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GABAergic Projection From the Basal Forebrain to the Visual Sector of the Thalamic Reticular Nucleus in the Cat

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ABSTRACT

We examined the projection from the basal forebrain to thalamic and cortical regions of the visual system in cats, with particular reference to the visual sector of the thalamic reticular nucleus, the lateral geniculate nucleus, and the striate cortex. First, we made injections of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) into the visual sector of the thalamic reticular nucleus and found cells labeled by retrograde transport in the lateral nucleus basalis magnocellularis. Injection of biocytin into the basal forebrain resulted in the anterograde labeling of a dense band of fibers and terminals within the entire thalamic reticular nucleus; this labeling extended through the visual sector including the perigeniculate nucleus. No orthograde labeling was found in the lateral geniculate nucleus. Next, we addressed the issue of putative neurotransmitters used by this pathway using a variety of immunocytochemical and histochemical markers. In this fashion, we identified two populations of cells in the nucleus basalis magnocellularis of the cat: large cholinergic cells that contain choline acetyltransferase, NADPH-diaphorase, and calbindin and that project to striate cortex and smaller cells that contain gamma-aminobutyric acid (GABA), glutamic acid decarboxylase, and parvalbumin and that project to the visual sector of the thalamic reticular nucleus. We also examined at the electron microscopic level terminals in the visual sector of the thalamic reticular nucleus that were labeled from a biocytin injection in the basal forebrain. Most of these terminals form symmetric contacts onto dendrites and were revealed by postembedding immunocytochemical staining to be positive for GABA.

Key words: NADPH-diaphorase, choline acetyltransferase, calbindin, parvalbumin, striate cortex

The thalamic reticular nucleus consists of a rim of cells that surrounds, and is reciprocally connected with, most of the dorsal thalamus (Minderhoud, 1971; Jones, 1975; Steriade et al., 1984; Harris, 1987; Cucchiaro et al., 1991; Conley and Diamond, 1990). Study of the thalamic reticular nucleus is thus essential to understanding the mechanisms by which any given thalamic nucleus processes and relays information to the cortex. Based on the pattern of its reciprocal connections with the dorsal thalamus and its inputs from the cortex, the thalamic reticular nucleus can be subdivided into sectors related to different functions and, in the case of sensory pathways, different modalities (Jones, 1975; Steriade et al., 1984; Conley and Diamond, 1990; Conley et al., 1991; Crabtree, 1992a,b). For example, the portion of the thalamic reticular nucleus that is connected with the lateral geniculate nucleus can be considered part of the visual sector of the thalamic reticular nucleus (Montero et al., 1977; Rodrigo-Angulo and Reinoso-Suárez, 1988; Crabtree and Killackey, 1989; Conley and Diamond, 1990; Cucchiaro et al., 1990). Each sector is further related to its dorsal thalamic target nuclei through its extrathalamic connections, the majority of which arise from the same areas that project to these same thalamic targets. For example, with the exception of the retina, all known inputs to the A-laminae of the lateral geniculate nucleus also project to the visual thalamic reticular nucleus, typically via branching of individual axons (Montero et al., 1977; Updyke, 1977; Smith et al., 1988; Uhlrich et al., 1988; Fitzpatrick et al., 1989; Montero, 1989; Tamamaki et al., 1989; Conley and Diamond, 1990; Cornwall et al., 1990; Asanuma, 1992; Cucchiaro et al., 1993; Uhlrich et al., 1993).

In contrast to these shared inputs, a unique source of input to the thalamic reticular nucleus from the basal forebrain has been described (Nauta, 1979; Levey et al., 1987; Steriade et al., 1987; Parent et al., 1988; Asanuma,

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1989; Jourdain et al., 1989; Asanuma and Porter, 1990, Cornwall et al., 1990; Chen and Bentivoglio, 1993). This projection is unique, because most of the dorsal thalamus does not receive projections from the basal forebrain (Nauta, 1979; Steriade et al., 1987; Asanuma, 1989; Asanuma and Porter, 1990; Cornwall et al., 1990). The basal forebrain includes several cholinergic cell groups that have been defined by Mesulam et al. (1983, 1984) as Ch1–4. In the cat, these cell groups are thought to provide all of the cholinergic input to the cortex (Fibiger, 1982; Irle and Markowitsch, 1984; Wahle et al., 1984). This widespread projection is implicated in cortical arousal mechanisms, because cholinergic transmission is involved in desynchronization of the electroencephalogram (EEG), and destruction of the basal forebrain area causes synchronization of the EEG (Buzsáki et al., 1988; Semba, 1991) However, because the thalamus is also involved in similar EEG and sleep phenomena (Steriade and Llinás, 1988), it is plausible that basal forebrain projections to the thalamic reticular nucleus are also involved in this process.

It is not known whether the basal forebrain projects uniformly to all sectors of the thalamic reticular nucleus, nor is it known to what extent these thalamic projections involve cholinergic cell groups. For instance, previous studies indicate that the projection from the basal forebrain to the rostral and medial portions of the thalamic reticular nucleus are at least partially cholinergic (Levey et al., 1987; Steriade et al., 1987; Chen and Bentivoglio, 1993), whereas the projections to more caudal or lateral areas of the thalamic reticular nucleus primarily contain gamma-aminobutyric acid (GABA) (Jourdain et al., 1989; Asanuma and Porter, 1990). Because the thalamic reticular nucleus is so important to the functioning of the entire dorsal thalamus, it is of great interest to determine the nature of the basal forebrain influence on the thalamic reticular nucleus.

One place to begin an investigation of this possible influence is with the projection from the basal forebrain to the visual sector of the thalamic reticular nucleus. This is because this region of the thalamic reticular nucleus is known to influence relay cells of the lateral geniculate nucleus (Steriade and Llinás, 1988), and many previous experiments have shown a clear effect of the state of the animal on the firing pattern of geniculate cells (Steriade and McCarley, 1990), which suggests a possible role of the basal forebrain. However, a projection from the basal forebrain to the visual sector of the thalamic reticular nucleus has not been previously documented. We believed it important to do so. Thus, we asked whether the basal forebrain projects to the visual sector of the thalamic reticular nucleus and, if so, to what extent this projection may be cholinergic or GABAergic. Finally, in addition to innervating the lateral geniculate nucleus, the parabrachial region of the brainstem provides a clear cholinergic input to the visual thalamic reticular nucleus (de Lima et al., 1985; de Lima and Singer, 1987; Uhlrich et al., 1988; Smith et al., 1988; Bickford et al., 1993). We therefore thought it would be useful to compare this parabrachial input to any possible cholinergic input from the basal forebrain.

Abbreviations

AC	anterior commissure
ACB	nucleus acumbens
BC	brachium conjunctivum
C	caudal
Cd	caudate nucleus
D	dorsal
ENT	entopeduncular nucleus
GP	globus pallidus
IC	internal capsule
Inf Col	inferior colliculus
LGN	lateral geniculate nucleus
MGN	medial geniculate nucleus
NB	nucleus basalis
NBM (ch4)	nucleus basalis magnocellularis
OT	optic tract
P	putamen
R	rostral
Sup Col	superior colliculus
TRN	thalamic reticular nucleus
V	ventral

MATERIALS AND METHODS

We used 12 cats for this study. In seven of them, we used retrograde transport to investigate the projection from the basal forebrain to the visual sector of the thalamic reticular nucleus. To do this, we injected wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) into the visual sector of the thalamic reticular nucleus bilaterally in four cats and unilaterally in three. In one cat, biocytin was injected bilaterally into the basal forebrain to label terminals in the thalamic reticular nucleus by anterograde transport. In another cat, HRP was injected unilaterally into the visual cortex to label retrogradely basal forebrain cells that project to cortex.

In addition to identifying the projection from the basal forebrain to the thalamic reticular nucleus and cortex, we attempted to determine which neuroactive substances may be involved in these pathways. Tissue from 11 cats, including the eight cats that received WGA-HRP injections plus three additional, uninjected cats, was stained for a variety of markers, and tissue from some cats was used for more than one marker. In all, staining for NADPH-diaphorase (the enzyme that produces nitric oxide) was done in each of the 11 cats; staining for choline acetyltransferase (ChAT; the synthesizing enzyme for acetylcholine) was done in four cats; staining for GABA was done in four cats; staining for glutamic acid decarboxylase (GAD; the synthesizing enzyme for GABA) was done in five cats; and staining for the calcium binding proteins calbindin and parvalbumin was done in one and six cats, respectively. Finally, tissue containing biocytin-labeled terminals was stained for GABA using postembedding immunocytochemical techniques.

Tracer injections

Cats that received tracer injections were deeply anesthetized with intravenous injections of sodium pentobarbital (initially 15 mg/kg, with 5–10 mg supplements as needed), placed in a stereotaxic apparatus, and prepared for surgery. We continuously monitored heart rate and maintained the rectal temperature at 38°C via a feedback-controlled heating blanket. We infused wound edges and pressure points with 2% lidocaine. A craniotomy was performed and the dura reflected. Iontophoretic injections of WGA-HRP were made in several sites in the thalamic reticular nucleus through a glass pipette (tip diameter 5–10 μm) filled with a solution of 5% WGA-HRP in saline. A combination of stereotaxic coordinates and visually evoked responses helped to guide placement of the pipette, and iontophoresis was achieved via DC current (3–5 μA for 15 minutes per site for four sites). Biocytin injections (5% in saline) into the basal forebrain were also done iontophoretically (5 μA for 1 hour)

after stereotaxic placement of the pipette (tip diameter 5 μm). Finally, we injected HRP [0.5 μl of 50% HRP in 2% dimethyl sulfoxide (DMSO) in saline] through a Hamilton syringe visually guided into the visual cortex.

Perfusion and sectioning

Following tracer injections, we maintained deep anesthesia for 24 hours before killing the cats with an overdose of sodium pentobarbital. We then perfused each cat through the aorta with saline followed by 2 liters of fixative solution. For cats with no injections or injections of WGA-HRP, the fixative solution was 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer; for cats injected with biocytin or HRP, the solution was 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer. The brain was removed and immersed in the appropriate fixative solution overnight. The next day, we cut sections on a vibratome at a thickness of 50 μm, or the brain was transferred to a sucrose solution for subsequent cutting of frozen sections at a thickness of 60 μm. All sections that contained HRP were reacted for tetramethybenzidine (TMB) and diaminobenzidine (DAB) first and then for NADPH-diaphorase (eight cats), ChAT (two cats), GABA (two cats), GAD (four cats), or parvalbumin (five cats). Sections from uninjected brains were either stained for a single histochemical label or a combination of two labels. For combinations, the NADPH-diaphorase reaction always followed immunohistochemical reactions, and parvalbumin staining followed GAD staining. Sections from the biocytin injected brain were reacted to reveal the location of the biocytin and processed for light or electron microscopy as described below.

Histochemistry

WGA-HRP and HRP. We processed sections containing WGA-HRP or HRP with TMB in 0.2 M sodium acetate buffer at pH 3.3 for 20 minutes at room temperature. The TMB crystals were stabilized first with 5% ammonium heptmolybdate in acetate buffer at pH 3.3 for 20 minutes and then with a solution containing 0.05% DAB, 0.025% cobalt chloride, and 0.01% hydrogen peroxide in 0.1 M sodium phosphate buffer at pH 7.4 for 5–8 minutes at room temperature (Horn and Hoffmann, 1987).

Biocytin. The sections that contained biocytin were placed in phosphate-buffered saline (PBS) after being cut on a vibratome. They were then incubated in a 1:200 dilution of avidin and biotinylated HRP (Vector, Burlingame, CA) in PBS with 1% normal goat serum overnight at 4°C. The next day, we rinsed them three times in PBS (10 minutes each), reacted them with nickel intensified DAB for 30 minutes, and rinsed them in phosphate buffer. We then either mounted them on slides for light microscopic examination or prepared them for electron microscopy. For the latter, we postfixed them in osmium, dehydrated and embedded them in Durcupan, cut them into thin (0.08 μm) sections, and mounted them on Formvar-coated slot grids; we then either stained them with uranyl acetate and lead citrate or treated them as described below for GABA analysis.

NADPH-diaphorase. To stain for NADPH-diaphorase activity, we rinsed sections in Tris buffer (pH 7.2) and then placed them in a solution of 0.1% NADPH, 0.025% nitroblue tetrazolium, and 0.2% Triton X-100 in Tris buffer (pH 7.2) for 30 minutes to 2 hours at 37°C (slightly modified from Hope and Vincent, 1989). The sections were subsequently rinsed in Tris buffer, mounted on glass slides, dehydrated briefly, and coverslipped with DPX (Fluka Chemika) or, if the tissue also contained a fluorescent label, Vectashield (Vector).

ChAT. To stain for the presence of ChAT, we incubated sections in 10% normal rabbit serum in PBS for 30 minutes and then placed them in a solution containing 1% normal rabbit serum, 0.2% Triton X-100, and a 1:50 dilution of a monoclonal anti-ChAT antibody (made in rat; Boehringer) for 40 hours at 4°C. The sections were then rinsed three times (10 minutes each) in PBS and placed in a 1:50 dilution of biotinylated rabbit anti-rat antibody (Vector) in PBS with 1% normal rabbit serum for 1 hour at room temperature. We then rinsed the sections three times (10 minutes each) in PBS and placed them either in a 1:200 solution of avidin and biotinylated HRP (Vector) in PBS or a 1:100 solution of avidin-D-fluorescein (Vector) in PBS for 1 hour at room temperature. After three rinses (10 minutes each) in PBS, the sections that contained the HRP tag were processed in DAB for 15 minutes, rinsed in phosphate buffer, and mounted on slides. Sections that contained the fluorescent tag were then reacted for NADPH-diaphorase as described above.

Calbindin. To stain for the presence of calbindin, we incubated sections in 10% normal horse serum in PBS for 30 minutes and then placed them in a solution containing 1% normal horse serum, 0.2% Triton X-100, and a 1:1,000 dilution of a monoclonal anticalbindin (made in mouse; Sigma, St. Louis, MO) for 16 hours at 4°C. The sections were then rinsed in PBS and placed in a 1:100 dilution of biotinylated horse anti-mouse antibody (Vector) in PBS with 1% normal horse serum for 1 hour at room temperature. We then rinsed the sections in PBS and tagged the antibody with DAB reacted HRP or fluorescein as described above for ChAT staining.

GABA for light microscopy. To stain for the presence of GABA, we incubated sections in 10% normal goat serum in PBS for 30 minutes and then placed them in a solution containing 1% normal goat serum, 0.2% Triton X-100, and a 1:1,000 dilution of a polyclonal anti-GABA antibody (made in rabbit; Sigma) for 16 hours at 4°C. The sections were then rinsed in PBS and placed in a 1:100 dilution of biotinylated goat anti-rabbit antibody (Vector) in PBS with 1% normal goat serum for 1 hour at room temperature. As described above, we then rinsed the sections, incubated them with avidin and biotinylated HRP, and reacted the sections with DAB.

GABA for electron microscopy. To stain for the presence of GABA in thin sections that contained biocytin-labeled terminals, we mounted sections on Formvar-coated nickel grids and etched the resin with periodic acid (1% for 10 minutes). After rinsing in H_2O, we then removed the osmium with sodium metaperiodate (1% for 7 minutes) and rinsed the grids in H_2O. We blocked nonspecific staining by incubating the grids in a solution of 0.8% bovine serum albumin, 0.1% fish gelatin (from the protocol in Amersham Auroprobe One GAR instruction booklet), and 5% normal goat serum in PBS. The grids were then washed for 5 minutes in a solution of 0.8% bovine serum albumin and 0.1% fish gelatin in PBS (washing buffer). We then incubated the grids 18 or 42 hours in a moist chamber at 4°C on drops of a 1:500 dilution of the Sigma anti-GABA antibody in a solution of 0.8% bovine serum albumin, 0.1% fish gelatin, and 1% normal goat serum in PBS (incubation buffer). Then, the grids were rinsed in washing buffer

(three times for 10 minutes each) and incubated in a 1:25 dilution of goat anti-rabbit IgG conjugated to 15 nm gold particles (Amersham; Auroprobe EM GAR) in incubation buffer for 2 hours at room temperature. The grids were then rinsed in washing buffer (three times for 15 minutes each), then in PBS (three times for 5 minutes each), and then were postfixed in 2% glutaraldehyde in PBS for 10 minutes. The grids were then rinsed in H_2O, stained with uranyl acetate and lead citrate, and examined with the electron microscope.

GAD. To stain for the presence of GAD, we incubated sections in 10% normal goat serum in PBS for 30 minutes and then placed them in a solution containing 1% normal goat serum and a 1:2,000 dilution of a polyclonal antibody directed against the 67 KD isoform of GAD (made in rabbit; Chemicon) for 16–40 hours at 4°C. The sections were then rinsed in PBS and placed in a 1:100 dilution of biotinylated goat anti-rabbit in PBS with 1% normal goat serum for 1 hour at room temperature. The sections were rinsed again in PBS and incubated in either a 1:200 dilution of avidin and biotinylated HRP (Vector) or a 1:100 dilution of avidin-D-rhodamine (Vector) in PBS for 1 hour. The sections were then rinsed, and those containing the HRP tag were reacted with DAB for 5–10 minutes. DAB-reacted sections that also contained retrograde HRP label were mounted on slides. Sections from uninjected brains, stained for GAD with DAB reacted HRP or rhodamine, were subsequently stained for parvalbumin as described below.

Parvalbumin. To stain for the presence of parvalbumin, we incubated sections in 10% normal horse serum in PBS for 30 minutes and then placed them in a solution containing 1% normal horse serum and a 1:2,000 dilution of a monoclonal antiparvalbumin antibody (made in mouse; Sigma) for 16 hours at 4°C. The sections were then rinsed in PBS, and then, if they had previously been stained for GAD, placed in a 1:100 dilution of fluorescein-labeled horse anti-mouse antibody in 1% normal horse serum and PBS for 1 hour at room temperature. These sections were then rinsed in PBS, mounted on slides, dehydrated, and coverslipped with Vectashield mounting medium (Vector). Sections that had not been previously stained for GAD were placed in a 1:100 dilution of biotinylated horse anti-mouse antibody (Vector) in 1% normal horse serum in PBS for 1 hour at room temperature. We then rinsed the sections and placed them either in a 1:200 dilution of avidin and biotinylated HRP (Vector) or a 1:100 dilution of avidin-D-fluorescein in PBS for 1 hour at room temperature. We rinsed the sections, reacted those containing the HRP label with DAB, rinsed them, and mounted them on slides. Those with fluorescein label were subsequently reacted for NADPH-diaphorase as described above.

Analysis

Light microscopy. In most cases, we plotted cell distributions with a computer-controlled microscope; in others, we used a drawing tube attached to a transmitted light or epifluorescent microscope. Cell counts were made while examining tissue sections either with transmitted or epifluorescent blue light used in sequence or with two wavelengths (blue and green) of epifluorescent light. We used various means of double labeling cells depending on specific experiments, and these are detailed in RESULTS. We measured soma sizes directly from tissue sections using the computer-controlled microscope system.

Electron microscopy. As noted above, gold particles were deposited via postembedding immunohistochemistry to label GABAergic processes. We identified these processes as follows: We created a montage of electron micrographs from each section, and, within these montages, we analyzed and identified terminal types. We outlined each terminal, measured its cross-sectional area, counted the number of gold particles within it, and computed its density of labeling (number of gold particles per μm^2). The further analysis based on the density of labeling is described in Results.

RESULTS

Because the experiments described are rather complicated, there is a danger that the reader will find it difficult to follow the significance of various results and how they might contribute to a consistent set of conclusions. To minimize this problem and provide a theoretical framework for the results to be described, we thought it best to start with our broad conclusions. These are summarized in Figure 1. Briefly, we conclude that, for the most part, cells of the basal forebrain can be divided into two groups: cells that project to visual cortex and that colocalize acetylcholine, NADPH-diaphorase, and calbindin and cells that project to the visual sector of the thalamic reticular nucleus and that colocalize GABA and parvalbumin. We further conclude that the parabrachial region of the brainstem provides the sole afferent source to the visual thalamic reticular nucleus and lateral geniculate nucleus of axons containing acetylcholine and NADPH-diaphorase. Below, we provide the evidence for these conclusions.

Projection of the basal forebrain to the visual thalamic reticular nucleus

Our first goal in this study was to determine the nature of any projection from the basal forebrain to the visual sector of the thalamic reticular nucleus. To accomplish this, we used both retrograde and anterograde pathway tracing techniques.

Retrograde tracing. To determine the distribution of basal forebrain cells that project to the visual thalamic reticular nucleus, we placed small injections of WGA-HRP into the thalamic reticular nucleus just dorsal to the lateral geniculate nucleus. This was done in seven cats, and, in each case, we found a population of retrogradely labeled cells in the basal forebrain. This region of caudal thalamic reticular nucleus overlying the lateral geniculate nucleus is a difficult target for injections, because it is comprised of a fairly narrow band of islands of cells and neuropil embedded in white matter. However, although the number of retrogradely labeled cells varied greatly in our experiments depending on the extent of the TRN that was injected, the general location of all the retrogradely labeled cells was very similar in all seven brains that received injections. Figure 2 shows a representative example of the labeling from one of the experiments. The basal forebrain cells labeled from these visual thalamic reticular nucleus injections were located just caudal to the putamen and in the region of the globus pallidus, placing them in the nucleus basalis of Meynert (or nucleus basalis magnocellularis).

The only other areas that contained retrogradely labeled cells were the dorsal division of the lateral geniculate nucleus, the pulvinar/lateral posterior complex, and regions that project to these structures. These regions include the visual cortex, hypothalamus, pretectum, superior collicu-

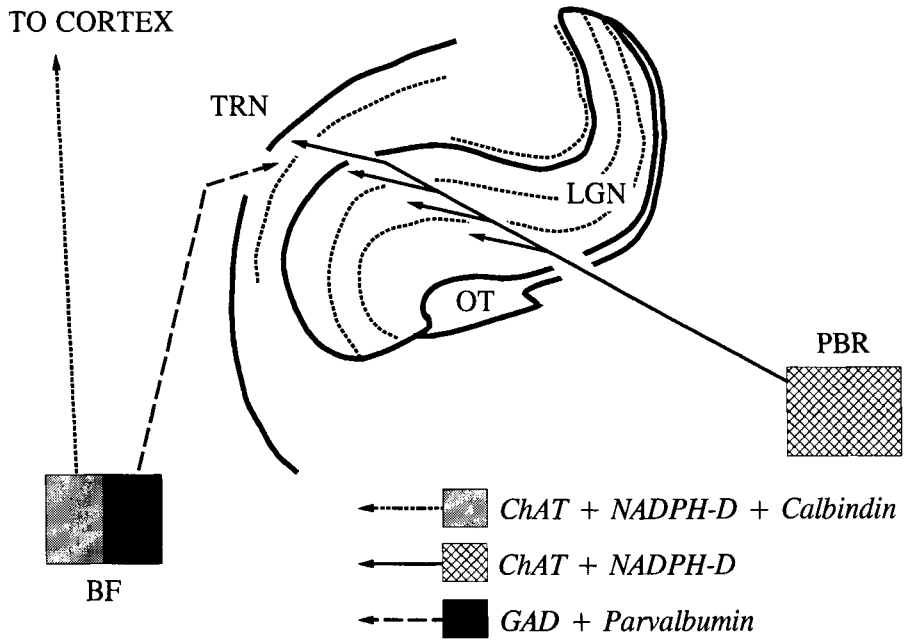


Fig. 1. Summary of major observations. Cells in the basal forebrain (BF) fall into two major groups. One is cholinergic and also contains NADPH-diaphorase and calbindin; it projects to visual cortex. The other is GABAergic and also contains parvalbumin; it projects to the visual thalamic reticular nucleus. Another cell group located in the brainstem parabrachial region (PBR) is cholinergic and also contains NADPH-diaphorase; it projects to both the lateral geniculate nucleus and visual thalamic reticular nucleus.

lus, parabrachial region, and dorsal raphé nucleus. Although a previous report states that the substantia nigra pars reticulata projects to the rostral pole of the thalamic reticular nucleus (Paré et al., 1990), we saw no evidence of any projection from the substantia nigra to the visual thalamic reticular nucleus, which is located more caudally.

Anterograde tracing. To then examine the distribution of basal forebrain terminals in the thalamic reticular nucleus, we placed an injection of biocytin into the basal forebrain. We specifically targeted the region in which we found retrogradely labeled cells following WGA-HRP injections into the visual thalamic reticular nucleus (see Fig. 2). As illustrated in Figure 3, this injection, which hit the nucleus basalis, globus pallidus, and part of the internal capsule, labeled a dense band of terminals within the entire thalamic reticular nucleus. The origin of the labeling in the more ventral regions of the thalamic reticular nucleus is unclear, because the internal capsule was labeled. However, we conclude that the terminal labeling in the thalamic reticular nucleus dorsal to the lateral geniculate nucleus must originate from the basal forebrain, because other inputs to the visual thalamic reticular nucleus do not pass through this region. This is indicated by the lack of terminal labeling in the lateral geniculate nucleus and the fact that all known inputs to the lateral geniculate nucleus other than the basal forebrain also innervate the visual thalamic reticular nucleus (see above).

Labeled terminals were also quite dense within the ventral lateral geniculate nucleus and the intergeniculate leaflet (see Fig. 3). Labeled axons seen in the optic tract did not enter the lateral geniculate nucleus but instead continued caudally to innervate the superior colliculus. These were most likely of cortical origin, labeled from the internal capsule.

Figure 4 shows a higher power view of labeled terminal arbors from basal forebrain within the visual thalamic reticular nucleus. These labeled arbors include dense clusters of terminals en passant (Fig. 4B) and were located primarily within the outermost layer of the thalamic reticular nucleus. However, we also saw sparse terminal labeling throughout more ventral regions including the perigeniculate nucleus lying immediately dorsal to the lateral geniculate nucleus.

Nature of cholinergic cells and their projections in the basal forebrain

After establishing that the basal forebrain indeed projects to the visual sector of the thalamic reticular nucleus, we wished to address the issue of what putative neurotransmitters are used by this pathway. Two particularly likely candidates are ACh and GABA (see above and Levey et al., 1987; Steriade et al., 1987; Jourdain et al., 1989; Asanuma and Porter, 1990; Chen and Bentivoglio, 1993). We shall first consider the issue of ACh. From a previous study (Bickford et al., 1993), we know that many axons and terminal arbors in the visual thalamic reticular nucleus are cholinergic and that many or all of these also contain NADPH-diaphorase. One source of these axons is the parabrachial region of the brainstem. Because a previous study in the cat reported that some cholinergic cells of the basal forebrain project to the rostral pole of the thalamic reticular nucleus (Steriade et al., 1987), we examined the possibility that some of the cholinergic axons in the visual thalamic reticular nucleus derive from the basal forebrain and that some of these may also colocalize NADPH-diaphorase.

Histochemistry and immunocytochemistry of cholinergic cells in the basal forebrain. As is shown in Figure 5, the cells of the nucleus basalis stain for ChAT, the synthesizing enzyme for acetylcholine. Such cells have also been identified by Mesulam et al. (1983, 1984) as part of the

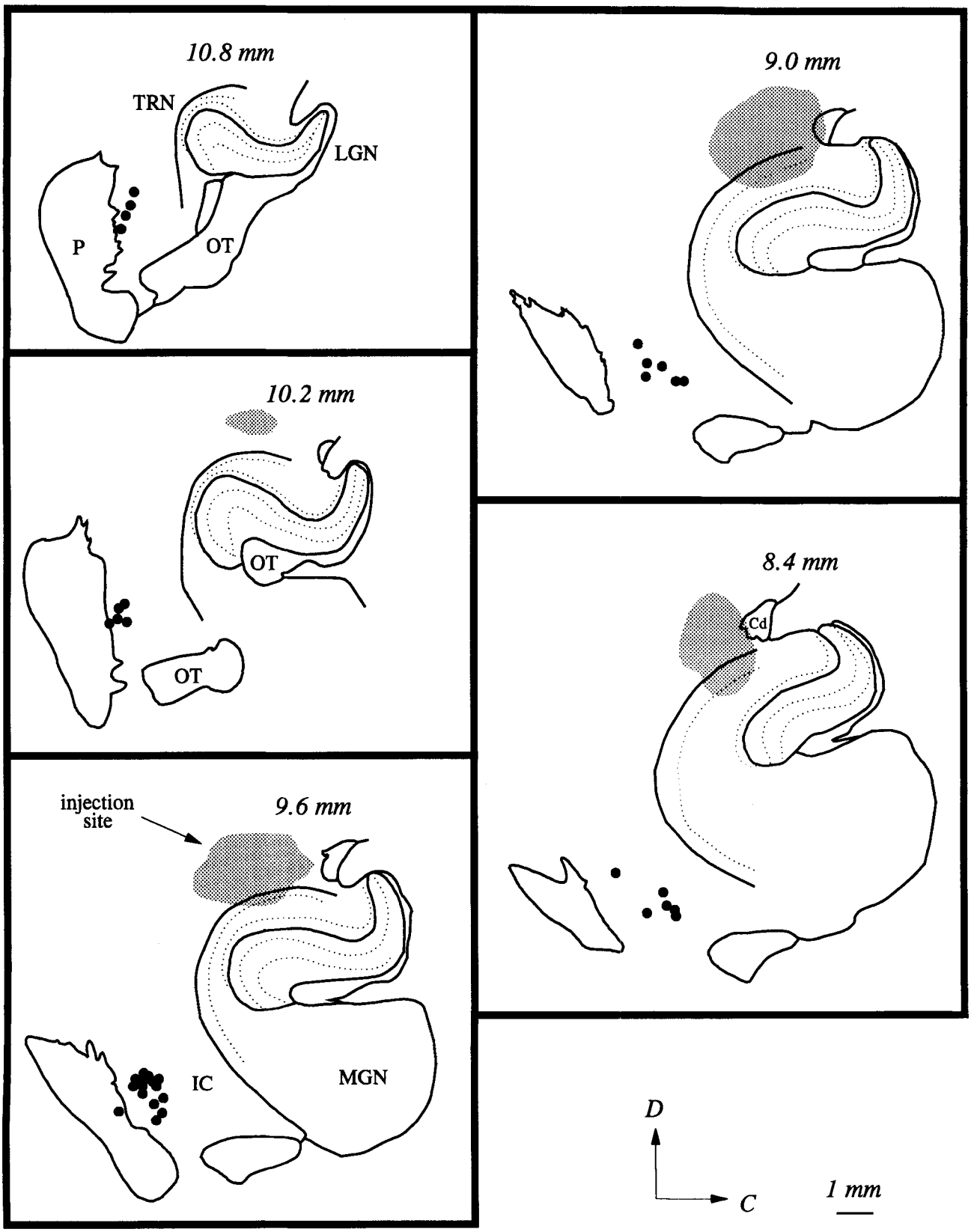
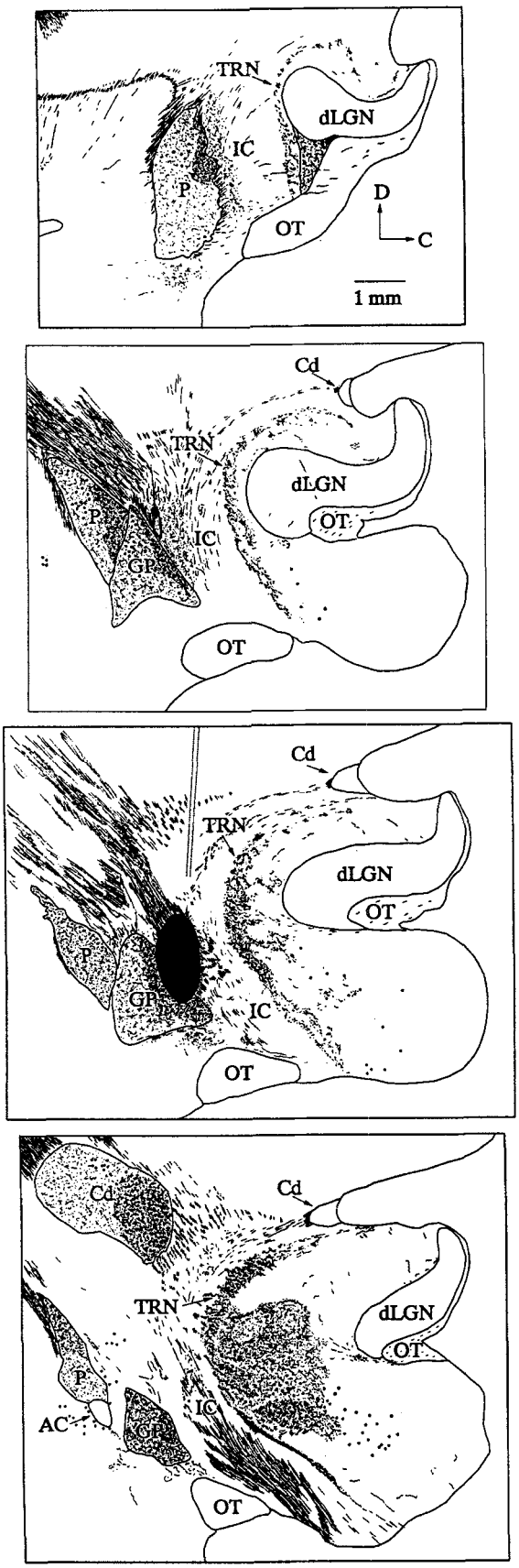


Fig. 2. Plot of cells (large circles) in the basal forebrain retrogradely labeled from an injection of WGA-HRP into the visual thalamic reticular nucleus (injection site). A series of five parasagittal sections is illustrated. Distance from the midline is indicated above each section. Retrogradely labeled cells were located in the peripallidal region of the nucleus basalis.



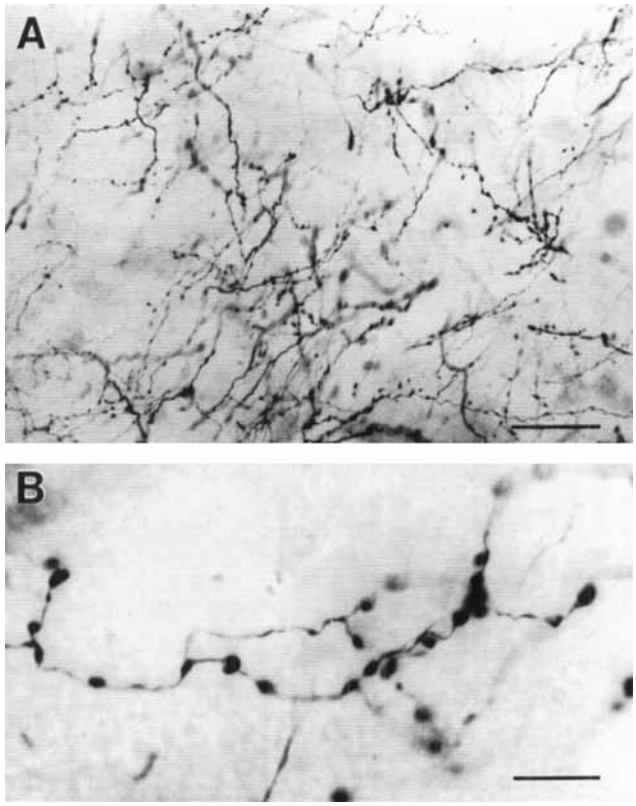


Fig. 4. Biocytin-labeled terminals in thalamic reticular nucleus following the injection illustrated in Figure 3. **A:** Lower power view. **B:** Higher power view. Scale bars = 50 μm in A, 10 μm in B.

cholinergic cell group Ch4. These cholinergic cells are located in a triangular region between the putamen and the internal capsule in lateral sections (Fig. 5A), whereas, in more medial sections, they form a ring around the globus pallidus (Fig. 5C). The cells retrogradely labeled from our visual thalamic reticular nucleus injections followed a similar pattern. Figure 5A also shows the dense cholinergic innervation of the thalamic reticular nucleus.

Figure 5 shows further that the pattern of staining for NADPH-diaphorase is essentially identical to that for ChAT. Figure 5B shows NADPH-diaphorase staining in a section similar to that stained for ChAT in Figure 5A, and, likewise, Figure 5D shows NADPH-diaphorase staining matched for the ChAT staining in Figure 5C. This raises the possibility that, as is the case for cells in the parabrachial region of the brainstem, cholinergic cells in the basal forebrain colocalize NADPH-diaphorase. Figure 6, which shows ChAT and NADPH-diaphorase staining in single sections at higher power, addresses this possibility. Nearly every nucleus basalis cell stained for ChAT (Fig. 6A,C) also stains posi-

Fig. 3. Distribution of labeled axons and terminals following an injection of biocytin into the basal forebrain. The four parasagittal sections are arranged from lateral (top) to medial (bottom), and the injection into the basal forebrain is shown as a solid black region, with the electrode track leading to it in the third section from the top. Labeled terminals are found throughout the thalamic reticular nucleus, including the visual sector overlying the lateral geniculate nucleus. No labeled terminals were seen within the lateral geniculate nucleus.

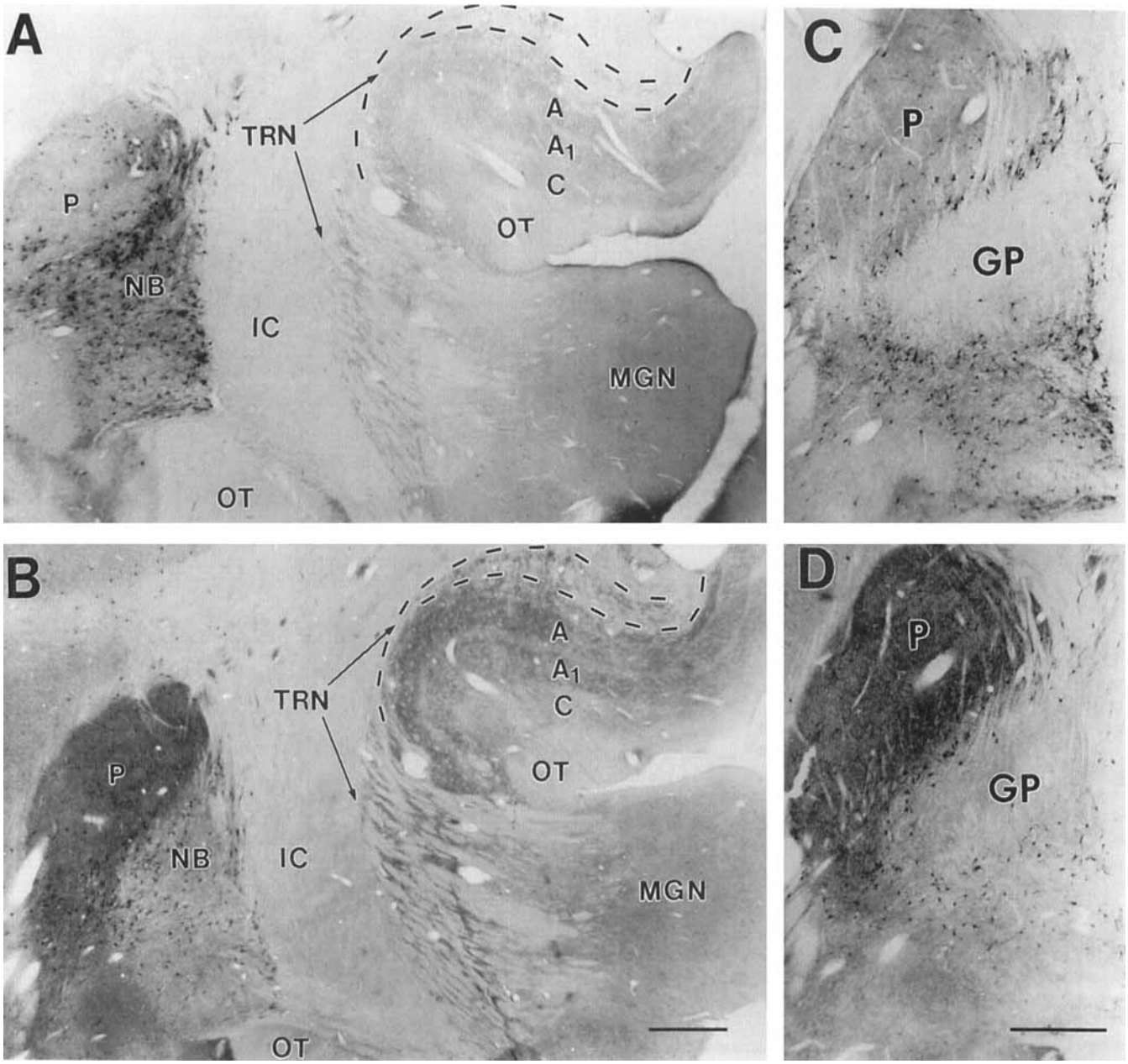


Fig. 5. Comparison of NADPH-diaphorase and ChAT staining in the basal forebrain and thalamic reticular nucleus. Shown are parasagittal sections, with dorsal up and rostral to the left. **A:** Lower power view stained for ChAT. The main laminae of the lateral geniculate nucleus (A, A_1, and C) are indicated. **B:** Lower power view, similar to that shown in A, stained for NADPH diaphorase. Axons in the thalamic reticular nucleus (outlined by dashed line) and cells in the basal forebrain show a very similar pattern of staining for ChAT and NADPH-diaphorase. **C:** Higher power view through the more medial basal forebrain stained for ChAT. **D:** Higher power view, similar to that shown in C, stained for NADPH-diaphorase. ChAT positive and NADPH-diaphorase positive cells form a ring around the globus pallidus. Scale bars = 1 mm.

tively for NADPH-diaphorase (Fig. 6B,D). Interestingly, Figure 6E,F shows that cholinergic cells in the putamen do not colocalize NADPH-diaphorase, indicating that such colocalization is not a general feature of cholinergic neurons, a conclusion also reached in our earlier study of the brainstem (Bickford et al., 1993). Figure 7, which plots the distribution of ChAT and NADPH-diaphorase staining through this region in one experiment, further illustrates this point. Overall, we found that 223 of the 253 (88%) ChAT positive cells (from four sections spaced 0.6 mm apart in one cat) in the peripallidal region of the nucleus basalis also contained NADPH-diaphorase (Fig. 6A–D). This pattern is very similar to that described for the parabrachial region (Bickford et al., 1993).

Although the vast majority of cells stained for ChAT also stain for NADPH-diaphorase, it appears qualitatively from our data that a lower percentage of NADPH-diaphorase positive cells stain for ChAT. It should be noted that ChAT staining involved immunocytochemistry, and, thus, limited penetration of the antibody leads to an unknown but

presumably large number of cholinergic cells deep in each section that will not be labeled for ChAT. Indeed, we noticed that ChAT positive cells were much more numerous near the surface of each section than near its middle. NADPH-diaphorase staining, however, is a simple histochemical reaction not involving antibodies and, thus, not limited by penetration problems. Because of the false-negative problem associated with ChAT but not NADPH-diaphorase staining, we did not bother to compute the number of NADPH-diaphorase-positive cells that also stain for ChAT.

Previous studies have also shown that NADPH-diaphorase and ChAT are located in separate cells in the caudate and putamen (Vincent et al., 1983a,b; Geula et al., 1993). However, there appear to be species differences in NADPH-diaphorase staining within the nucleus basalis. On the one hand, in species such as monkey, baboon, and human, cholinergic cells of the nucleus basalis do not show NADPH-diaphorase staining, but these cells do stain positively for the calcium-binding protein calbindin. On the other hand, in the rat, in which some basal forebrain cells do colocalize ChAT and NADPH-diaphorase, these same cells do not stain positively for calbindin (Chang and Kuo, 1991; Pasqualotto and Vincent, 1991; Geula et al., 1993; Kitchener and Diamond, 1993). As a further characterization of the cholinergic cells of the nucleus basalis in the cat, we combined staining for calbindin and NADPH-diaphorase for these cells. Figure 8 shows representative examples, and nearly every cell stained for calbindin (Fig. 8A,C) also stains for NADPH-diaphorase (Fig. 8B,D). Figure 9 further illustrates this point by showing the pattern of calbindin and/or NADPH-diaphorase staining in a section through the region of the basal forebrain. Overall, we found that 185 of the 244 (76%) calbindin-positive cells (from seven sections spaced 0.6 mm apart in one cat) in the nucleus basalis of seven sections also stained for NADPH-diaphorase. As is the case with double labeling for ChAT and NADPH-diaphorase (see above), we did not compute the percentage of NADPH-diaphorase-positive cells that colocalize calbindin. Nonetheless, we conclude that, in the cat, most cholinergic cells of the nucleus basalis contain both NADPH-diaphorase and calbindin, and this does not seem to be the case for other species (see above).

Many cells in the putamen were lightly stained for calbindin. However, as has been reported for other species (Geula et al., 1993), we found no evidence for colocalization of calbindin and NADPH-diaphorase in the putamen. This is also illustrated in Figure 9.

Projection of cholinergic cells from the basal forebrain. To determine whether the projection to the visual thalamic reticular nucleus arises from the cholinergic (and NADPH-diaphorase- plus calbindin-positive) cells of the basal forebrain, we performed double-labeling experiments: We injected WGA-HRP into the thalamic reticular nucleus to label cells retrogradely in the basal forebrain, and, following the TMB/DAB reaction to reveal these cells, we stained the same sections for NADPH-diaphorase or ChAT. In such sections, we found that, although the retrogradely labeled cells were interspersed among the ChAT- or NADPH-diaphorase-positive cells, very few retrogradely labeled cells stained for both substances. Figure 10A,B shows examples of basal forebrain cells retrogradely labeled from the WGA-HRP injection into the visual thalamic reticular nucleus. Many cells were found labeled with WGA-HRP or NADPH-diaphorase but not both (Fig. 10A), and many were found with WGA-HRP or ChAT labeling, but not both (Fig. 10B). Figure 11 shows the pattern of WGA-HRP and NADPH-diaphorase labeling after our largest injection centered on the visual thalamic reticular nucleus. In this case, we found that only 16 of the 337 (4.7%) retrogradely labeled basal forebrain cells (from 20 sections spaced 0.6 mm apart in one cat) also contained NADPH-diaphorase. Likewise, after another large injection illustrated in Figure 12, four of 49 (8.2%) retrogradely labeled cells (from 22 sections spaced 0.6 mm apart in one cat) also stained positively for ChAT. However, after smaller injections more clearly limited to the thalamic reticular nucleus (see, e.g., the injection illustrated in Fig. 1), we found no double-labeled cells. This suggests that even the small minority of cholinergic and NADPH-diaphorase-positive cells retrogradely labeled from our larger injections may not actually terminate in the visual thalamic reticular nucleus (see below).

Another major target of basal forebrain is cerebral cortex, and we tested the possibility that this is the chief target for the cholinergic cells in the basal forebrain. We performed studies analogous to those described in the paragraphs above but injected WGA-HRP into visual cortex and reacted basal forebrain tissue for both TMB and either NADPH-diaphorase or ChAT. We frequently found double-labeled cells in these experiments. An example of WGA-HRP and NADPH-diaphorase double labeling is illustrated in Figure 10C. Figure 13 shows a plot of the distribution of retrogradely labeled cells in the basal forebrain after a cortical injection, showing as well those also labeled for NADPH-diaphorase. Note that the cells were distributed in and around the nucleus basalis, similar to those retrogradely labeled from visual thalamic reticular nucleus injections. Overall, we found that 74 of 104 (71%) retrogradely labeled basal forebrain cells (from 11 sections spaced 0.6 mm apart in one cat) colocalized NADPH-diaphorase.

It is important to emphasize that our finding that cholinergic basal forebrain cells do not innervate the visual thalamic reticular nucleus, though a negative finding, seems robust for two reasons. First, as described above, we found many cortically projecting cells in the basal forebrain that were cholinergic, so we are able to visualize such cells after retrograde labeling. Second, we have previously reported with techniques similar to those used here that cholinergic cells of the parabrachial region innervate the visual thalamic reticular nucleus (Bickford et al., 1993), so our failure here is not due to a failure of labeling cholinergic cells retrogradely from this region. However, to drive home this second point more explicitly, we analyzed the parabrachial region from the same individual cases in which we found very few cholinergic basal forebrain cells projecting to the visual thalamic reticular nucleus. Unlike the basal forebrain, the parabrachial region in these experiments contained many retrogradely labeled cells that also stained positively for NADPH-diaphorase or ChAT. One example is illustrated in Figure 10D. In one case, illustrated in Figure 14 (the same case as illustrated in Fig. 11), we show the distribution of retrogradely labeled parabrachial cells including those also labeled for NADPH-diaphorase. In this example, 39 of 55 (71%) retrogradely labeled cells (from three parasagittal sections spaced 1.2 mm apart in one cat) stained positively for NADPH-diaphorase. In another case in which the WGA-HRP injection site was confined to the visual thalamic reticular nucleus and did not encroach the lateral geniculate nucleus (not illustrated), we found that 27 of 41 (66%) retrogradely labeled parabrachial cells (from

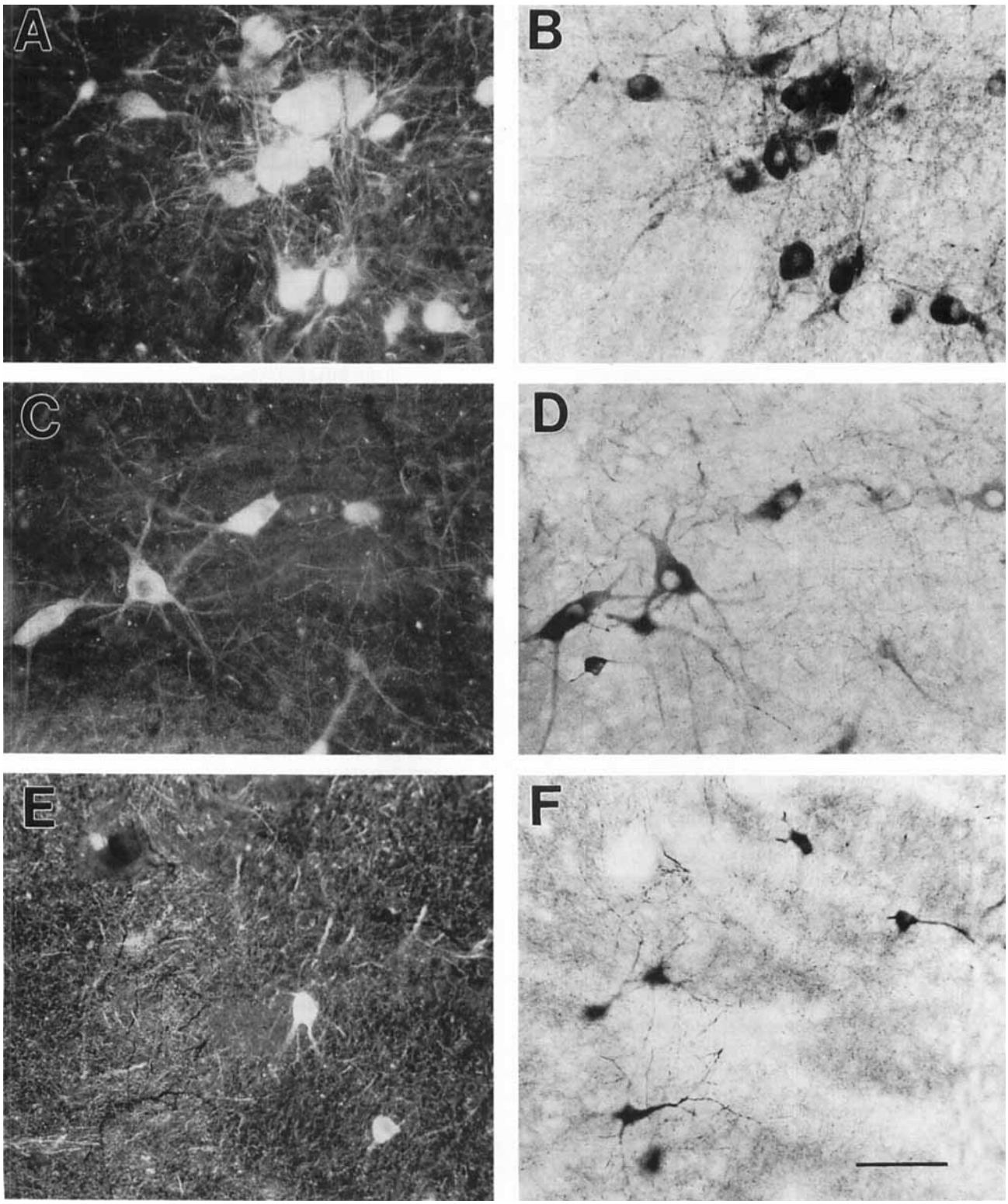


Fig. 6. Cells labeled for ChAT with fluorescein on the left (**A,C,E**; photographed with epifluorescent blue light) with the same fields stained for NADPH-diaphorase on the right (**B,D,F**; photographed with transmitted white light). Almost every cell in the nucleus basalis (A–D) contains both labels. In contrast, very few cells in the putamen (E,F) are double labeled. Scale bar = 50 μm.

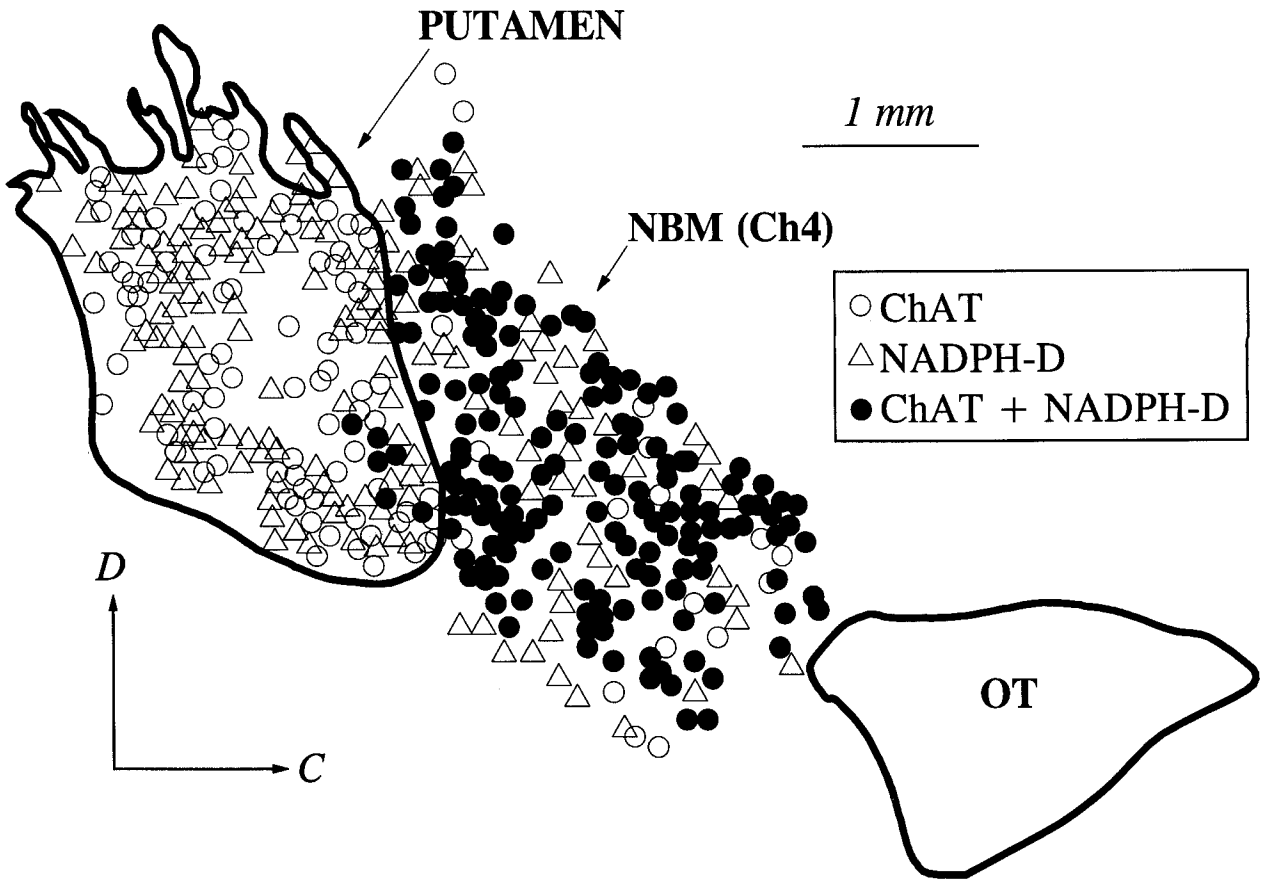


Fig. 7. Plot of single- and double-labeled cells stained for ChAT and NADPH-diaphorase in a parasagittal section through the basal forebrain. Most of the cells in the nucleus basalis that are positive for ChAT also contain NADPH-diaphorase. Few of the ChAT positive cells in the putamen contain NADPH-diaphorase.

26 adjacent coronal sections in one cat) also stained positively for ChAT (see also Bickford et al., 1993).

As was noted above, the cholinergic cells of the basal forebrain and parabrachial region both also contain NADPH-diaphorase, but this is not a general feature of cholinergic cells elsewhere in the brain. Because most of these cells in the basal forebrain also contain calbindin (see above), we looked for this in the parabrachial region. Specifically, we treated sections containing the parabrachial region for both NADPH-diaphorase and calbindin and looked for double labeled cells (not illustrated). In four sections spaced 0.6 mm apart, we counted 130 parabrachial cells positive for calbindin, 560 positive for NADPH-diaphorase, and only one double labeled for both neuroactive substances. Thus, although cholinergic cells in the basal forebrain and parabrachial region are alike in their possession of NADPH-diaphorase, only the basal forebrain cell population also contains calbindin. Finally, we also treated sections through the parabrachial region for parvalbumin, and, although we saw some labeled fibers in this region, we found not a single parabrachial cell labeled for parvalbumin (not illustrated).

Nature of GABAergic cells and their projections in the basal forebrain

Because we found that the basal forebrain does project to the visual thalamic reticular nucleus and that its cells of origin are primarily or exclusively noncholinergic, we next examined whether this projection uses GABA as a neurotransmitter.

Identification of GABAergic cells of the basal forebrain. To identify GABAergic cells of the basal forebrain, we first used an antibody directed towards GABA. Although this antibody produced excellent cellular staining in nearby areas such as the putamen, entopeduncular nucleus, and thalamic reticular nucleus, we saw very light cellular staining in the nucleus basalis or globus pallidus. Such light staining is hard to interpret, and perhaps we could have enhanced it with prior colchicine treatment. However, we instead examined staining with an antibody directed against the 67 KD isoform of GAD, the synthesizing enzyme for GABA. As illustrated in Figure 15, this antibody darkly stained terminals, but it only lightly stained cell somata in the areas of the globus pallidus and nucleus basalis. Although this light cellular staining was adequate in isolation to identify GABAergic neurons, when combined with retrograde labeling, it was often too light to permit confident identification of double labeling. Identification of double-labeled cells was also made difficult, because the intensely labeled terminals, previously termed wooly fibers (Haber and Nauta, 1983), densely surround most cells in this area, and these obscured the outer borders of the neurons.

To overcome this problem, we sought another means of identifying the GABAergic cells of the basal forebrain. Our

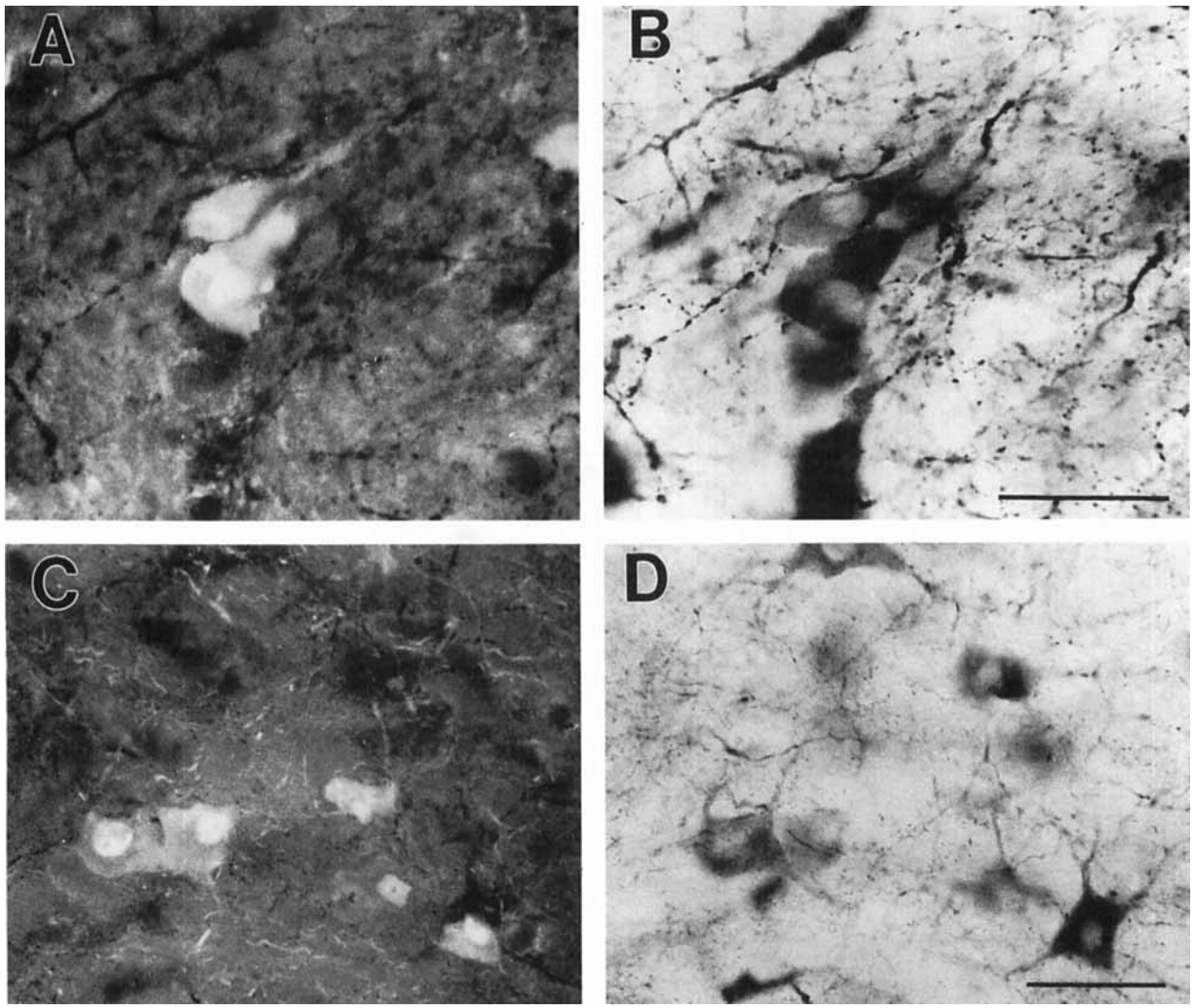


Fig. 8. Basal forebrain cells labeled for calbindin with fluorescein, on the left (**A,C**; photographed with epifluorescent blue light), with the same fields stained for NADPH-diaphorase, on the right (**B,D**; photographed with transmitted white light). Most cells contain both labels. Scale bars = 50 μm; bar in B also applies to A, bar in D also applies to C.

first candidate was an antibody directed against the calcium-binding protein parvalbumin, which has been shown to stain GABAergic neurons in many areas of the brain (Celio, 1990). Our goal here was to determine if parvalbumin also labeled all or most GABAergic cells of the nucleus basalis in the cat, and, to test this, we combined GAD staining (tagged with either rhodamine or DAB-reacted HRP) with parvalbumin staining (tagged with fluorescein). We found that most or all GABAergic cells of the nucleus basalis indeed contain parvalbumin. Figure 16 illustrates a typical example: The cell here is stained both for GAD with rhodamine (Fig. 16A) and parvalbumin with fluorescein (Fig. 16B). Figure 17 shows another cell labeled both with fluorescein-tagged parvalbumin (Fig. 17A) and with GAD visualized in this case with DAB-reacted HRP (Fig. 17B). Figure 18 shows an example of the distribution of cells through this area labeled with GAD, parvalbumin, or both, and such labeling was seen in the putamen as well as the nucleus basalis and globus pallidus. Overall, we sampled 302 labeled cells in the nucleus basalis (from five sections spaced 0.6 mm apart in one cat). We found that, of these basal forebrain cells, 238 of 258 (92%) positive for parvalbumin also contained GAD and that 238 of 282 (84%) GAD-containing cells stained positively for parvalbumin. A somewhat smaller percentage of the GAD-positive cells also contained parvalbumin than the converse, because the penetration of the GAD antibody was greater than the penetration of the parvalbumin antibody. In any case, because two antibodies were used, each with a finite but unknown penetration limitation, these estimates of double labeling should be viewed as minimal estimates. It thus seems reasonable to conclude that parvalbumin can be used to identify the GABAergic cells of the basal forebrain with minimal error.

We found that none of the parvalbumin-containing cells in the basal forebrain or putamen also stains positively for NADPH-diaphorase. Figure 19 shows examples of this, Figure 19A–D for the nucleus basalis and Figure 19E,F for

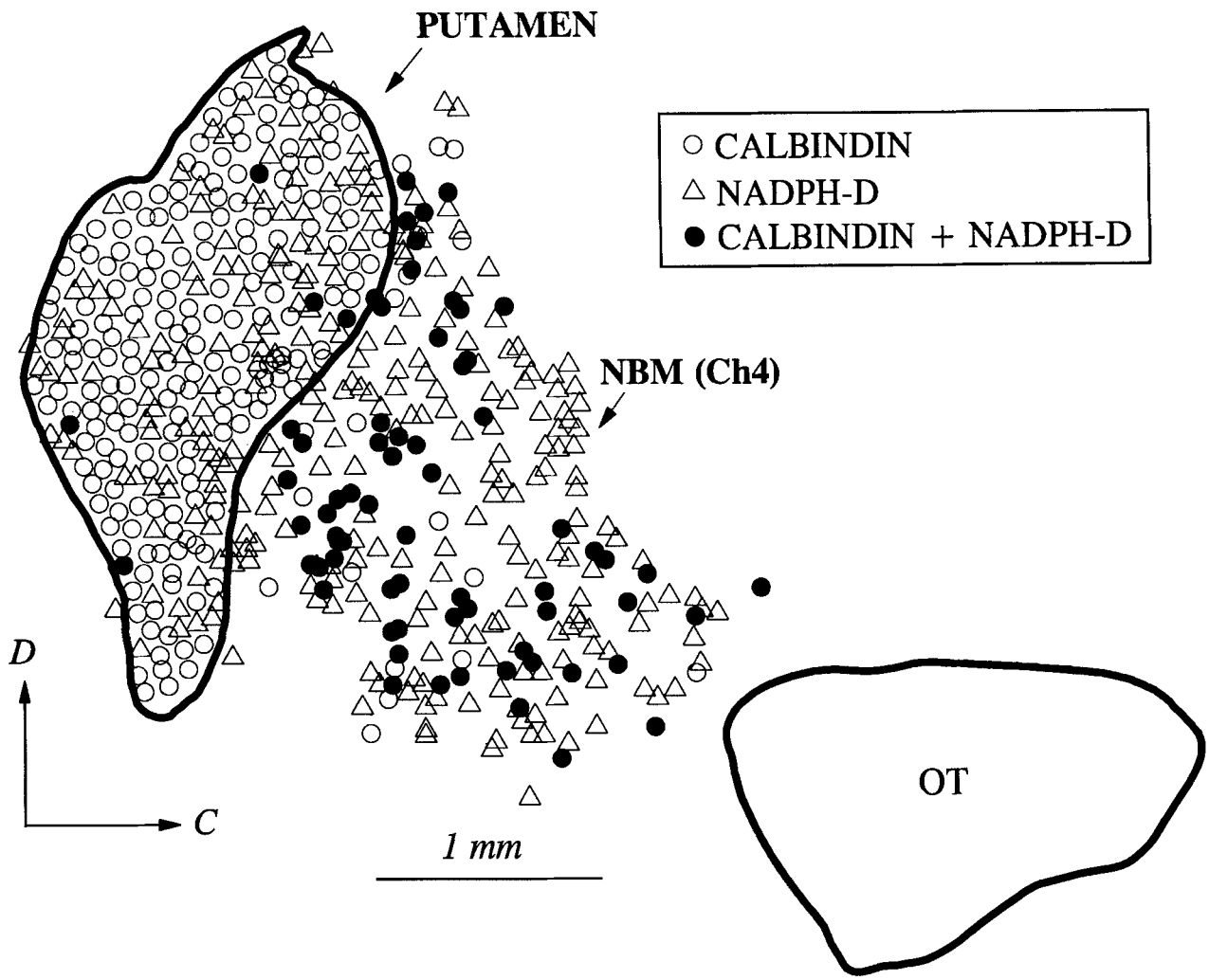


Fig. 9. Plot of single- and double-labeled cells stained for calbindin and NADPH-diaphorase in a parasagittal section through the basal forebrain. Most of the cells in the nucleus basalis that are positive for calbindin also contain NADPH-diaphorase. Few of the calbindin-positive cells in the putamen contain NADPH-diaphorase.

the putamen. Figure 20 shows the distribution of cells labeled for parvalbumin or NADPH-diaphorase in a more medial section through the globus pallidus. At this medial level, the NADPH-diaphorase-containing cells of the nucleus basalis form a ring around the globus pallidus. We conclude that at least two distinct populations of basal forebrain neurons can be identified: a cholinergic population that also contains NADPH-diaphorase and calbindin and a GABAergic population that also contains parvalbumin.

Further evidence that these two cell populations are distinct can be gleaned from their different soma sizes. Figure 21 shows the size distributions of 100 parvalbumin-positive and 100 NADPH-diaphorase-positive cells in the nucleus basalis. These were obtained from two adjacent series of sections stained for one or the other substance; within each series, the sections were spaced 0.3 mm apart. The cells containing NADPH-diaphorase are significantly larger, on average, with a mean and standard deviation of 794.2 ± 180.2 μm^2 vs. 374.6 ± 155.5 μm^2 ($P < 0.001$ by a Mann-Whitney U test).

Projection of GABAergic cells from the basal forebrain. We used parvalbumin as a marker for GABAergic cells of the basal forebrain in order to determine the extent to which they innervate the visual thalamic reticular nucleus. The projection, as for the cholinergic cells, was determined by retrograde transport of WGA-HRP injected into the visual thalamic reticular nucleus. We found that the TMB reaction product for retrograde transport combined with immunocytochemistry for parvalbumin readily revealed double labeled cells. Figure 22 shows two examples of such double labeling, and Figure 23 illustrates the distribution of basal forebrain cells that project to the visual thalamic reticular nucleus plus the subset that clearly contain parvalbumin. We found that 68 of 79 (86%) retrogradely labeled basal forebrain cells (from 27 sections spaced 0.3 mm apart in one cat) contain parvalbumin. Given the limited penetration of the parvalbumin antibody, these results suggests that most, if not all, of the projection from the basal forebrain to the visual thalamic reticular nucleus emanates from GABAergic cells.

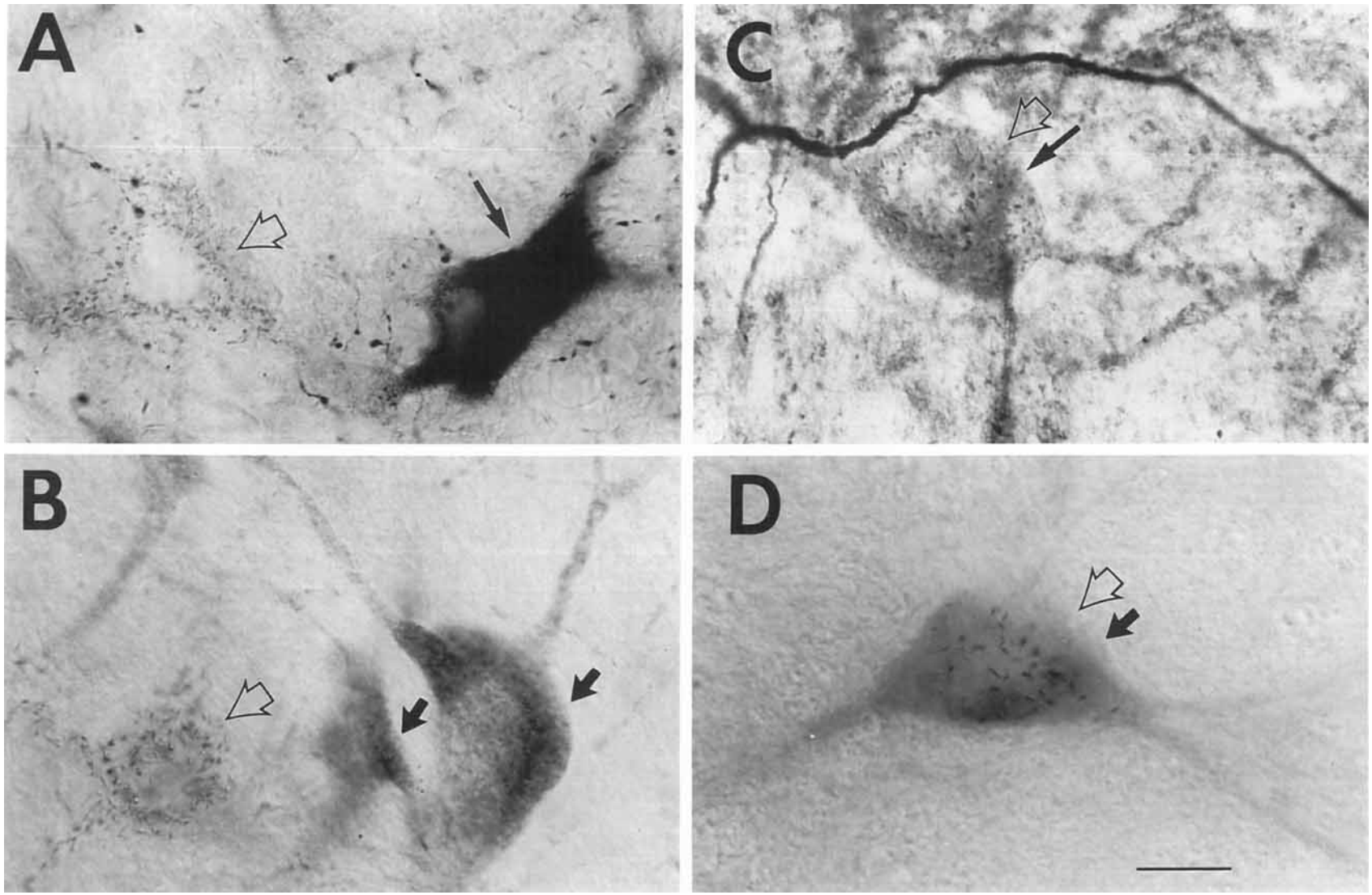


Fig. 10. Cells retrogradely labeled with TMB/DAB reacted HRP and subsequently stained with NADPH-diaphorase or ChAT. **A,B:** Single-labeled cells in the basal forebrain. Some cells were retrogradely labeled following a WGA-HRP injection into the visual thalamic reticular nucleus, and others were single labeled with either NADPH-diaphorase (A) or ChAT (B). Open arrows indicate retrogradely labeled cells, and solid arrows indicate cells stained for NADPH-diaphorase or ChAT. **C:** Double-labeled cell (open and solid arrows) in the basal forebrain. The cell was retrogradely labeled following an injection of HRP into the visual cortex, and it also stained positively for NADPH-diaphorase. **D:** Double-labeled cell (open and solid arrows) in the parabrachial region of the brainstem that was retrogradely labeled following an injection of WGA-HRP in the visual thalamic reticular nucleus and stained for ChAT. Scale bar = 50 μm.

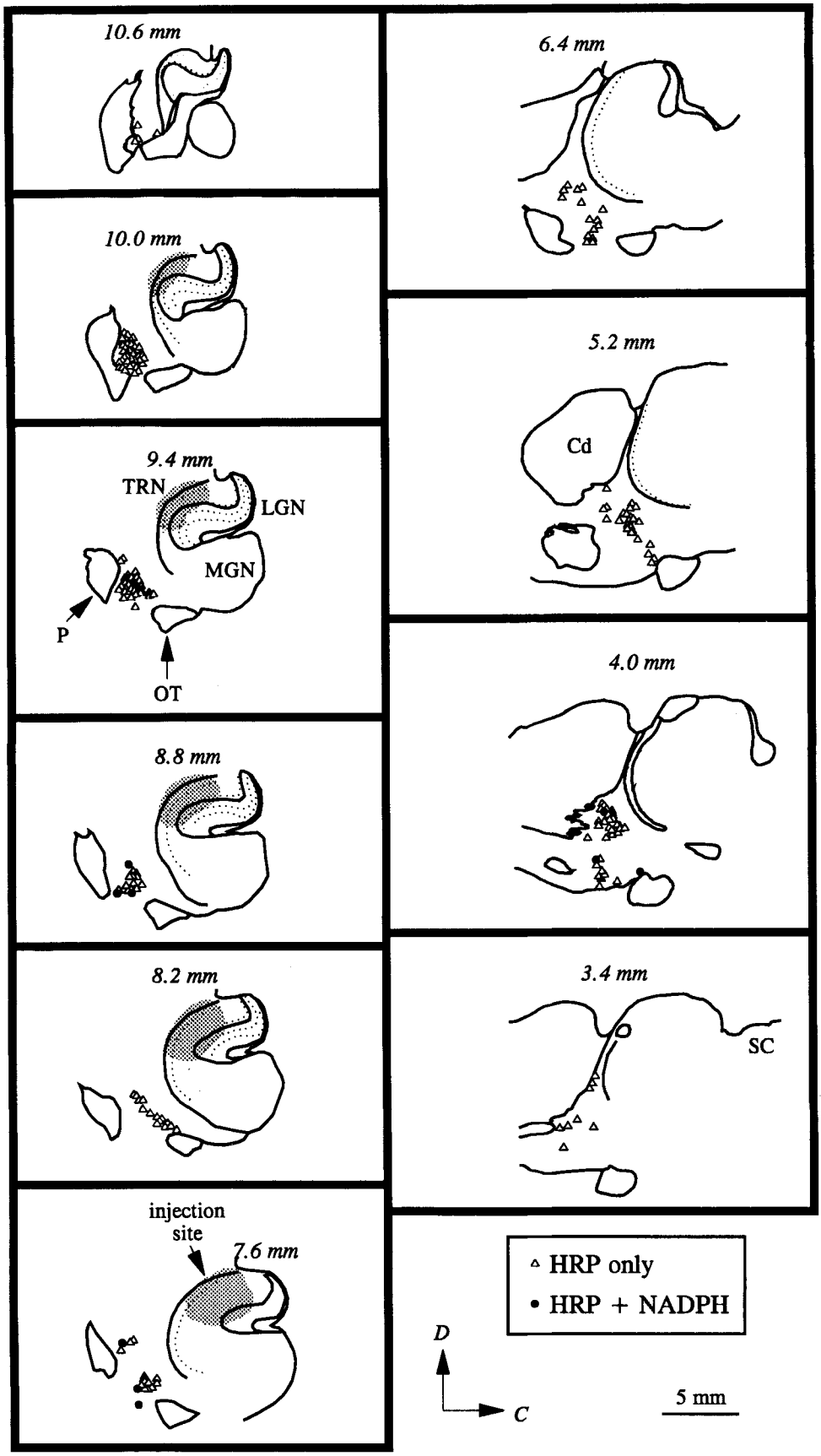
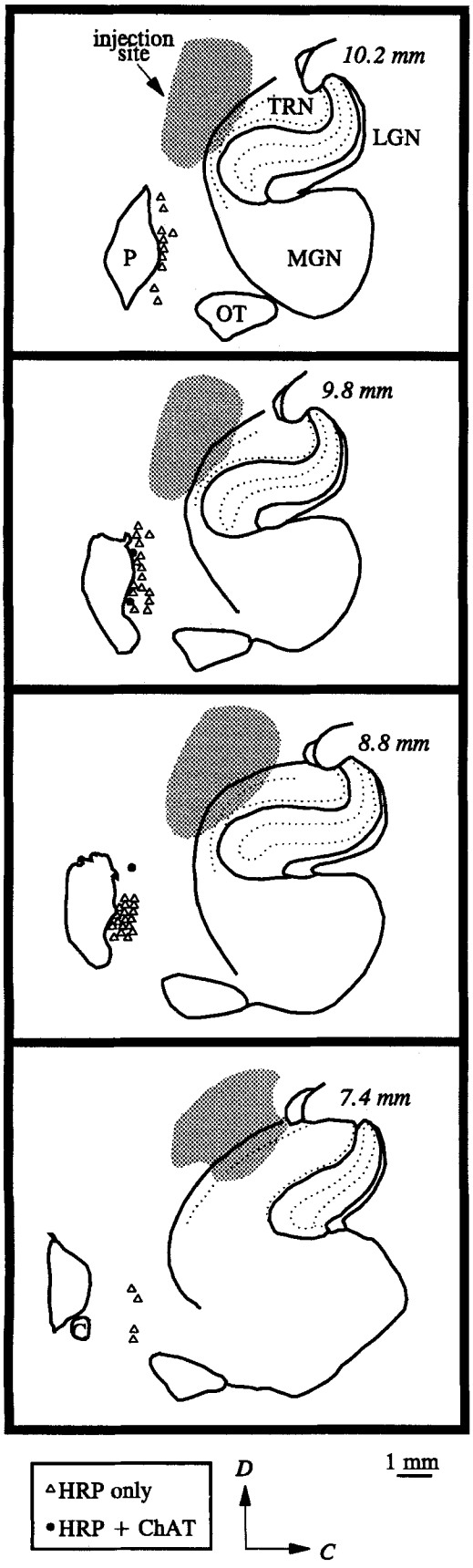


Fig. 11. Plot of cells in the basal forebrain retrogradely labeled from an injection of WGA-HRP into the visual thalamic reticular nucleus (injection site). A series of ten parasagittal sections is illustrated. Distance from the midline is indicated above each section. Retrogradely labeled cells are shown, including the subset that also contains NADPH-diaphorase.



Electron microscopic observations of labeled basal forebrain terminals in the visual thalamic reticular nucleus

We used electron microscopy to obtain more complete proof that axons from the basal forebrain actually form contacts onto cells of the visual thalamic reticular nucleus. Terminals from these axons were labeled anterogradely from biocytin injected into the basal forebrain (see above and Figs. 3, 4). We found in our sample that all but one of the 27 labeled terminals contacted dendrites of thalamic reticular neurons, and the remaining terminal contacted a soma. To minimize the distortion caused by oblique sectioning of dendrites, which we assumed to be roughly circular in cross section, we measured the minimum path across the dendritic profile in our sections to estimate dendritic diameter. By these measures, the diameter of the postsynaptic dendrites varied considerably, with a mean and standard deviation of 1.06 ± 0.52 μm and a range of 0.18–2.33 μm. This suggests that both proximal and distal dendrites received innervation from the basal forebrain.

In keeping with the evidence described above that most or all of the basal forebrain cells making up this pathway are GABAergic, the synaptic contacts formed by their terminals had modest postsynaptic densities. Thus, the synapses formed were mostly symmetric in appearance, which is to be expected for synaptic contacts from GABAergic terminals. To demonstrate more directly that many of these terminals are GABAergic, we employed postembedding immunocytochemistry for GABA in thin sections through the visual thalamic reticular nucleus, choosing regions that contained terminals labeled anterogradely with biocytin from the basal forebrain. Gold particles were used to visualize the anti-GABA antibody in synaptic terminals (see Materials and Methods). We analyzed the biocytin-labeled terminals for the added presence of these gold particles. Figure 24 shows examples of this double labeling.

To place identification of double labeling on a more quantitative footing, we determined the density of the gold particles found within all of the 64 biocytin-labeled terminals in two thin sections. Section 1 was incubated in the primary antibody for 18 hours, and section 2 was incubated in the primary antibody for 42 hours. As a control from the same thin sections, we also measured the particle density in a sample of terminals that were not labeled with biocytin. For the unlabeled control population, we chose RSD terminals (see Fig. 24D), so named because they have round vesicles, small profiles, and dark mitochondria and form asymmetric contacts (Guillery, 1969a,b; Ide, 1982). RSD terminals may derive from brainstem axons, from geniculocortical axons, or from corticogeniculate axons (Jones and Powell, 1969; Roberston and Rinvik, 1973; Ohara and Lieberman, 1981; Robson, 1983; de Lima et al., 1985; Van Horn et al., 1987; Weber and Kalil, 1987; Cucchiaro et al., 1988; Montero, 1989; Raczkowski and Fitzpatrick, 1989). RSD terminal morphology is quite distinct from that usually associated with GABAergic terminals, which are thought to contain flattened or pleomorphic vesicles and form symmetrical synaptic contacts. RSD terminals have

Fig. 12. Plot of cells in the basal forebrain retrogradely labeled from the visual thalamic reticular nucleus that also contain ChAT; conventions as in Figure 11 for the series of four parasagittal sections.

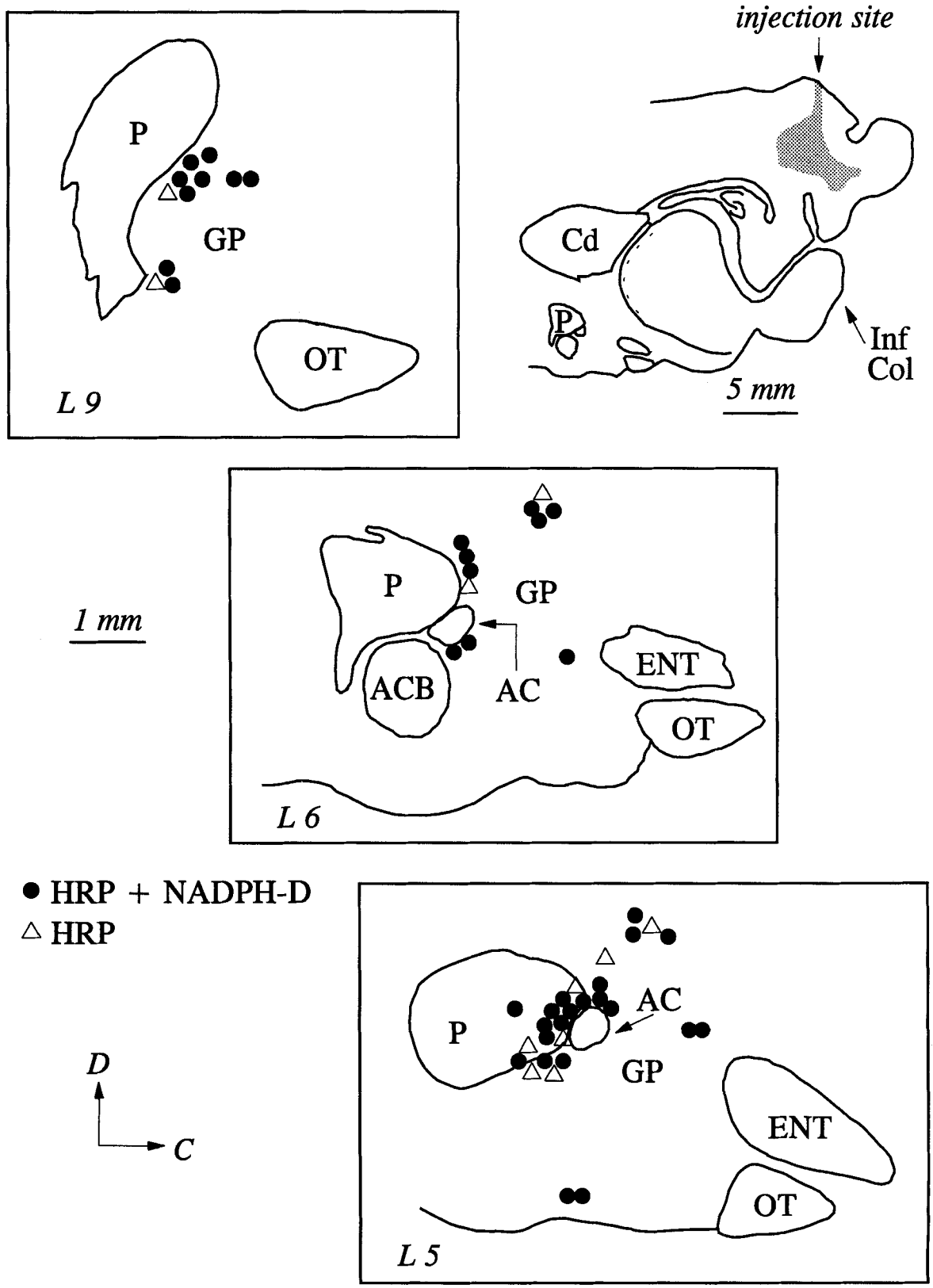


Fig. 13. Distribution of cells in a series of three parasagittal sections through the basal forebrain that are retrogradely labeled following an injection of HRP into the visual cortex (injection site) including the subset positive for NADPH-diaphorase.

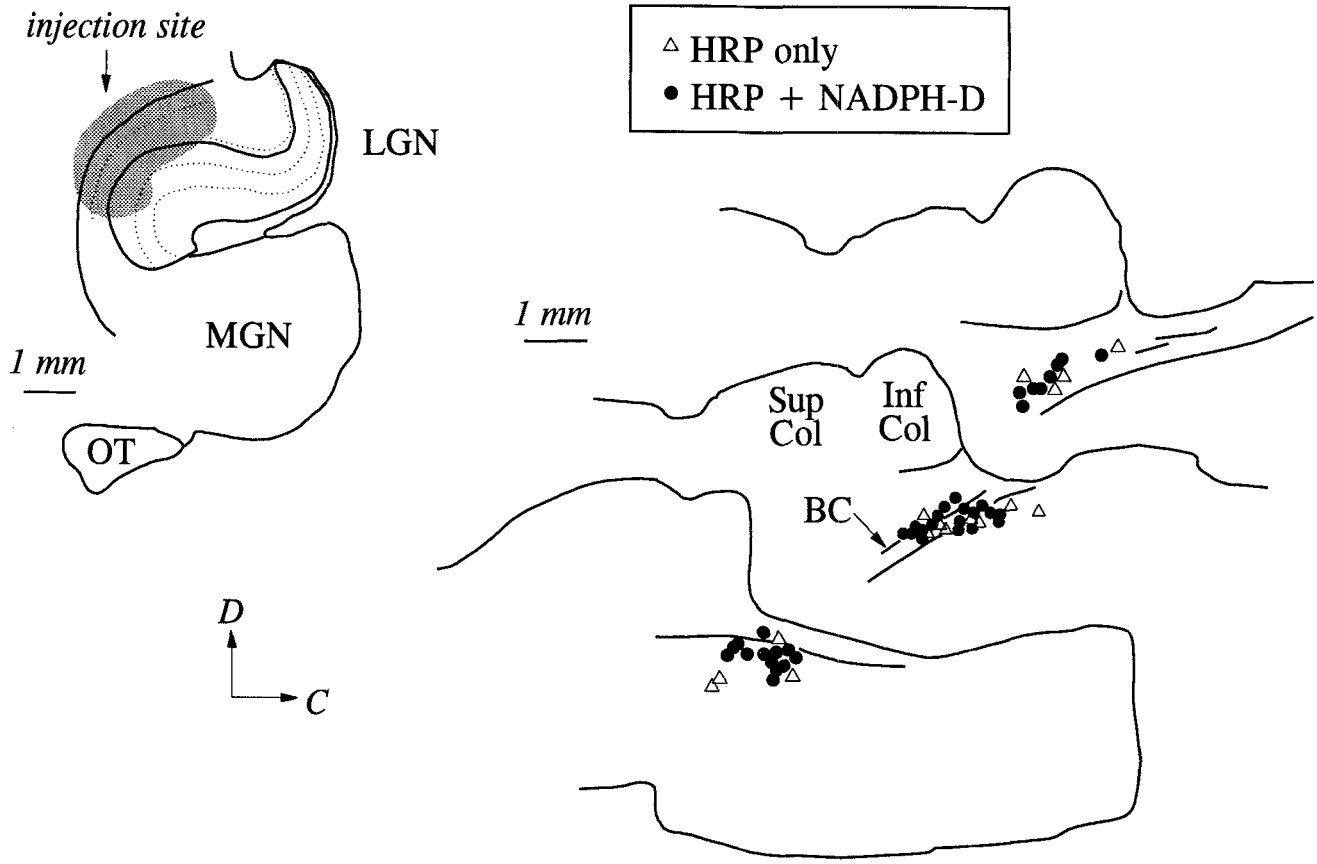


Fig. 14. Distribution of cells in a series of three parasagittal sections through the parabrachial region that are retrogradely labeled following an injection of HRP into the visual thalamic reticular nucleus (injection site) including the subset positive for NADPH-diaphorase positive. This is the same case as illustrated in Figure 11.

been shown to be GABA and GAD negative (Montero and Singer, 1984; De Biasi et al., 1988; Rinvik and Ottersen, 1988), and part of this population stains intensely for glutamate (Rinvik and Ottersen, 1988; Montero, 1990). Thus, the unlabeled RSD population serves as a baseline against which labeling positive for GABA can be detected. For each section, we collected a montage of photographs from a 3,060 μm^2 area and measured the gold particle density in unlabeled RSD terminals. This was compared to the density of gold particle in all the biocytin-labeled terminals within the section.

Figure 25 summarizes this analysis. The RSD terminals (72 from section 1 and 62 from section 2) indeed show a low density of GABA labeling, and many of the terminals identified as emanating from the basal forebrain exhibit GABA labeling well above background. Overall, the density of GABA labeling is significantly greater in the basal forebrain terminals than in the RSD terminals ($P < 0.001$ by a Mann-Whitney U test for each section). To estimate the percentage of biocytin-labeled terminals that are positive for GABA in each section, we adopted a 95% confidence level as follows: The density level below which are found $\geq 95\%$ of the RSD population is taken as the labeling threshold, and all GABA labeling above this level is considered positive. This threshold level for section 1 is 15.7 grains/μm^2, and 15 of the 22 (68%) biocytin-labeled terminals are deemed GABAergic. For section 2, the threshold is 13.7 grains/μm^2, which is exceeded by 34 of the 42 (81%) biocytin-labeled terminals. It is worth noting that section 2 was incubated in the antibody longer than was section 1, and, although this did not produce more GABA labeling for the RSD terminals ($P > 0.1$ by a Mann-Whitney U test), it did lead to more labeling for the biocytin-labeled terminals ($P < 0.01$ by a Mann-Whitney U test). This may mean that even longer incubation periods would have pulled more of the biocytin-labeled terminals out of the baseline GABA labeling represented by the RSD terminals.

DISCUSSION

Summary

Our results, which describe relationships between the basal forebrain and thalamic plus cortical regions of the visual system, are summarized in Figure 1. Our general conclusion is that at least two types of projection cell exist in the basal forebrain. One is GABAergic and also contains the calcium-binding protein parvalbumin. It projects not to visual cortex or the lateral geniculate nucleus but only to the thalamic reticular nucleus. This is the only known input to the visual thalamic reticular nucleus that does not also innervate the lateral geniculate nucleus; it is thus

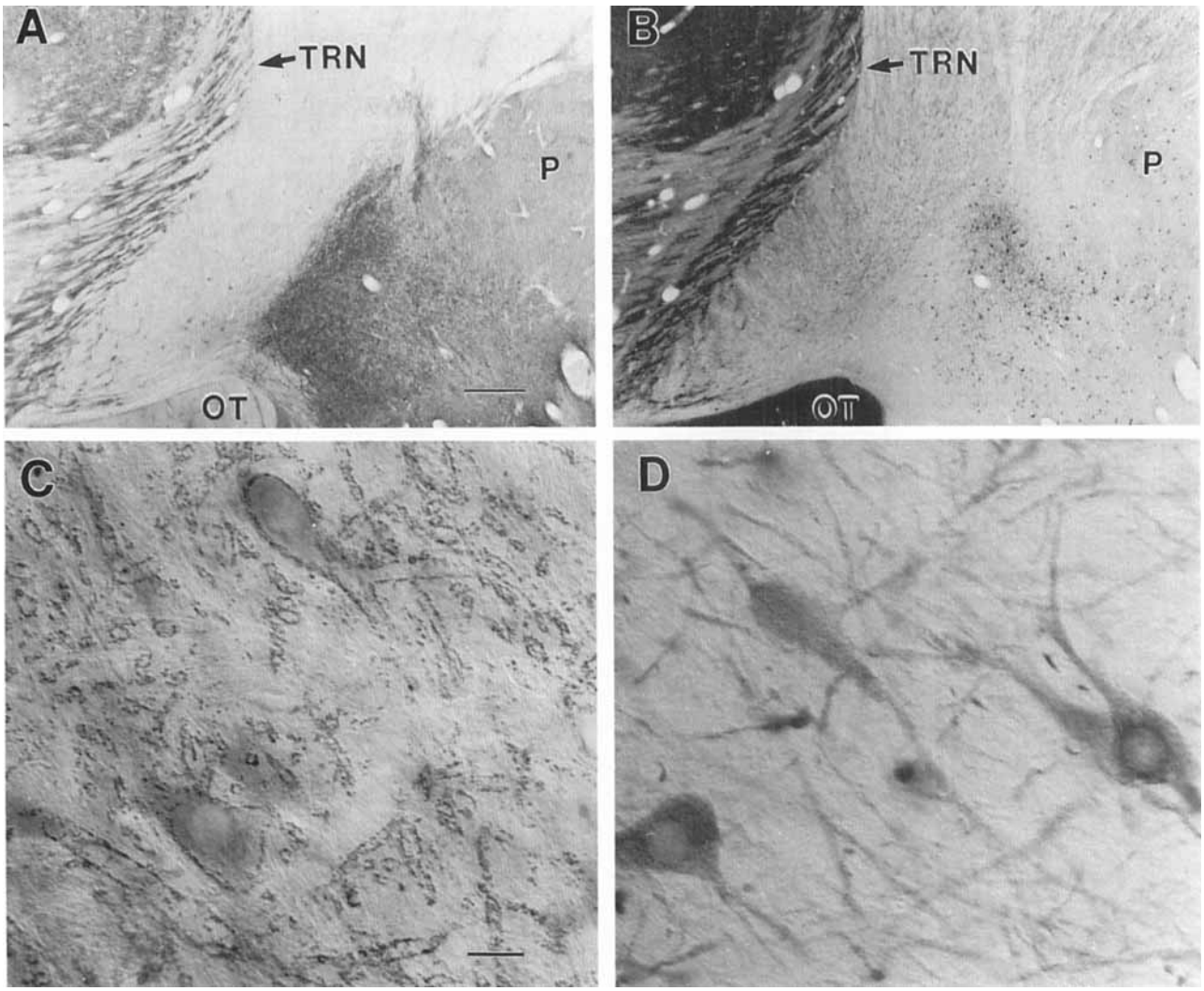


Fig. 15. GAD and parvalbumin staining in the basal forebrain. **A:** Lower power view of a parasagittal section through the basal forebrain stained for GAD. Dense terminal staining is seen within the nucleus basalis and globus pallidus. **B:** Section similar to that shown in A stained for parvalbumin. Many parvalbumin-positive cells are seen within the nucleus basalis and globus pallidus. **C:** Higher power view of GAD positive cells and terminals in the basal forebrain. Lightly stained cells are surrounded by darkly stained terminals. **D:** Section similar to that shown in C stained for parvalbumin. Somata and dendrites are darkly stained for parvalbumin. Scale bars = 1 mm in A, which also applies to B, 20 μm in C, which also applies to D.

comparable to the retinal input to the lateral geniculate nucleus, because the retina provides the only known input to the lateral geniculate nucleus that does not also innervate the visual thalamic reticular nucleus. The other basal forebrain projection cell type is cholinergic, and it also contains NADPH-diaphorase, an enzyme involved in the production of nitric oxide, and calbindin, a calcium-binding protein. It does not project to the thalamic reticular nucleus or lateral geniculate nucleus but instead innervates visual cortex. The cholinergic cells are, on average, considerably larger than are the GABAergic cells. Finally, we conclude that the parabrachial region of the brainstem provides the sole source of cholinergic inputs to the visual thalamic reticular nucleus, and these inputs also contain NADPH-diaphorase. Although our observations are clearly limited to the visual pathways, it is tempting to extrapolate these observations more generally to basal forebrain, thalamus, and cortex.

Nomenclature

The basal forebrain cells that project to the visual thalamic reticular nucleus are interspersed among the large, cortically projecting cholinergic cells of the nucleus basalis. However, virtually all of the smaller thalamic reticular nucleus projecting cells are GABAergic (see below), like the adjacent cells of the globus pallidus. What is the proper nomenclature for these projection neurons?

Previous studies have used different terms to describe the cells of origin of this projection (Nauta, 1979; Levey et al., 1987; Steriade et al., 1987; Asanuma, 1989; Jones and Cuello, 1989; Jourdain et al., 1989; Asanuma and Porter, 1990; Cornwall et al., 1990; Hazrati and Parent, 1991b; Chen and Bentivoglio, 1993). Given the location amidst the globus pallidus of the GABAergic cells that innervate the visual thalamic reticular nucleus, one might consider this cell group to be part of the globus pallidus. However,

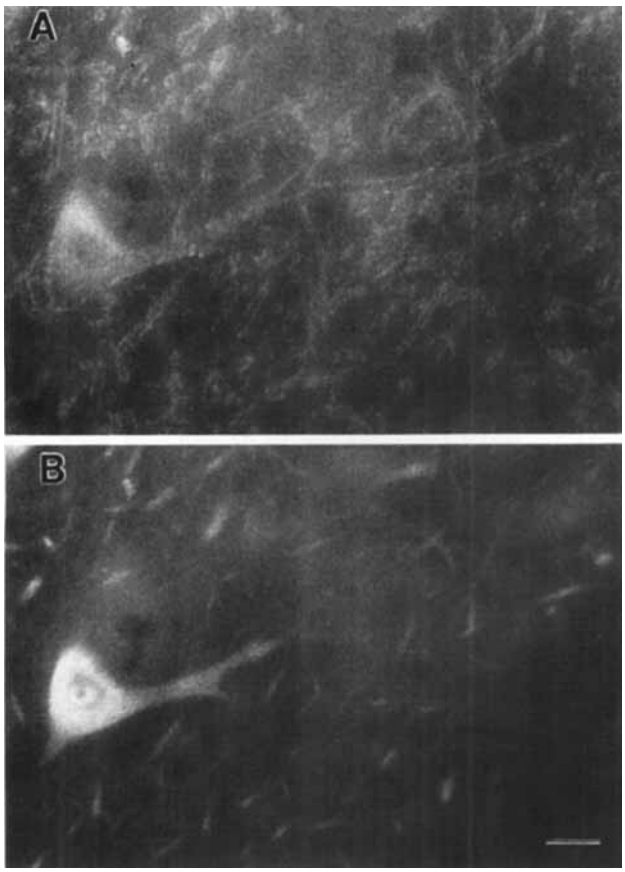


Fig. 16. Basal forebrain cell double labeled for GAD and parvalbumin. **A:** Staining for GAD with rhodamine (photographed with epifluorescent green light). **B:** Staining for parvalbumin with fluorescein (photographed with epifluorescent blue light). Scale bar = 20 μm.

Asanuma and Porter (1990) have recently argued that basal forebrain cells in the rat that project to the thalamic reticular nucleus are more related to the cholinergic system based both on their location within the nucleus basalis as well as on their projections, which are separate from the more ventral pallidothalamic fiber tract. Our study agrees with these observations. Of particular importance is the observation from our material that cholinergic cells projecting to visual cortex are interspersed with GABAergic cells projecting to the visual thalamic reticular nucleus. This raises the possibility that some afferents to this region of the basal forebrain may innervate both the cholinergic and GABAergic projection cells. In any case, we favor Asanuma and Porter's (1990) resolution of this question and consider the nucleus basalis magnocellularis to be composed of at least two subsets that include the cholinergic cells (Ch4), which project to the cortex, and the GABAergic cells, which project to the thalamic reticular nucleus. Although this may be regarded as a question of semantics, we think it sensible to refer to both cell groups as part of nucleus basalis. Further resolution of this matter will await more detailed anatomical or physiological studies to determine whether the inputs and response properties of the GABAergic projection cells of the basal forebrain are more similar to the Ch4 cells or to the cells of the globus pallidus.

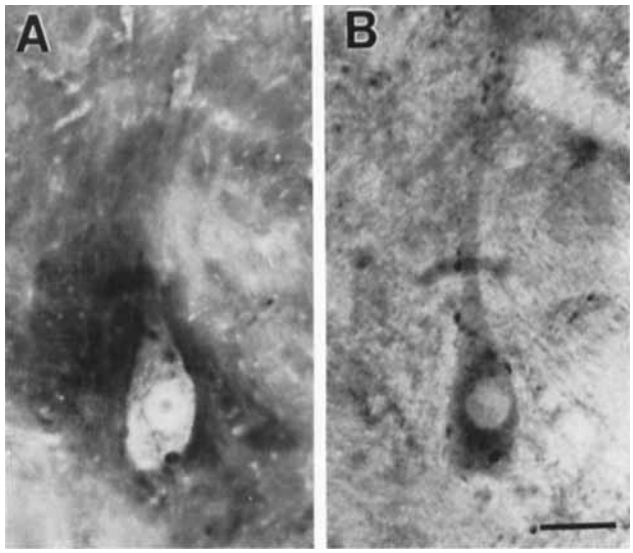


Fig. 17. Basal forebrain cell double labeled for GAD and parvalbumin. **A:** Staining for parvalbumin with fluorescein (photographed with epifluorescent blue light). **B:** Staining for GAD with DAB reacted HRP (photographed with transmitted white light). Scale bar = 20 μm.

Qualifications to the main conclusions

Figure 1 is obviously a simplified schema. It is important to emphasize that, although the majority of our observations support it, some do not. It seems worthwhile to consider each of the conclusions embodied by Figure 1 in more detail.

Projections of the basal forebrain. Retrograde tracing experiments make it quite clear that the basal forebrain projects to both visual cortex and the region of the visual thalamus. Because most WGA-HRP injections centered on the thalamic reticular nucleus spilled over slightly into the lateral geniculate nucleus (although not all did), and because of the possibility of fibers passing through the thalamic reticular nucleus to other targets (although all known to pass through do innervate cells there via collaterals) picking up the label, it is hard from retrograde tracing alone to determine the specificity of the projection from the basal forebrain to the visual thalamus. However, our orthograde tracing makes reasonably clear the conclusion that the basal forebrain innervates all of the visual thalamic reticular nucleus but not the lateral geniculate nucleus. Orthograde tracing also enabled us to comment on the nature of terminal arbors from the basal forebrain in the visual thalamic reticular nucleus: Terminal boutons are located en passant, and they form symmetrical contacts, mostly onto dendrites of cells in the thalamic reticular nucleus.

Colocalization of neuroactive substances. We found that the vast majority (88%) of basal forebrain cells labeled for ChAT also contained NADPH-diaphorase. Likewise, 76% of the calbindin-labeled cells contained NADPH-diaphorase. False negatives are clearly a possibility in such experiments, meaning that the majority of the cholinergic cells, perhaps even 100%, also contain both calbindin and NADPH-diaphorase, but our methods were not sufficiently sensitive to see this in every case. In double-labeling experiments, one label (e.g., NADPH-diaphorase) may partially obscure the other (e.g., ChAT or calbindin), and we

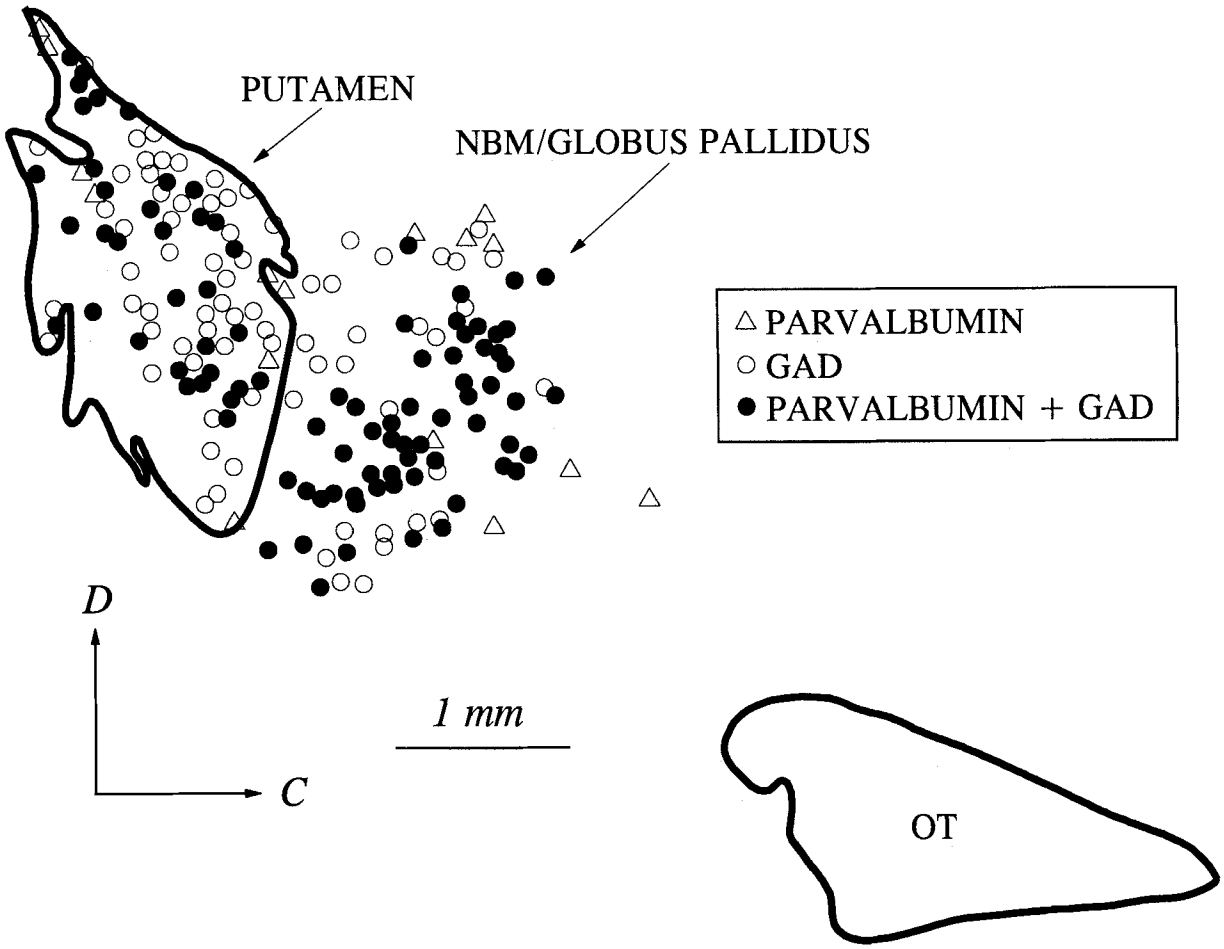


Fig. 18. Plot of cells stained for GAD and parvalbumin in a parasagittal section through the basal forebrain, including the putamen and globus pallidus. Most parvalbumin-positive cells also contained GAD.

took care to consider a cell to be double labeled only when both labels were quite clear. If one was questionable, we counted it as single labeled. Although it seems reasonable to conclude that practically every cholinergic cell in the basal forebrain also contains calbindin and NADPH-diaphorase, we certainly cannot rule out the possibility that a minority do not contain all three neuroactive substances, which implies that two or more types of cholinergic cells may exist in the basal forebrain.

A similar argument holds for our conclusion that GABAergic basal forebrain cells also contain parvalbumin. False negatives for double labeling with two antibodies (GAD and parvalbumin) due to limited penetration are especially likely. Thus, our findings that 92% of the parvalbumin containing cells contain GAD and 84% of the GAD containing cells contain parvalbumin are both almost certainly underestimates. It seems safe to conclude that practically all GABAergic cells in the basal forebrain also contain parvalbumin, and vice versa.

Projection of cholinergic cells from the basal forebrain. We found that 71% of the basal forebrain cells retrogradely labeled from HRP placed into the visual cortex also contain NADPH-diaphorase. The possibility exists that dense HRP labeling in some of these cells obscures clear verification of NADPH-diaphorase that might be present. Thus, the actual fraction of projection neurons positive for NADPH-diaphorase might be somewhat higher. We assume that the NADPH-diaphorase staining also identifies cholinergic cells, and provisos to this are noted above. Thus, most, and perhaps all, of the basal forebrain projection to visual cortex emanates from cholinergic cells.

We found very little evidence to support the conclusion that cholinergic cells from the basal forebrain innervate the thalamic reticular nucleus. Again, we used double labeling of retrogradely transported WGA-HRP and NADPH-diaphorase or ChAT. Only after the largest injections of WGA-HRP centered on the visual thalamic reticular nucleus did we see any retrogradely labeled cells positive for NADPH-diaphorase or ChAT, and these represented only about 5–10% of the retrogradely labeled cells. After smaller injections more clearly limited to the thalamic reticular nucleus, none of the retrogradely labeled cells stained positively for NADPH-diaphorase or ChAT. We conclude that virtually none of the cholinergic cells in the basal forebrain innervates the visual sector of the thalamic reticular nucleus; the few seen after larger injections that extend beyond the thalamic reticular nucleus probably label fibers of passage en route to other targets, such as visual cortex. Cholinergic cells containing NADPH-diaphorase from the parabrachial region do innervate the visual sector

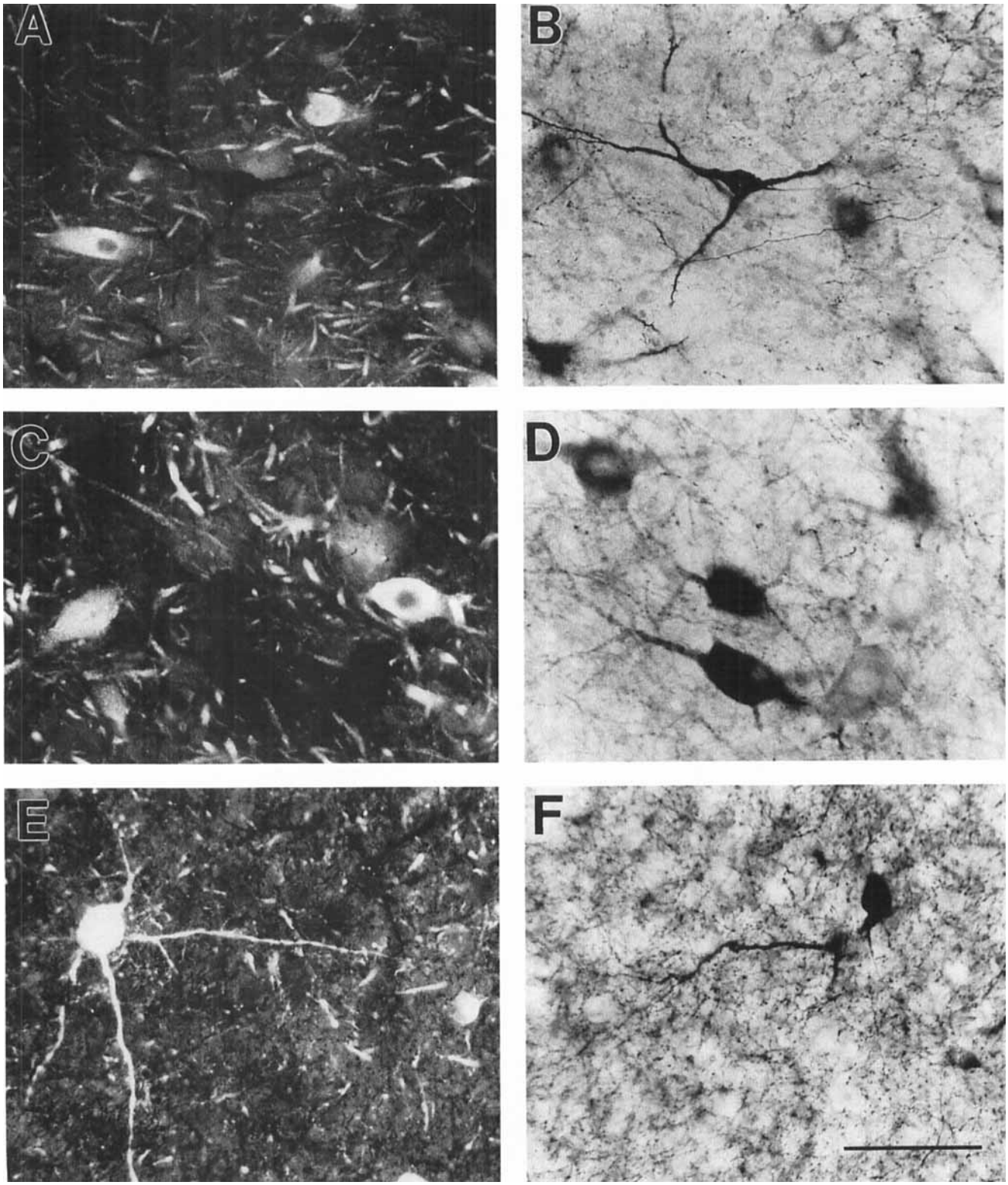


Fig. 19. Nucleus basalis and putamen cells stained for parvalbumin or NADPH-diaphorase. No cells in either structure contain both labels. Parvalbumin staining with fluorescein is shown on the left (**A,C,E**; photographed with epifluorescent blue light), and the same fields stained for NADPH-diaphorase are shown on the right (**B,D,F**; photographed with transmitted white light). A–D: Cells in the peripallidal nucleus basalis. E,F: Cells in the putamen. Scale bar = 50 μm.

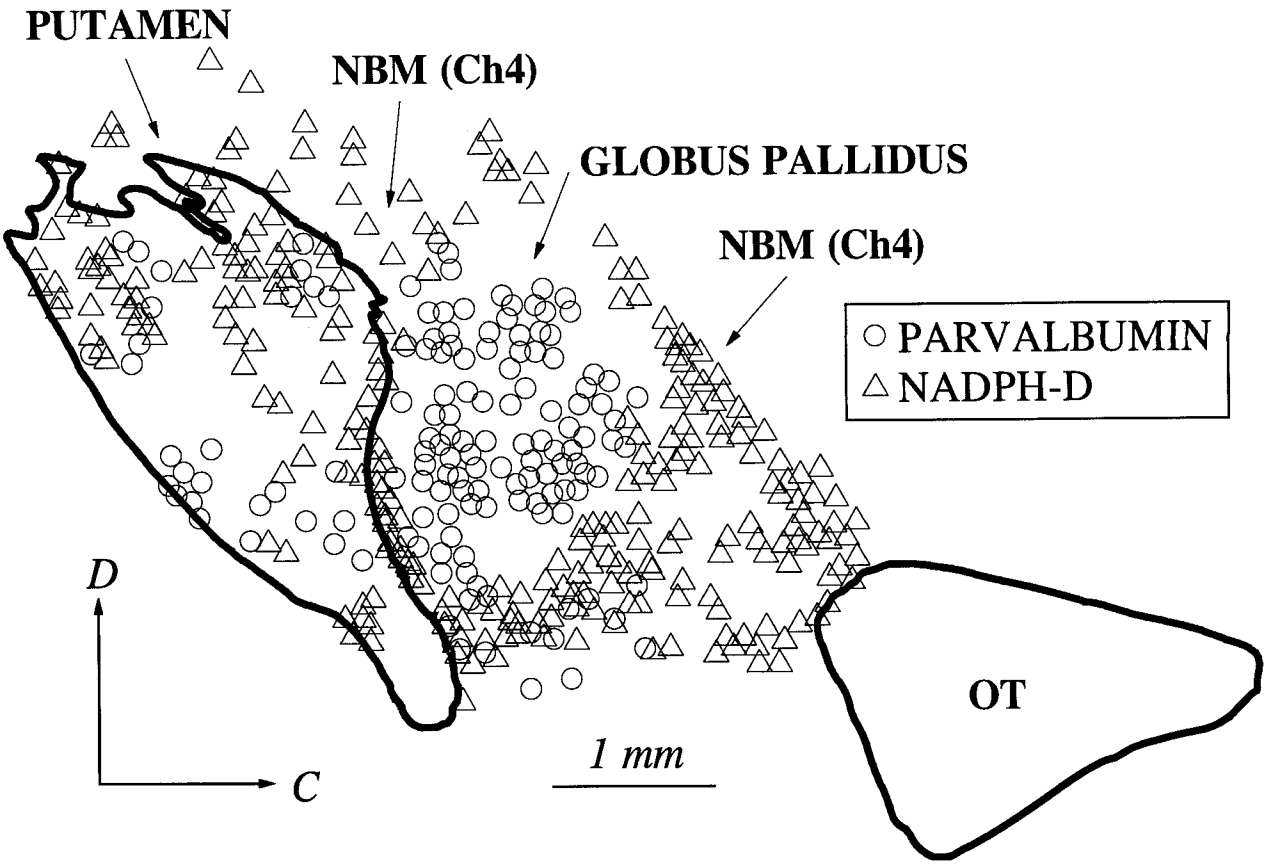


Fig. 20. Plot of cells stained for NADPH-diaphorase and parvalbumin in a parasagittal section through the basal forebrain, including the putamen and globus pallidus. The NADPH-diaphorase-positive cells form a ring around the parvalbumin-positive cells of the globus pallidus. No cells were double labeled.

of the thalamic reticular nucleus, and it is plausible that these parabrachial axons provide the only cholinergic innervation to this structure (de Lima and Singer, 1987; Smith et al., 1988; Fitzpatrick et al., 1989; Bickford et al., 1993).

Projection of GABAergic cells from the basal forebrain. As was noted in Results, we resorted to using immunocytochemistry for parvalbumin as a marker for GABAergic cells in the basal forebrain, and we discuss provisos to this strategy above. Most of the basal forebrain cells labeled by retrograde transport of HRP from visual cortex contained NADPH-diaphorase; parvalbumin, which is found in GABAergic cells of the basal forebrain, is not found in cells containing NADPH-diaphorase. Taken together, these observations render the visual cortex an unlikely target for GABAergic cells of the basal forebrain. However, after WGA-HRP injections into the visual thalamic reticular nucleus, 86% of retrogradely labeled basal forebrain cells were deemed GABAergic due to their positive staining for parvalbumin. As was noted above, this is probably an underestimate.

Our electron microscopic studies using orthograde labeling with biocytin combined with postembedding immunocytochemistry for GABA confirm the conclusion that basal forebrain cells form synaptic contacts onto dendrites of thalamic reticular nucleus cells and that many of these contacts are GABAergic. This is an important observation for our main conclusion that GABAergic basal forebrain cells form synaptic contacts onto visual thalamic reticular nucleus cells.

However, we do not believe that this approach provides an accurate quantitative estimate of the relative number of parent cells forming this pathway. There are two reasons for this. First, although we found a number of terminals orthogradely labeled from basal forebrain that did not stain positively for GABA, many of these could well have been false-negative observations. It is interesting in this context that the section with a longer incubation time in the anti-GABA antibody had more of the terminals from basal forebrain clearly identified as positive for GABA (see Results). Second, perhaps many of the basal forebrain terminals negative for GABA indeed represent a non-GABAergic pathway. In this context, it was noted in a previous study in the rat that, although the majority of terminals in the thalamic reticular nucleus labeled from the basal forebrain formed symmetric contacts, a small number formed asymmetric contacts (Asanuma and Porter, 1990). However, there is no reason to assume that the number of terminals formed by a pathway correlates with the number of its parent cells, because this assumes that all cells form equal numbers of terminals. In other systems, we know that different cell types typically form different numbers of terminals. For instance, the smaller X cells of the retina form fewer terminals from their axons than do the larger Y cells (Sur et al., 1987). Indeed, the observation that GABAergic cells are among the smallest of the nucleus basalis (see Fig. 21) raises the distinct possibility that the relative number of GABAergic terminals they support in the visual thalamic reticular nucleus is relatively low. For this reason,

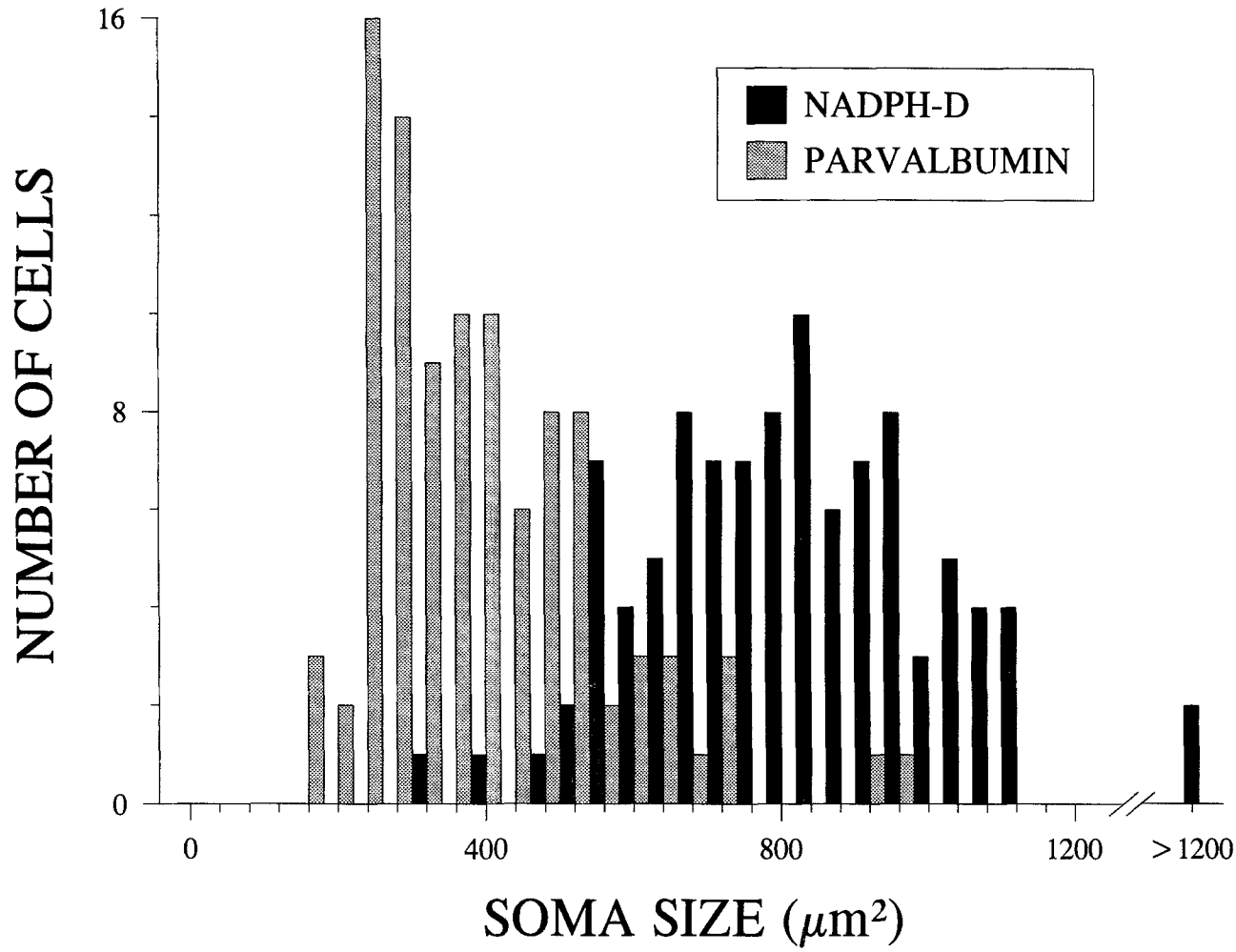


Fig. 21. Soma sizes of basal forebrain cells positive for parvalbumin or NADPH-diaphorase.

we depend more on retrograde labeling to obtain estimates of the relative number of these GABAergic cells that innervate the visual thalamic reticular nucleus.

Classes of projection cells in the basal forebrain. Our data clearly indicate that most basal forebrain cells projecting to visual cortex are cholinergic (and also contain calbindin and NADPH-diaphorase); conversely, most projecting to the visual thalamic reticular nucleus are GABAergic (and also contain parvalbumin), and few if any are cholinergic. However, in none of the double-labeling experiment in which retrograde labeling was combined with immunocytochemistry designed to detect cholinergic or GABAergic projection cells were all projection cells identified with regard to putative neurotransmitters. This raises the clear possibility that more than just cholinergic and GABAergic cells exist in the basal forebrain. A minority, for instance, may use an excitatory amino acid or some other neurotransmitter, and this deciding issue will require further experimental evidence.

Comparison with previous studies

Although our results conform to many observations of basal forebrain organization that have been previously reported, there are certain differences that deserve attention.

Neuroactive substances in basal forebrain cells. Certain previous observations seem at odds with our findings that the majority of the cholinergic cells of the cat's nucleus basalis contain NADPH-diaphorase. In the rat, NADPH-diaphorase labeling has been reported for cholinergic cells of the basal forebrain (Pasqualotto and Vincent, 1991; Kitchener and Diamond, 1993). However, the double-labeled cells in these studies were found primarily in the medial septum and the vertical and horizontal limbs of the diagonal band of Broca; few of the cholinergic cells of the caudal nucleus basalis contained NADPH. Further differences are seen in other species. For example, the cholinergic basal forebrain cells of the monkey, baboon, and human do not stain for NADPH-diaphorase (Geula et al., 1993). Staining in the basal forebrain for the calcium-binding protein calbindin also seems to vary across species. Our results in the cat are similar to those in the monkey, baboon, and human (Geula et al., 1993), where the cholinergic basal forebrain cells contain calbindin. This is unlike the situation in the rat, where these cholinergic cells do not stain for calbindin (Chang and Kuo, 1991; Geula et al., 1993).

Projections of basal forebrain cells. Previous studies have reported both cholinergic and GABAergic projections

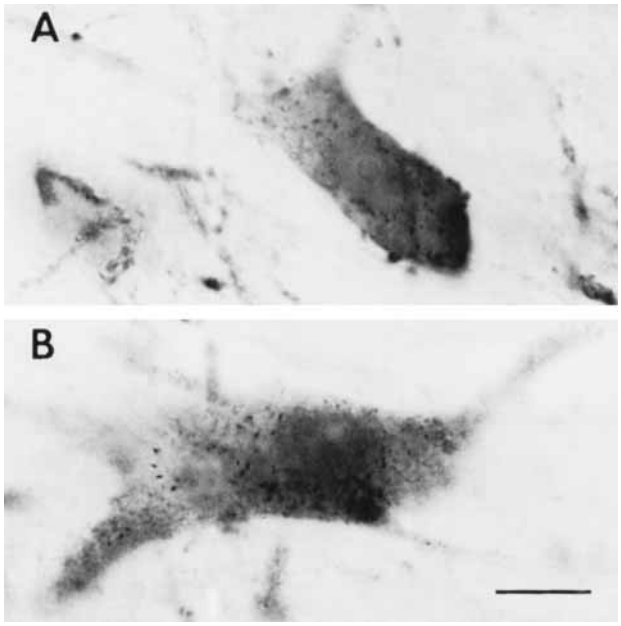


Fig. 22. **A,B:** Basal forebrain cells retrogradely labeled from the visual thalamic reticular nucleus and stained for parvalbumin. Scale bar = 20 μm.

from the basal forebrain to the thalamic reticular nucleus (Levey et al., 1987; Steriade et al., 1987; Jourdain et al., 1989; Asanuma and Porter, 1990; Chen and Bentivoglio, 1993). The percentage of retrogradely labeled cells that stain for ChAT or for GABAergic markers and their location seem to depend to some extent on the location of the injections within the thalamic reticular nucleus. For example, following an injection into the rostral pole of the thalamic reticular nucleus in the cat, Steriade et al. (1987) found that 17% of the retrogradely labeled cells stained for ChAT. We never saw more than 8% of such cells following large injections centered on the more caudal and lateral visual thalamic reticular nucleus, but these injections extended beyond the thalamic reticular nucleus; smaller injections clearly limited to the visual thalamic reticular nucleus never labeled such cholinergic cells.

The location of retrogradely labeled basal forebrain cells may also vary with the location of the injection within the thalamic reticular nucleus. In our study, we consistently found retrogradely labeled cells in the lateral nucleus basalis. In contrast, Steriade et al. (1987) found most of their retrogradely labeled cells in the horizontal limb of the diagonal band of Broca and the substantia innominata, which are medial and ventral to the location of our retrogradely labeled cells. It is possible that the basal forebrain projection to the thalamic reticular nucleus follows a somewhat topographic arrangement similar to that of cortically projecting basal forebrain cells (Mesulam et al., 1986). In support of this idea, we saw that the location of basal forebrain cells that project to the visual sector of the thalamic reticular nucleus is very similar to that of basal forebrain cells that project to the visual cortex.

There are, however, several previous results that are frankly inconsistent with our data. A study in the rat reported that some basal forebrain cells project to both the cortex and the thalamic reticular nucleus (Jourdain et al., 1989). As was noted above, our results strongly suggest that, in the cat, these projections arise primarily from separate neuron populations. This may be due to another difference between species, and it should be explored further. In addition, several previous studies have reported that both cholinergic and GABAergic basal forebrain cells project to the cortex, and that, occasionally, both ChAT and GAD are located within the same basal forebrain cells (Brashear et al., 1986; Fisher et al., 1988; Kosaka et al., 1988; Fisher and Levine, 1989). Although we did not stain for ChAT and GAD in the same sections, the combined results of our experiments indicate that the cholinergic and GABAergic cells within the nucleus basalis form separate populations with separate axonal targets. Further data are needed to resolve this question; for instance, it would be worthwhile to establish whether basal forebrain terminals in the cortex contain GABA.

Functional implications

Although this was not the primary focus of this study, our results do provide two interesting details concerning nitric oxide in the cat's visual system. First, we have found that the cholinergic basal forebrain cells that project to the visual cortex contain NADPH-diaphorase. Second, the results of this and a previous study (Bickford et al., 1993) allow us to conclude that the parabrachial region is the sole source of ACh and NADPH-diaphorase input to the lateral geniculate nucleus and visual thalamic reticular nucleus. This implies that, during arousal, the basal forebrain cells will release both ACh and nitric oxide in the visual cortex, whereas the parabrachial cells release both neuroactive substances in the lateral geniculate nucleus and visual thalamic reticular nucleus. Therefore, although NADPH-diaphorase staining is not a general feature of cholinergic cells, it is present in the majority of the cholinergic input to the visual thalamus and cortex. The reciprocal connections between the basal forebrain and the parabrachial region (Haring and Wang, 1986; Jones and Beaudet, 1987; Parent et al., 1988; Semba et al., 1988; Jones and Cuello, 1989; Hazrati and Parent, 1991a; Losier and Semba, 1993) further suggest that the release of ACh and nitric oxide in the visual thalamus and cortex may be synchronized during arousal. Furthermore, because nitric oxide apparently acts by stimulating soluble guanyl cyclase to increase levels of cyclic guanosine 3′,5′-monophosphate in target cells (Knowles et al., 1989), cholinergic arousal mechanisms may include changes in the states of cells in the visual system via second messenger systems.

Our results indicate that the basal forebrain provides a major input to the dendrites of cells of the visual thalamic reticular nucleus. These anatomical data suggest that, when active, GABAergic basal forebrain cells will inhibit the cells of the visual thalamic reticular nucleus. Cells in the thalamic reticular nucleus are themselves GABAergic (Houser et al., 1980) and provide an inhibitory input to the lateral geniculate nucleus. In the cat, axons from the perigeniculate nucleus, which is part of the visual thalamic reticular nucleus, seem mostly to contact dendrites of relay cells (Cucchiarro et al., 1991). Similarly, the sites of termination of the visual thalamic reticular nucleus in other species are consistent with the idea that the main output of this nucleus is directed to geniculocortical relay cells (Ohara et al., 1980; Montero and Scott, 1981; Harting et al., 1991).

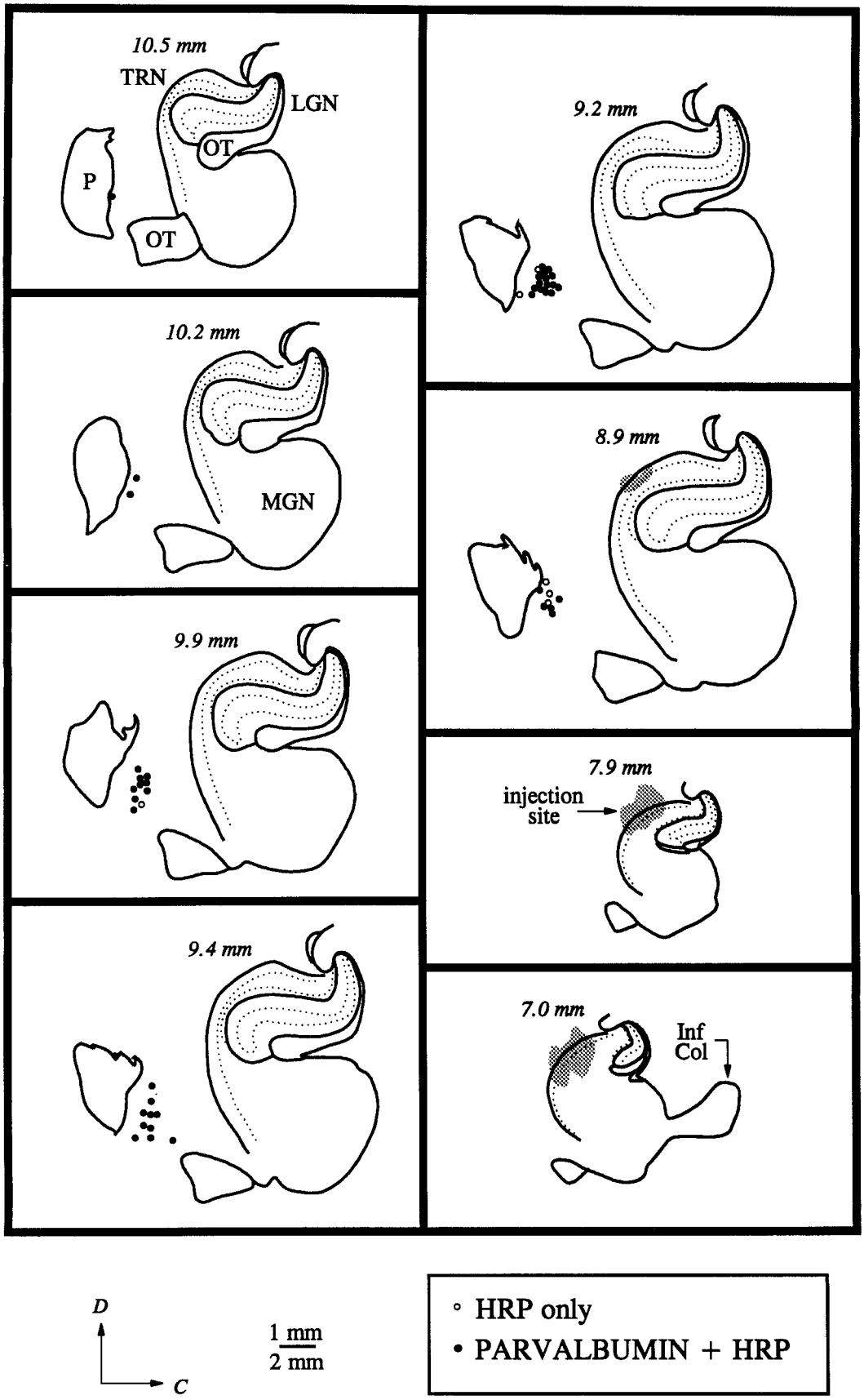


Fig. 23. Plot of cells in the basal forebrain retrogradely labeled from an injection of WGA-HRP into the visual thalamic reticular nucleus (injection site); most conventions as in Figure 11. Retrogradely labeled cells are shown, including the subset that also contains parvalbumin. A series of six parasagittal sections through the basal forebrain (at 8.9–10.5 mm from the midline) are illustrated along with two parasagittal sections through the injection site (at 7.0 and 7.9 mm; bottom two in right column). Scale bar = 1 mm (sections through the basal forebrain), 2 mm (sections through the injection site).

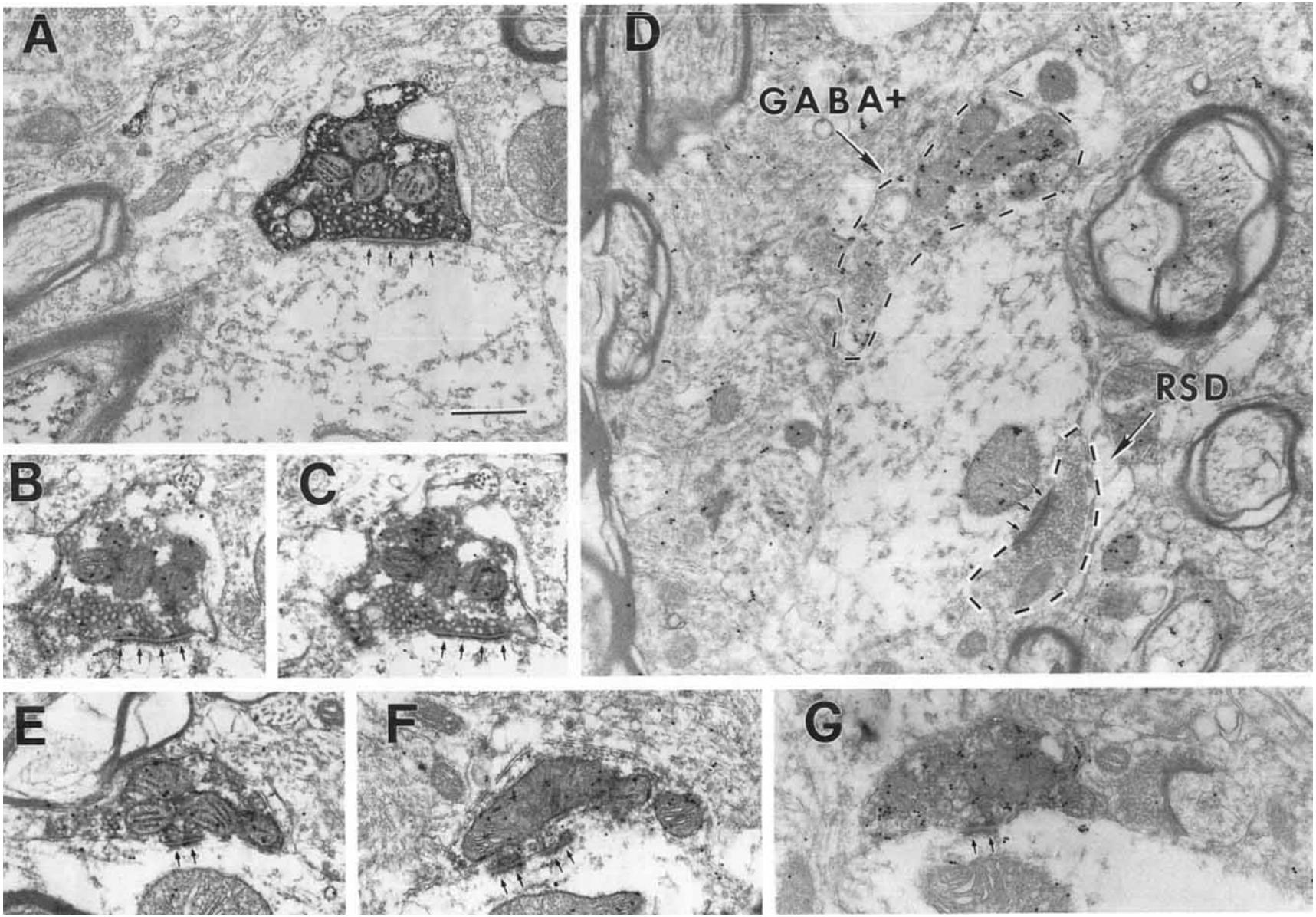


Fig. 24. Terminals in the visual sector of the thalamic reticular nucleus. Shown are terminals orthogradely labeled with biocytin from the basal forebrain as well as terminals labeled with postembedding immunocytochemistry for GABA, which is revealed by gold particles and unlabeled RSD terminals (see text for details). The etching used for immunocytochemistry also leaches the biocytin labeling, and this makes it easier to visualize the gold particles in biocytin labeled terminals. **A:** Biocytin-labeled terminal before immunocytochemistry and etching. Arrows point to a synaptic contact. **B,C:** Consecutive sections through the biocytin-labeled terminal shown in A after immunocytochemistry. Gold particles are clearly visible in this biocytin-labeled terminal. Arrows point to the same synaptic contact as in A. **D:** Section showing both a terminal positive for GABA as well as an RSD terminal with no gold particles present. **E–G:** Three different terminals labeled with biocytin and positive for GABA. Arrows in each point to a synaptic contact, although some may be puncta adhaerentia. Scale bar = 0.5 μm.

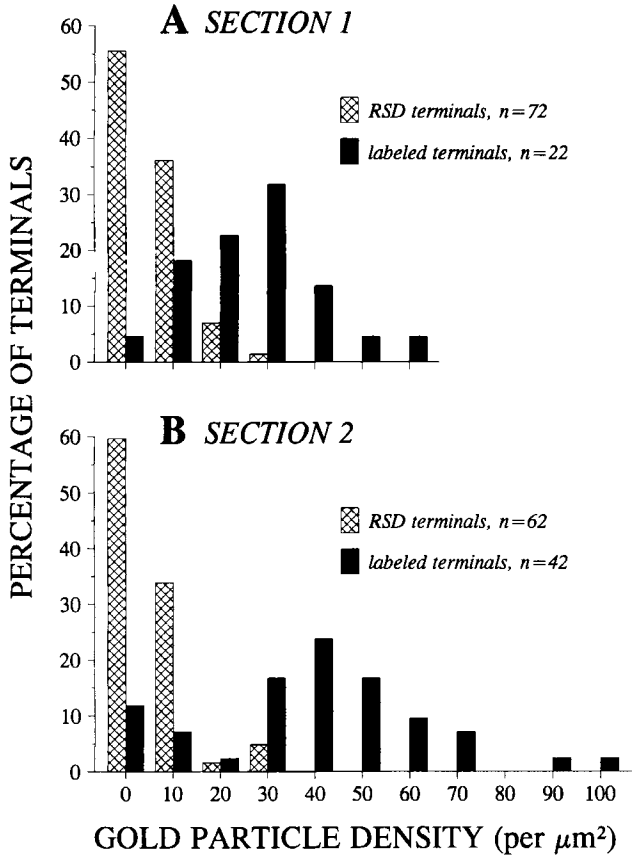


Fig. 25. Distribution of gold particles signaling the presence of GABA in terminals within the visual thalamic reticular nucleus. Separately shown are terminals orthogradely labeled from the basal forebrain and RSD terminals, which are known to be non-GABAergic and serve as a control population. **A:** Terminals in section 1, which was incubated in the anti-GABA antibody for 18 hours. **B:** Terminals in section 2, which was incubated in the anti-GABA antibody for 42 hours.

It thus follows that activity in the GABAergic projection cells of the basal forebrain, by inhibiting cells of the thalamic reticular nucleus, will disinhibit geniculate relay cells, leading to an increased flow of visual information to the cortex.

There is abundant evidence that changes in the activities of both the cholinergic parabrachial cells that project to the visual thalamus as well as the cholinergic basal forebrain cells that project to the cortex are correlated with changes in states of arousal (Detári et al., 1984, 1987; Detári and Vanderwold, 1987; Buzsáki et al., 1988; El Mansari et al., 1989; Szymusiak and McGuinty, 1989; Steriade et al., 1990; Semba, 1991; Kayama et al., 1992), but the response properties of the GABAergic basal forebrain cells that project to the visual thalamic reticular nucleus are unknown. If the cholinergic and GABAergic basal forebrain cells fire in synchrony, then the basal forebrain projection to the thalamic reticular nucleus may provide an additional mechanism by which the transfer of visual information is increased during arousal. Further elucidation of the function of this pathway will await studies to determine how the firing patterns of the GABAergic thalamic reticular nucleus-projecting cells are correlated with the activity of the cholinergic cortically projecting cells.

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