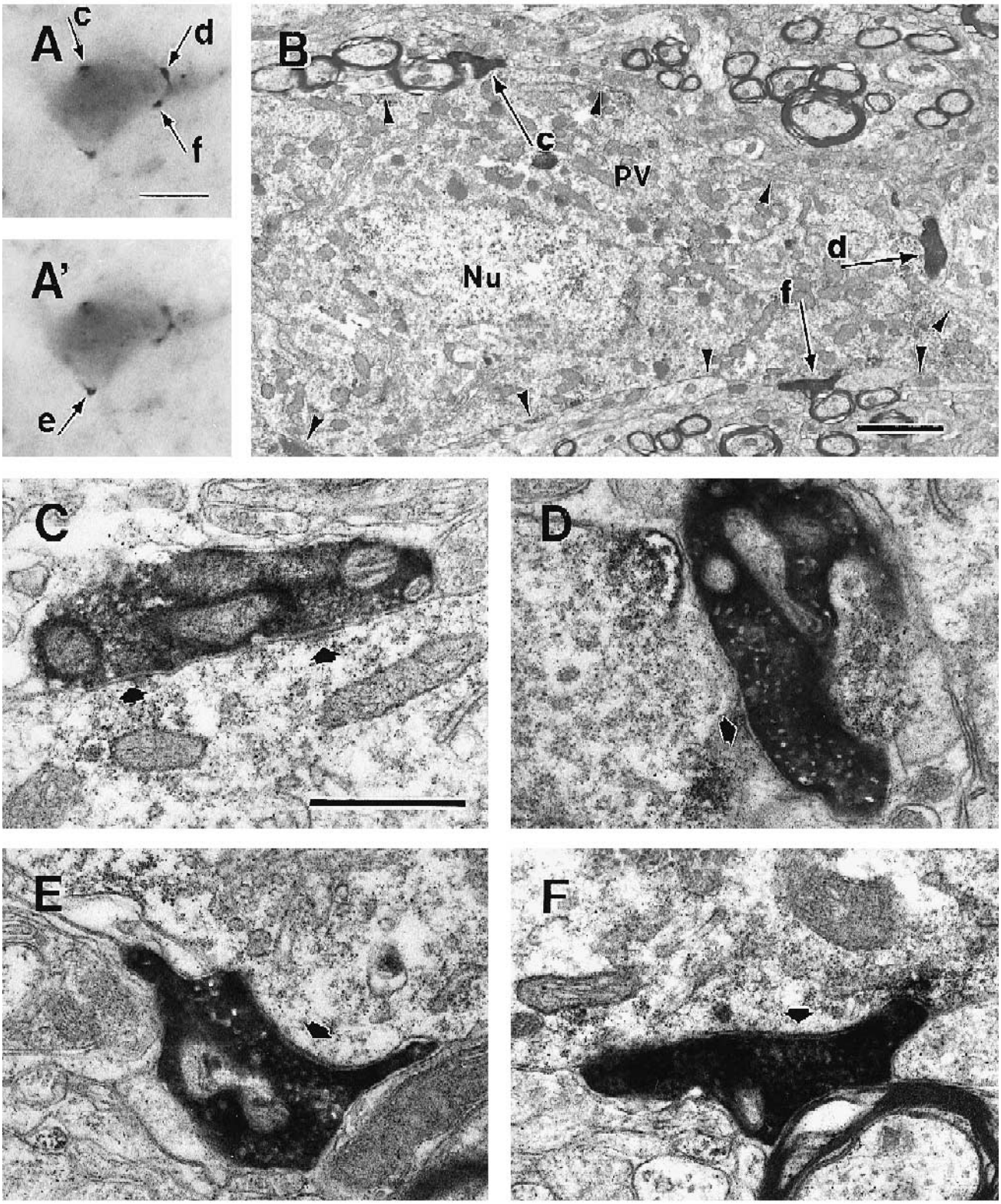

Fig. 3. A and A′: PV-ir cell in the ventromedial corner of the GP contacted by multiple PHA-L-labeled boutons, of which four are shown (labeled c–f indicating respective panels below). B: low magnification electron micrograph of this PV-ir neuron with 3 PHA-L-labeled boutons present at this level of transection (arrows, indicated c–f). Small arrowhead point to boundaries of outer membrane of PV-ir soma. Nu, nucleus. C–F: high magnification of PHA-L-labeled terminals. Boutons in C–E are taken in a different section than shown in B. All boutons make symmetric contacts (short filled arrows) with the PV-ir soma. Scale bars in A: 10 μm, B: 2 μm, C: 0.5 μm (also applies to D–F).

boutons abutting brown PV-immunoreactive (PV-ir) neuronal cell bodies in the SI (Fig. 2), the VP, the ventromedial corner of the GP (Fig. 3) and the internal capsule (Fig. 4A,B). Several PV-ir cells received multiple labeled contacts distributed around the soma (Fig. 3). This latter mode of innervation was predominant in the ventromedial GP and the adjacent internal capsule. Electron microscopic analysis of selected profiles revealed that the PV-ir neurons contained deeply indented nuclei, and a large volume of cytoplasm, rich in organelles (Fig. 2F and Fig. 3B), as described previously for the PV-ir neurons in the GP [13]. PHA-L-labeled presynaptic terminals were found to establish, if the postsynaptic membrane was not precluded by immunoprecipitate, symmetrical synapses with the PV-ir cell bodies (Fig. 2E–G, Fig. 3 and Fig. 4C), and occasionally with proximal dendrites. The PHA-L-labeled terminals were 0.8–1.5 μm in size and contained several mitochondria and numerous oval-shaped or round small vesicles. The selected PV-ir cell bodies were also contacted by several other, unlabeled, and occasionally PV-ir, boutons establishing both symmetrical and asymmetrical synapses.

Both in light and electron microscopy, PV-ir boutons were easily distinguishable from the PHA-L-labeled ones (brown vs. black color in the light microscope, and different texture and density under the electron microscope, see Fig. 4). Postembedding immunogold labeling for GABA revealed that PV-ir terminals, some of which synapse on PV-containing cell bodies (Fig. 4D), contained immunogold labeling (Fig. 4E). Neighboring unlabeled dendrites and terminals establishing asymmetric synapses were devoid of gold particles. The DAB reaction product of PV immunoreactivity was completely removed by treatment with periodic acid and sodium metaperiodate (Fig. 4E). The intense PHA-L immunoprecipitate (i.e., nickel-intensified DAB) partially remained in the ultrathin sections after this treatment. Few gold particles were encountered

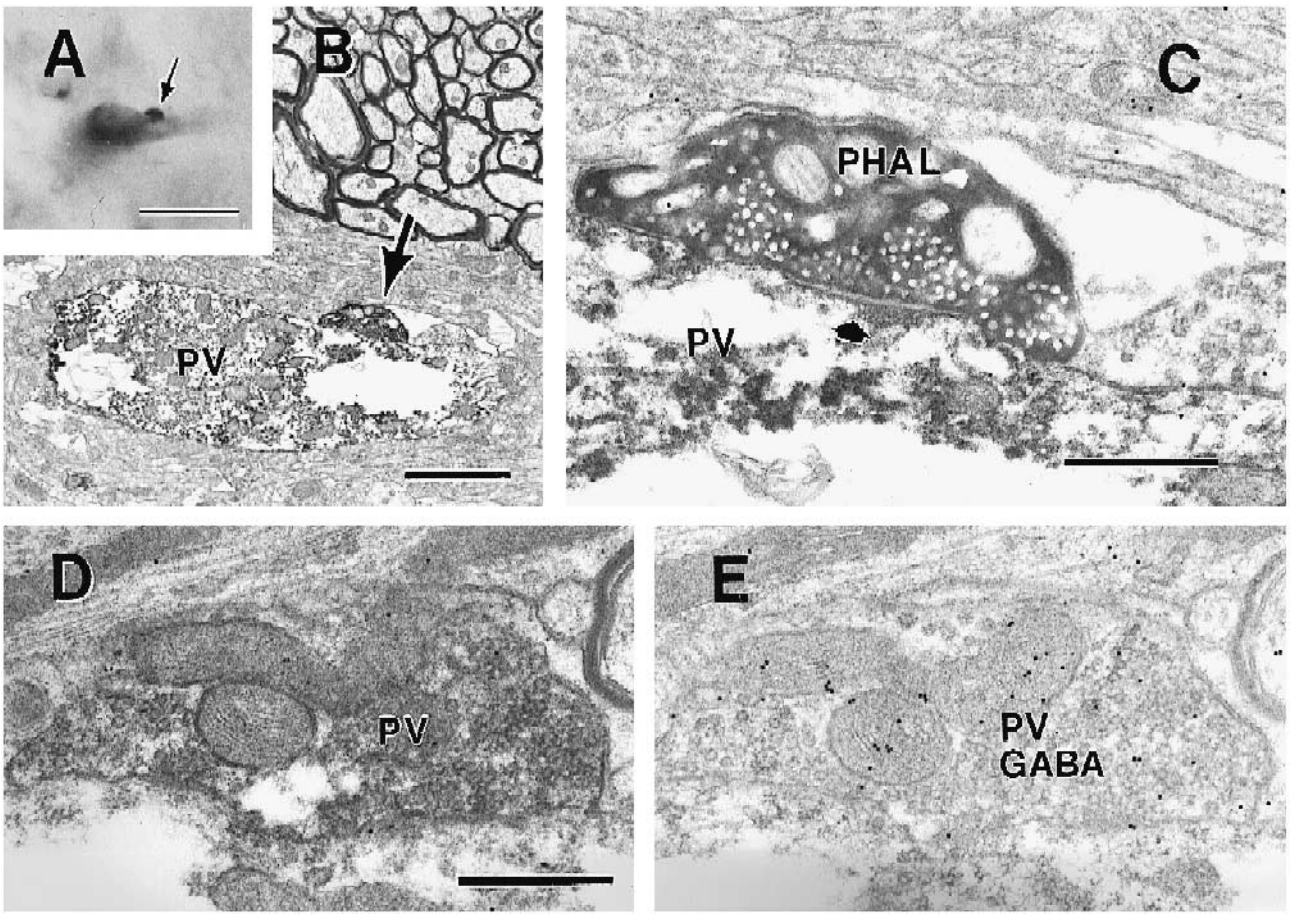

Fig. 4. A and B: light and electron micrographs of a PHA-L-labeled terminal (arrows) establishing a synaptic contact on a PV-ir soma located in the internal capsule. C: detail of the synapse (thick arrow) between the PHA-L-labeled terminal (PHA-L) and the PV-ir soma (PV). D and E: two sections of a PV-ir bouton (PV) terminating on a PV-ir soma in the dorsal half of the ventral pallidium. The second section (E) was exposed to sodium metaperiodate (loss of electron dense DAB signal) and postembedding staining procedure for GABA, revealing accumulation of gold particles (PV/GABA). Note the different appearance and intensity of the DAB signal in D in comparison to the NiDAB signal in the PHA-L-labeled bouton (C), the latter could not be removed by sodium metaperiodate treatment.

on PHA-L-labeled terminals, but no conclusion could be drawn since false-negative results of the GABA immunogold labeling cannot be excluded.

These results show that PV-containing, presumably GABAergic, neurons in pallidal and adjacent basal forebrain territories are directly targeted by projections originating in the dopaminergic neuron-rich areas of the ventral midbrain. Electrophysiological studies show that dopamine appears to be a functional neuromodulator in the pallidal areas and the SI [19]. The SN-VTA afferents to these cells therefore may represent a dopamine-GABA interaction in the basal forebrain. It is not known whether the identified PV-ir neurons represent a population of interneurons, as in the striatum and cortex [2,14], or represent a subset of projection neurons, as in the globus pallidus [14] or in the medial septum [9]. On the one hand, it can be speculated that these PV-ir, presumably GABAergic, interneurons subserve a feedforward inhibition of, e.g., the cholinergic projection neurons in the basal forebrain. PV-ir boutons in the basal forebrain were found to be positively labeled for GABA. GABAergic terminals on cholinergic neurons have been described [27], but there is no evidence of the existence of PV-ir input to cholinergic cells. On the other hand, the PV-ir neurons found to receive input form the SN-VTA may represent projection neurons of which the efferent connections remain to be determined. These cells may either project to the striatum [16], the mediodorsal thalamus [12,17,28], the cerebral cortex [10], or may be part of the basal ganglia output system [20].

The observation that the SN-VTA afferents terminate with multiple boutons on PV-ir somata indicates a strong influence of this projection on these neurons. This mode of termination on PV-ir cells differs from that of the SN-VTA afferents on the cholinergic neurons in the basal forebrain, that we observed terminating with predominantly one single bouton on the proximal dendritic shafts [11]. The question is whether the described projection is dopaminergic in nature. Axosomatic contacts between TH-positive boutons and medium and large aspiny GAD-positive neurons in the striatum have been described [15], but those boutons seem smaller in diameter (0.4–1.0 μm) than the PHA-L-labeled ones in this study (0.8–1.5 μm). A great proportion of the SN-VTA afferents to the basal forebrain has been shown to arise from dopaminergic neurons, although a non-dopaminergic, including GABAergic, component has been suggested [6,18,21,24].

The nigrostriatal and mesocorticolimbic dopaminergic pathways have been extensively studied with regard to their role in various behavioral phenomena, such as motor behavior, motivation, and reward [5,8]. This study and related reports [11,26] show that an additional ventral midbrain-basal forebrain pathway exists with terminals on basal forebrain cholinergic and parvalbumin-containing neurons. Ultimately, an understanding of the impact of this putative dopaminergic/GABAergic interaction in the basal forebrain may come from the determination of the output relationships of the dopaminoceptive PV neurons in multiple tracing/immunocytochemical studies, as well as from physiological and pharmacological characterizations of these connections.

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