

Parvalbumin, Calbindin, or Calretinin in Cortically Projecting and GABAergic, Cholinergic, or Glutamatergic Basal Forebrain Neurons of the Rat

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ABSTRACT

The basal forebrain (BF) plays an important role in modulating cortical activity and facilitating processes of attention, learning, and memory. This role is subserved by cholinergic neurons but also requires the participation of other noncholinergic neurons. Noncholinergic neurons include γ -amino butyric acidergic (GABAergic) neurons, some of which project in parallel with the cholinergic cells to the cerebral cortex, others of which project caudally or locally. With the original aim of distinguishing different subgroups of GABAergic neurons, we examined immunostaining for the calcium binding proteins (CBPs) parvalbumin (Parv), calbindin (Calb), and calretinin (Calret) in the rat. Although the CBP⁺ cell groups were distributed in a coextensive manner with the GABAergic cells, they were collectively more numerous. Of cells retrogradely labeled with cholera toxin (CT) from the prefrontal or parietal cortex, Parv⁺ and Calb⁺ cells, but not Calret⁺ cells, represented substantial proportions (~35–45% each) that collectively were greater than that of GABAergic projection neurons. From dual immunostaining for the CBPs and glutamic acid decarboxylase (GAD), it appeared that the vast majority (>90%) of the Parv⁺ group was GAD⁺, whereas only a small minority (<10%) of the Calb⁺ or Calret⁺ group was GAD⁺. Significant proportions of Calb⁺ (>40%) and Calret⁺ (>80%) neurons were immunopositive for phosphate-activated glutaminase, the synthetic enzyme for transmitter glutamate. The results suggested that, whereas Calret⁺ cells predominantly comprise caudally or locally projecting, possibly glutamatergic BF neurons, Parv⁺ cells likely comprise the cortically projecting GABAergic BF neurons and Calb⁺ cells the cortically projecting, possibly glutamatergic BF neurons that would collectively participate with the cholinergic cells in the modulation of cortical activity. *J. Comp. Neurol.* 458:11–31, 2003. © 2003 Wiley-Liss, Inc.

Indexing terms: calcium binding proteins; medial septum–diagonal band; substantia innominata; preoptic nuclei

The basal forebrain (BF) plays a critical role in the modulation of cortical activity across sleep–wake states (for review, see Jones, 2000). Constituent cholinergic neurons stimulate cortical activation during waking and paradoxical sleep and play an important role in the processes of attention, learning, and memory (Metherate et al., 1988, 1992; Smith, 1988; Huerta and Lisman, 1993; Jones, 1993; Muir et al., 1994; Kilgard and Merzenich, 1998; Cape and Jones, 2000; Cape et al., 2000). In addition to cholinergic cells, other neurons constitute important elements of the BF cell population. Among these, γ -amino butyric acidergic (GABAergic) neurons are twice as numerous and more heterogeneous than the cholinergic neurons (Brashear et al., 1986; Kiss et al., 1990a; Gritti et al., 1993). Relatively small GABAergic neurons project cau-

dally and most probably locally (Gritti et al., 1994) to the diencephalon and brainstem, and relatively large

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GABAergic neurons project in parallel with the cholinergic neurons to the cerebral cortex (Gritti et al., 1997). Subgroups of GABAergic neurons projecting to different targets may play different roles in the modulation of cortical activity and behavioral state (for review, see Jones and Muhlethaler, 1999). Multiple subgroups of GABAergic neurons have been distinguished by their morphology, projections, and physiology in the neocortex and hippocampus. These have also been found to differ in their content of the calcium binding proteins (CBPs) parvalbumin (Parv), calbindin-D28k (Calb), and calretinin (Calret) that has accordingly provided a means to distinguish them by immunohistochemical staining (Celio, 1986; Kawaguchi and Hama, 1988; Kawaguchi and Kubota, 1993; Freund and Buzsaki, 1996; DeFelipe, 1997). In the neocortex and hippocampus, these CBPs are contained within largely nonoverlapping groups of nonpyramidal GABAergic interneurons. Within the BF of the rat, the CBPs have been visualized in noncholinergic, presumed GABAergic neurons (Celio, 1990; Kiss et al., 1990a, 1997; Chang and Kuo, 1991; Zaborszky et al., 1999). In the medial septum–diagonal band nuclei, Parv-containing neurons have been shown to correspond to the septohippocampal projection GABAergic neurons (Freund, 1989; Kiss et al., 1990b), whereas Calret-containing neurons appeared to correspond to locally or caudally projecting presumed GABAergic neurons (Kiss et al., 1997). The CBPs thus appeared to be a useful tool for identifying and distinguishing different subgroups of noncholinergic, presumed GABAergic neurons in the BF.

The original aims of the present study were to determine in the rat brain whether the CBPs are contained

within subgroups of GABAergic BF neurons and to investigate which CBP(s) is(are) contained within the cortically projecting GABAergic neurons. With immunoperoxidase staining, we examined the distribution of all Parv-, Calb-, and Calret-immunostained neurons and the distribution of those projecting to the prefrontal or parietal cortex visualized by retrograde transport of cholera toxin (CT). Given unanticipated larger numbers of CBP-containing neurons as compared with GABAergic neurons (Gritti et al., 1993, 1997) in estimated total and cortically projecting cell contingents, we subsequently examined the colocalization of the CBPs with the synthetic enzyme for GABA, glutamic acid decarboxylase (GAD), by dual-immunofluorescent staining. We also investigated whether Calb might be colocalized with choline acetyl transferase (ChAT) in the rat brain, as documented in the monkey brain (Chang and Kuo, 1991). We also examined whether CBPs might be colocalized with glutamate by dual immunostaining for phosphate activated glutaminase (PAG), the synthetic enzyme for the transmitter pool of glutamate (Kaneko and Mizuno, 1988, 1994; Akiyama et al., 1990; Kaneko et al., 1992; Manns et al., 2001). We previously presented some of the results in abstract form (Gritti et al., 1999, 2001).

MATERIALS AND METHODS

Animals and surgery

Fourteen adult male Wistar rats (Charles River, St. Constant, Canada), weighing approximately 250 g were used in this study. The rats were anesthetized with sodium pentobarbital (Somnotol, 60–67 mg/kg, intraperito-

Abbreviations

AAA	anterior amygdaloid area	MCPO	magnocellular preoptic nucleus
ac	anterior commissure	MD	mediodorsal thalamic nucleus
Acb	nucleus accumbens	MeA	medial amygdaloid nucleus
AHA	anterior hypothalamic area	MO	medial orbital cortex
AM	anteromedial thalamic nucleus	MPA	medial preoptic area
AO	anterior olfactory nucleus	MS	medial septum
BF	basal forebrain	mt	mammillothalamic tract
BLA	basolateral amygdaloid nucleus	oc	optic chiasm
BST	bed nucleus of the stria terminalis	ot	optic tract
CeA	central amygdaloid nucleus	OTu	olfactory tubercle
Cg1	cingulate cortex, area 1	Par1	parietal cortex, area 1
Cg3	cingulate cortex, area 3	Par2	parietal cortex, area 2
CL	centrolateral thalamic nucleus	PaV	paraventricular hypothalamic nucleus
CM	centromedial thalamic nucleus	PC	paracentral thalamic nucleus
CPu	caudate putamen	Pir	piriform cortex
DBB	diagonal band of Broca nucleus	PO	posterior thalamic nuclear group
DHA	dorsal hypothalamic area	POAH	preoptic-anterior hypothalamic region
EP	entopeduncular nucleus	PT	paratenial thalamic nucleus
f	fornix	PV	paraventricular thalamic nucleus
Fr1	frontal cortex, area 1	Re	reuniens thalamic nucleus
Fr2	frontal cortex, area 2	Ret	reticular thalamic nucleus
FL	Forelimb area of the cortex	Rh	rhomboid thalamic nucleus
FStr	fundus striati	SCh	suprachiasmatic nucleus
GP	globus pallidus	SIa	substantia innominata pars anterior
IAM	interanteromedial thalamic nucleus	SIp	substantia innominata pars posterior
ic	internal capsule	sm	stria medullaris
IL	infralimbic cortex	SO	nucleus supraopticus
IMD	intermediodorsal thalamic nucleus	VLO	ventrolateral orbital cortex
LH	lateral hypothalamic area	VM	ventromedial thalamic nucleus
LHa	anterior lateral hypothalamic area	VMH	ventromedial hypothalamic nucleus
LO	lateral orbital cortex	VO	ventral orbital cortex
LOT	lateral olfactory tract nucleus	VPL	ventral posterolateral thalamic nucleus
LPOA	lateral preoptic area	VPM	ventral posteromedial thalamic nucleus
LS	lateral septum		

neally) for all surgical procedures and for perfusion. The McGill University Animal Care Committee and the Canadian Council on Animal Care approved all procedures.

For the study of the CBP-containing cells that project to the cerebral cortex with the use of immunoperoxidase techniques, eight rats were first operated on under general anesthesia for injection of the retrograde tracer, CT b subunit (cholera toxin; LIST Biological Laboratories, Campbell, CA). The tracer was placed into the prefrontal cortex in four rats (CT22, CT25, CT26, and CT27) and into the parietal cortex in four rats (CT24, CT29, CT30, and CT31). Injections were performed under stereotaxic guidance according to coordinates for the right prefrontal cortex: anterior–posterior (AP), +11.5; vertical (V), +5.5; lateral (L), –0.75 (with reference to ear bar zero); and for the right parietal cortex: AP, –1.0; V, –3.0; L, –4.6 (with reference to bregma; see Paxinos and Watson, 1986). CT was dissolved as a 1% solution in Tris buffer (pH 7.5) and injected in a volume of 100 nl over 5–10 minutes through a 1- μ l Hamilton microsyringe that was fitted with an oil-filled micropipette with a tip diameter of 50–100 μ m. Two rats (CT22 and CT24) also received colchicine treatment approximately 24 hours after the CT injections and approximately 24 hours before death. All rats were killed under anesthesia by perfusion with a paraformaldehyde fixative approximately 48 hours after the CT injections.

For the study of the colocalization of CBPs and the transmitter enzymes using immunofluorescent staining, six rats were used (CT80, CT81, CT82, CT83, PP1, and PP2), four of which (CT80, CT81, CT82, CT83) received colchicine treatment approximately 24 hours before death and were killed under anesthesia by perfusion with a Zamboni fixative.

As previously described (Gritti et al., 1997), colchicine (Sigma, St. Louis, MO) was injected in a dose of 50 μ g in 25 μ l of saline solution into the left ventricle of anesthetized rats according to stereotaxic procedures (coordinates AP –0.8, V –4.4, L +1.5, with reference to bregma). Colchicine was found to enhance GAD immunostaining within nerve cell bodies but to not alter that of CBPs for which it was deemed unnecessary. Although it was also found to be unnecessary for revealing GAD when using the anti-GAD67 antibody in paraformaldehyde-fixed tissue (Gritti et al., 1997), colchicine was important for revealing GAD with this antibody in the Zamboni-fixed tissue used for the immunofluorescent staining of PAG.

Perfusion and fixation

Brains used for immunoperoxidase staining of CBP and CT (from eight rats) were fixed with a paraformaldehyde solution. After a brief rinse with phosphate buffered saline (0.1 M, containing 20 mM MgCl₂, pH 7.4), the animals were perfused through the ascending aorta with 1,000 ml of 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4 over ~30 minutes), followed by 10% sucrose in phosphate buffer (pH 7.4, over ~15 minutes), as previously described (Gritti et al., 1993, 1997). The brains were removed and immersed overnight in 30% sucrose in phosphate buffer for cryoprotection. Brains were subsequently frozen at –50°C with isopentane and stored at –80°C.

Brains used for immunofluorescent staining of CBP and GAD, PAG, or ChAT (from six rats) were fixed with Zamboni's solution, according to a slight modification of the procedure developed by Kaneko and colleagues for immunostaining of the PAG enzyme (Kaneko and Mizuno, 1988;

Kaneko et al., 1989; Manns et al., 2001). After a brief rinse with phosphate buffered saline, the animals were perfused through the ascending aorta with 500 ml of a modified Zamboni's solution containing 0.3% paraformaldehyde and 75% saturated picric acid in 0.1 M sodium phosphate buffer (pH 7.0, over ~30 minutes). The brains were postfixed overnight at 4°C in a solution of 3% paraformaldehyde with 75% saturated picric acid. Brains were subsequently immersed in a solution of 30% sucrose at 4°C for approximately 48 hours for cryoprotection. Brains were then frozen at –50°C and stored at –80°C.

Immunohistochemistry

Immunoperoxidase staining. Brains processed for immunoperoxidase staining (by L.M.) of CBP and CT were cut in the coronal plane on a freezing microtome at a thickness of 25 μ m (CT22 and CT24) or 20 μ m (CT25, CT26, CT27, CT29, CT30, and CT31). Sections were collected at 400- μ m intervals in phosphate buffer (pH 7.4, 0.1 M) to produce 16 or 20 adjacent series.

With the peroxidase–antiperoxidase (PAP) technique employed previously (Gritti et al., 1997), adjacent series from several brains were processed for single or dual immunostaining of the CBPs and CT. Single-immunostained series were processed for Parv (CT22, CT24, and CT25), Calb (CT22, CT24, and CT25), or Calret (CT29 and CT30). For Parv, a monoclonal antibody from mouse (Sigma) was used at a dilution of 1:1,000; for Calb, a monoclonal antibody from mouse (Sigma) was used at a dilution of 1:200; and for Calret, a polyclonal antibody from rabbit (Chemicon International, Temecula, CA) was used at a dilution of 1:10,000. Sections were incubated in the primary antibodies overnight at room temperature. They were subsequently placed for 30 minutes in an anti-mouse (for Parv and Calb) or anti-rabbit (for Calret) secondary antibody (both from donkey, diluted 1:50 or 1:100, respectively; Jackson Immuno Research, West Grove, PA) and then for 30 minutes in mouse or rabbit PAP (diluted 1:200; Jackson Immuno Research). PAP was revealed by 3, 3'-diaminobenzidine (DAB) in the presence of 0.1% glucose oxidase for 14 minutes at room temperature. Adjacent series of sections were processed with sequential, dual-immunoperoxidase staining for CT and one CBP, according to previously published procedures (Gritti et al., 1994, 1997). Sections were incubated overnight in goat anticholera toxin antiserum (at a dilution of 1:40,000; LIST Biological Laboratories). They were subsequently placed in an anti-goat antiserum (from donkey at 1:100) and goat PAP (1:200; both from Jackson Immuno Research). PAP was revealed with DAB using nickel intensification in the presence of 0.01% H₂O₂ for 6 minutes or in the presence of 0.1% glucose oxidase for 15 minutes (in CT22 and CT24). After prolonged rinsing, series of sections were incubated for dual immunostaining with one of the CBP antibodies: the mouse antibody for Parv (1:1,000; in all brains), the mouse antibody for Calb (1:200; in CT22, CT24, CT25, CT26, CT27, and CT31), or the rabbit antiserum for Calret (1:10,000; in CT29 and CT30), as described above. PAP was revealed by using DAB in the presence of 0.1% glucose oxidase in Tris buffer (pH 7.4) for 13–15 minutes at room temperature.

In all single- or dual-immunoperoxidase staining procedures, a Tris saline solution (TS; 0.1 M) containing normal donkey serum at 1% concentration was used for diluting the primary antibody and for rinsing, and one containing

normal donkey serum at 6% was used for blocking before incubations. Controls were routinely run with every procedure by omitting the primary antibodies and for antisera, replacing them with normal sera from the same species to ensure that nonspecific staining due to cross-reactivity was not occurring. At the end of processing, all sections were washed in phosphate buffer, mounted out of water, dehydrated, and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ).

Immunofluorescent staining. To assess dual immunostaining for the CBPs and GAD, PAG, or ChAT enzyme proteins (EPs), we found that immunoperoxidase techniques were not suitable due to the colocalization of the CBPs and EP in the cytoplasm of the cells and thus used immunofluorescent staining (by L.M. and I.D.M.) in six brains (CT80, CT81, CT82, CT83, PP1, and PP2). Sections were cut on a freezing microtome at 20 μm thickness, and 20 adjacent series of sections were collected at 400- μm intervals.

Dual immunostaining for CBPs and GAD was performed in series from four brains (CT80, CT81, CT82, and CT83). For Parv or Calb with GAD, series of sections were incubated overnight at room temperature with the mouse anti-Parv (1:1,000) or mouse anti-Calb (1:200) antibodies in conjunction with a rabbit polyclonal antibody for GAD67 (1:3,000; Chemicon International). The sections were subsequently incubated for 2 hours with a Cy2-conjugated anti-mouse antiserum (1:200) for revelation of the Parv or Calb and with a Cy3-conjugated anti-rabbit antiserum (1:1,000) for revelation of the GAD enzyme (both secondary antibodies were from goat and supplied by Jackson Immuno Research). Similarly, the dual immunostaining of Calret with GAD was performed by incubating sections overnight in the rabbit anti-Calret antiserum (1:10,000) with a sheep anti-GAD antiserum (1:1,000; supplied by E. Mugnaini, Chicago, IL; Oertel et al., 1982). These sections were then incubated with Cy2-conjugated anti-rabbit antiserum (1:50) for revelation of the Calret and a Cy3-conjugated anti-sheep antiserum (1:1,000) for revelation of the GAD enzyme (both secondaries from donkey; Jackson Immuno Research).

Dual immunostaining for CBPs with PAG (in series from CT80, CT81, CT82, CT83, PP1, and PP2) was performed by incubating adjacent series of sections with antibodies to the respective CBPs in conjunction with a rabbit anti-PAG antiserum (1:6,000, developed and kindly supplied by T. Kaneko, Kyoto, Japan; Akiyama et al., 1990). The sections were incubated in the mouse anti-Parv antibody (1:1,000) with the rabbit anti-PAG antibody (1:6,000) and subsequently in a biotinylated anti-mouse secondary antibody (1:1,000) followed by Cy2-conjugated streptavidin (1:1,000) for Parv with a Cy3-conjugated anti-rabbit antiserum (1:1,000) for PAG (both secondary antibodies were from donkey and supplied by Jackson Immuno Research). In another series, sections were incubated with the mouse anti-Calb antibody (1:200) with the rabbit anti-PAG antibody, followed by a Cy2-conjugated anti-mouse antiserum (1:200) for revelation of Calb and a Cy3-conjugated anti-rabbit antiserum (1:1,000) for revelation of PAG (both secondary antibodies were from goat; Jackson Immuno Research). Dual immunostaining of Calret with PAG was performed by incubating an adjacent series of sections with a goat polyclonal antibody for Calret (1:2,500; Chemicon International) and rabbit anti-PAG (1:6,000). The sections were subsequently incubated with

biotinylated anti-goat antiserum (1:50) followed by a Cy2-conjugated streptavidin (1:1,000) for revelation of Calret and a Cy3-conjugated anti-rabbit antiserum (1:1,000) for revelation of PAG (with both secondaries from donkey; Jackson Immuno Research).

Dual immunostaining for Calb with ChAT was performed in two brains (CT81 and CT82) by incubating sections with the mouse anti-Calb antiserum (1:200) and a rabbit anti-ChAT antiserum (1:3,000; Chemicon International). The sections were subsequently incubated with Cy2-conjugated anti-mouse antiserum (1:200) for revelation of Calb and a Cy3-conjugated anti-rabbit antiserum (1:1,000) for revelation of the ChAT enzyme (both secondaries were from goat; Jackson Immuno Research).

For all series of immunofluorescent staining, the primary and secondary antibodies were diluted in TS (0.1 M, pH 7.4) that contained 1% bovine serum albumin and was also used for rinses between incubations. Before incubation with the primary antibody, the sections were placed in TS with 6% bovine serum albumin for blocking. Controls were routinely run with every dual-immunostaining procedure by omitting the primary antibodies and for antisera, replacing them with normal sera from the same species. At the end of processing, all sections were washed in TS, mounted from TS, dehydrated, and coverslipped with Permount (Fisher Scientific).

Analysis

Immunoperoxidase material. For the analysis of immunoperoxidase-stained material (performed by I.G.), a computer-based two-dimensional image analysis system (Biocom, Les Ulis, France) was employed. It comprised a Leitz Orthoplan microscope equipped with an x/y-sensitive stage and video camera connected to a Compaq 386 computer. For assessment of CT injection sites, the region of the deposit in the cortex was outlined, and the retrogradely labeled neurons were mapped in the cortex and thalamus. For morphologic and numerical assessments of CBP-immunopositive (+) and CT⁺ cells in the BF, cells were drawn or plotted through the basal forebrain cholinergic cell area. For this purpose, a previously designed and published computer atlas (Gritti et al., 1993) was used that allowed fitting of each section to the atlas and plotting of cells into the contours of the cytoarchitectonically and immunohistochemically delineated nuclei of the BF at six levels (separated by 800 μm) from ~A10.9 to ~A6.9 (with reference to Paxinos and Watson, 1986). For mapping, all profiles of nerve cell bodies that were immunopositive were plotted (by using a 40 \times objective) through the entire area of each nucleus at each level (Gritti et al., 1993). For measurement of cell soma length, nerve cell bodies were selected at relatively equally spaced intervals through the full area of each nucleus and drawn in contour by using a 40 \times objective. In those series dual immunostained for CBPs and CT, cells were drawn or plotted as CBP/CT immunopositive or immunonegative to compute averages or totals for the CBP and CT cell groups. Accordingly, cells were classified as: Parv⁺/CT⁺, Calb⁺/CT⁺, or Calret⁺/CT⁺; Parv⁺/CT⁻, Calb⁺/CT⁻, or Calret⁺/CT⁻; or Parv⁻/CT⁺, Calb⁻/CT⁺, or Calret⁻/CT⁺.

Total cell counts were computed per nucleus in one brain for single-labeled CBP cell types and in multiple brains (three or four) for double-labeled CBP/CT cell types. The estimated total numbers of cells were calculated according to Abercrombie's (1946) formula by multi-

plication of the raw cell counts by the number of sections per interval and by a correction factor for cell size (mean large diameter) in relation to section thickness, i.e., section thickness (μm)/(section thickness [μm] + cell diameter [μm]), calculated per nucleus in the same manner performed previously for estimation of GAD⁺ and ChAT⁺ cell numbers (Gritti et al., 1993, 1994, 1997).

Immunofluorescent material. For assessment of dual-immunostaining for CBP and the EPs, GAD, PAG, or ChAT, series of sections were examined (by I.D.M.) under fluorescent illumination for double labeling of the CBP cells with the EP. The sections were examined with a Leitz Dialux EB20 microscope equipped with a Ploemopak 2 reflected light fluorescence illuminator and Leica filter cubes for fluorescein (I3 with excitation between 450 and 490 nm and long pass emission above 515 nm) and rhodamine (N2.1 with excitation between 515 and 560 nm and long pass above 590 nm). Because the fluorescent images could not be transmitted by video camera and mapped with our image analysis system (as for the immunoperoxidase material), fluorescent cells were examined under the microscope by using a 63 \times oil objective and their single or dual immunostaining was tabulated by hand. In each microscopic field, all CBP⁺ cell body profiles were evaluated for double labeling with the respective EPs (EP⁺). Because of fewer labeled cells due to lower sensitivity in immunofluorescence than in immunoperoxidase material, cells were examined on all sections of each series. By progressing systematically through the nuclei (except the olfactory tubercle) of the BF, CBP⁺ cell body profiles were accordingly judged as EP⁺ or EP⁻ and tabulated per microscopic field starting at \sim A10.9 and moving caudally to \sim A6.9 at 400- μm intervals through the BF nuclei (to yield \sim 60–160 profiles sampled for the different series across nuclei). Three series were examined for the CBPs dual immunostained with GAD or PAG, and two series were examined for Calb with ChAT. Results were tabulated for calculation and presentation of the average proportion of the respective CBP⁺ cells examined that were GAD⁺, PAG⁺, or ChAT⁺.

Statistical analysis was performed with Systat 9.0 (SPSS, Chicago, IL). Color photographs were prepared with Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA). Atlas diagrams (from Biocom) were prepared as composite illustrations with Corel Draw 8.0 (Ottawa, Canada).

RESULTS

Distribution of CBP-containing neurons

Through the cholinergic cell area of the BF, where cholinergic and GABAergic cells are codistributed, there were neurons containing the CBPs Parv, Calb, or Calret. The CBP⁺ cells were of different sizes and shapes, from small to large and oval, fusiform, or polygonal (Fig. 1A,C,E). The Parv⁺ BF cells often were multipolar in shape and appeared to be commonly and relatively large in size (Fig. 1A; with a maximum large diameter of 29.6 μm and mean \pm standard deviation of 16.9 \pm 3.7 μm ; Table 1). Calb⁺ BF cells were highly variable in shape and size but, in addition to many small cells, included relatively large multipolar cells (Fig. 1C; with a maximum large diameter of 27.7 μm and 14.8 \pm 3.6 μm ; Table 1). The Calret⁺ BF cells also were variable, often oval or fusiform and bipolar (Fig. 1E) and, in addition to many small or medium cells,

included some large cells (with a maximum large diameter of 25.8 μm and 15.5 \pm 3.7 μm ; Table 1).

Through the BF, the CBP⁺ cell groups were distributed throughout the cholinergic cell nuclei (Figs. 2, 3, 4), where they appeared to be more numerous than GAD⁺ cells (Gritti et al., 1993). According to estimates of total numbers of cells in one brain (Table 1), Parv⁺ (\sim 23,000), Calb⁺ (\sim 72,000), and Calret⁺ (\sim 27,000) cells greatly outnumbered GAD⁺ and ChAT⁺ cells in the BF nuclei (estimated at \sim 39,000 and \sim 18,000 according to similar calculations by Gritti et al., 1993). This also was the case with the exclusion of cells in the olfactory tubercle, where many small Calb⁺ cells are located, for BF totals of 18,243 Parv⁺, 34,690 Calb⁺, 22,527 Calret⁺, 30,791 GAD⁺; and 15,056 ChAT⁺.

Distribution of cortically projecting CBP-containing neurons

To determine whether the different CBP-containing cell groups comprised cortically projecting neurons, retrograde labeling with CT was examined in CBP⁺ neurons by dual immunostaining using immunoperoxidase in brains with CT injections into the prefrontal or parietal cortex.

CT injection sites. The position and extent of the CT injections in the prefrontal and parietal cortices were assessed by the CT deposit in the cortex (Fig. 5) and then by the distribution of retrogradely labeled projection neurons in the contralateral cortex and in the ipsilateral thalamus within each brain, as previously documented (Gritti et al., 1997).

Injections were centered in the medial prefrontal cortex in four rats. The CT deposit was commonly located in the medial/ventral orbital cortex (MO/VO) and extended into the prelimbic cingulate cortex, area 3 (Cg3), more dorsally (Fig. 5A), similarly to previously published prefrontal cortex injection sites (Gritti et al., 1997). Across rats the injection sites also overlapped to varying degrees into the ventrolateral orbital (VLO) cortex laterally (CT25 and CT26), the infralimbic (IL) cortex caudally (CT25 and CT26) and the prelimbic cingulate cortex, area 1 (Cg1), cortex dorsally (CT27). Retrogradely labeled corticocortical projection neurons were located in the contralateral homotypic cortex, including MO/VO (CT22, CT25, CT26, and CT27), VLO (CT25 and CT26), Cg3 (CT22, CT25, CT26, and CT27), IL (CT25 and CT26), and Cg1 (CT27). Retrogradely labeled thalamocortical projection neurons were commonly located in the mediodorsal nucleus (MD), the centrolateral nucleus (CL), and the midline, reuniens (Re), and rhomboid (Rh) nuclei. A few retrogradely labeled neurons were also observed in different animals in the paratenial (PT), paraventricular (PV), centromedial (CM), paracentral (PC), anteromedial (AM), interanteromedial (IAM), intermediodorsal (IMD), and ventromedial (VM) nuclei.

Injections were centered in the parietal cortex in four rats. The CT deposit was located over the primary somatosensory cortex, parietal cortex 1 (Par1, Fig. 5B), similarly to previously published parietal cortical injection sites (Gritti et al., 1997). All injections (CT24, CT29, CT30, and CT31) were centered in the dorsomedial region of Par1. The retrogradely labeled corticocortical projection neurons were located predominantly in the contralateral homotypic Par1 cortex. In all rats, the retrogradely labeled thalamocortical neurons were located predominantly in the ventroposterior lateral and medial nuclei (VPL and

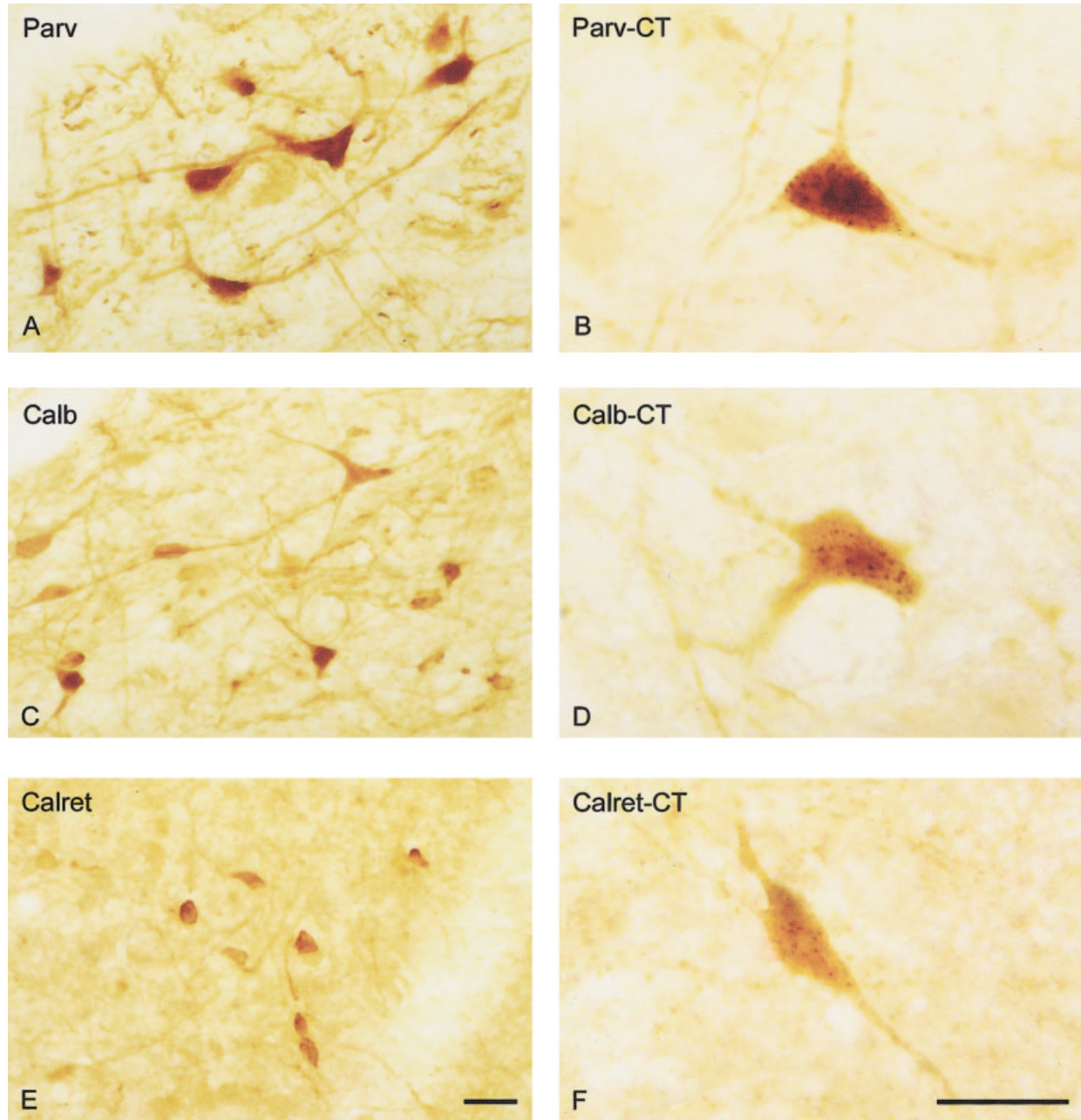


Fig. 1. Photomicrographs of sections taken through the basal forebrain (BF) cholinergic cell area and processed by immunoperoxidase staining for parvalbumin (Parv), calbindin (Calb), or calretinin (Calret) by single immunostaining (A, C, E) or for Parv and cholera toxin (CT), Calb-CT, or Calret-CT by dual immunostaining (B, D, F) by using 3, 3'-diaminobenzidine (DAB) for the calcium binding proteins (CBPs) and DAB with nickel intensification for CT. The retrograde

labeling is evident by the darkly stained CT⁺ granules in the soma (B, D, F). Images are taken from the region of the magnocellular preoptic nucleus and substantia innominata pars anterior (near the perforating blood vessels seen in A, C, and E; near A8.5, Figs. 2-4) to show single- (A, C, E) and dual- (B, D, F) immunostained CBP⁺ and CBP⁺/CT⁺ cells, respectively (Parv⁺ and Parv⁺/CT⁺ cells; Calb⁺ and Calb⁺/CT⁺ cells; and Calret⁺ and Calret⁺/CT⁺ cells). Scale bars = 20 μ m.

VPM) and in the posterior (PO), centromedial (CM), centrolateral (CL), and PC (paracentral) nuclei.

Retrogradely labeled CBP⁺ neurons. Across the BF cholinergic cell area, CBP⁺ cells were retrogradely labeled with CT from the prefrontal cortex and the parietal cortex (Fig. 1). Substantial numbers of Parv⁺/CT⁺ cells were retrogradely labeled and often darkly labeled with many

prominent CT⁺ granules in their soma (Fig. 1B). Similarly, many Calb⁺/CT⁺ cells were retrogradely labeled and often darkly so, indicating many prominent CT⁺ granules (Fig. 1D). Very few Calret⁺ cells could be found that were retrogradely labeled, and these were very lightly labeled, indicating small numbers of light CT⁺ granules (Fig. 1F). Given their rarity and minimal label-

TABLE 1. Calcium Binding Protein Immunopositive Cells in Immunoperoxidase Material and Their Retrograde Labeling With Cholera Toxin from the Prefrontal Cortex and Parietal Cortex Across the Basal Forebrain¹

Cell type	CBP+/CT+ cells										
	Prefrontal cortex					Parietal cortex					
	Large diameter (n)	Counts CBP+	Est. Total CBP+	Large diameter (n)	Counts CBP+/CT+	Est. Total CBP+/CT+	% CT+ (CBP+/CT+)	Counts CBP+/CT+	Est. Total CBP+/CT+	Est. Total CT+	% CT+ (CBP+/CT+)
Parv+ cells											
MS	15.3 ± 3.2 (33)	163	3272	16.1 ± 4.2 (10)	12.0 ± 2.0	257.2 ± 41.9	43.0	21.3 ± 21.7	466.9 ± 484.1	745.6 ± 598.8	56.8
DBB	17.8 ± 3.7 (18)	110	2188	19.8 ± 4.8 (5)	9.8 ± 5.9	502.5 ± 184.1	38.4	33.5 ± 32.4	670.6 ± 655.1	1523.9 ± 1340.7	41.1
OTu	17.2 ± 4.4 (16)	228	4400	18.5 ± 4.6 (5)	11.8 ± 2.1	238.2 ± 50.6	35.9	30.3 ± 28.4	625.0 ± 593.9	1359.7 ± 1185.9	45.7
MCPO	18.8 ± 4.1 (15)	79	1511	19.9 ± 3.8 (3)	12.5 ± 3.7	245.0 ± 79.8	30.0	20.8 ± 15.0	412.1 ± 306.0	1139.8 ± 583.5	33.9
Slα	17.2 ± 3.5 (31)	220	3943	16.8 ± 3.9 (17)	29.8 ± 10.1	618.8 ± 173.1	42.5	53.8 ± 26.2	1140.5 ± 588.3	2110.5 ± 530.7	52.2
Slp	19.4 ± 4.7 (6)	17	391	14.1 ± 0.0 (1)	6.3 ± 4.4	144.4 ± 106.1	28.8	15.5 ± 7.4	351.6 ± 174.6	943.8 ± 412.7	37.1
GP	16.2 ± 2.9 (42)	353	7007	16.9 ± 3.4 (12)	17.8 ± 6.2	375.8 ± 142.4	43.8	80.0 ± 54.1	1713.1 ± 1199.0	4163.8 ± 3312.0	44.3
Avg/Total	16.9 ± 3.7 (161)	1170	22643	17.3 ± 4.0 (53)	99.8 ± 6.9	2077.3 ± 209.4	38.4	255.0 ± 154.5	5395.6 ± 3404.3	11966.3 ± 6656.6	44.8
Calb+ Cells											
MS	15.1 ± 3.2 (47)	449	9048	17.2 ± 2.6 (3)	5.0 ± 1.8	105.6 ± 42.1	35.8	3.5 ± 3.5	75.3 ± 76.0	199.7 ± 224.9	44.4
DBB	15.7 ± 3.8 (30)	178	3678	18.3 ± 0.4 (3)	10.3 ± 5.3	212.3 ± 113.0	43.3	28.0 ± 15.6	584.9 ± 324.9	1045.0 ± 285.1	53.7
OTu	13.3 ± 3.3 (73)	1760	37028	15.1 ± 1.1 (4)	11.3 ± 5.1	253.6 ± 122.2	36.6	6.5 ± 4.9	148.1 ± 112.8	786.8 ± 825.9	25.2
MCPO	15.3 ± 3.2 (57)	315	6567	16.9 ± 3.2 (8)	23.8 ± 11.1	507.1 ± 252.8	47.6	41.5 ± 34.6	899.7 ± 751.2	1653.7 ± 1343.5	53.7
Slα	14.6 ± 4.0 (68)	357	7283	17.3 ± 6.3 (4)	5.5 ± 3.1	115.4 ± 68.5	22.0	5.5 ± 3.5	118.0 ± 75.8	1059.7 ± 167.6	11.8
Slp	14.5 ± 3.4 (75)	318	6371	16.6 ± 2.0 (4)	4.5 ± 1.3	95.1 ± 27.3	22.3	7.0 ± 2.8	153.0 ± 61.8	486.3 ± 14.2	31.7
GP	16.0 ± 3.8 (41)	146	2909	14.1 ± 0.0 (1)	3.3 ± 2.2	79.7 ± 56.1	21.0	1.5 ± 2.1	37.2 ± 52.5	615.8 ± 210.5	4.9
Avg/Total	14.8 ± 3.6 (391)	3523	71718	16.5 ± 3.1 (27)	63.5 ± 23.3	1370.7 ± 548.5	35.9	93.5 ± 57.3	2049.3 ± 1255.4	5806.9 ± 2255.3	33.6
Calret+ cells											
MS	14.3 ± 3.7 (14)	188.0	4397								
DBB	17.1 ± 3.7 (23)	131.0	2850								
OTu	15.8 ± 3.7 (21)	189.0	4264								
MCPO	16.0 ± 3.8 (21)	265.0	5903								
Slα	15.5 ± 3.9 (18)	176.0	4015								
Slp	15.0 ± 3.7 (19)	222.0	5074								
GP	15.1 ± 1.7 (7)	15.0	344								
Avg/Total	15.5 ± 3.7 (123)	1186.0	26791								

¹Cell counts on one side of the brain, ipsilateral to CT injections in the cortex, are presented for Parv⁺ (CT22), Calb⁺ (CT22), and Calret⁺ cells (CT30) and as the mean ± standard deviation for Parv⁺/CT⁺ cells, Calb⁺/CT⁺ cells, and CT⁺ cells for the prefrontal cortex (CT22, CT25, CT26, and CT27 for Parv-CT and Calb-CT series) and the parietal cortex (CT24, CT29, CT30, and CT31 for Parv-CT series and CT24 and CT31 for Calb-CT series). Cell counts represent the total number of labeled cells mapped in each nucleus across the appropriate levels from ~A10.9 to ~A6.9 (Figs. 2-4, 6-9) at 800- μ m intervals (in sections 25 μ m thick in CT22 and CT24 or 20 μ m thick in CT25-CT31). The estimated total numbers of cells were calculated according to Abercrombie's (1946) formula by multiplying the raw cell counts by the number of sections per interval (32 for CT22 and CT24 and 40 for CT25-CT31) and correcting for section thickness (μ m) and cell size (μ m); mean large diameter per cell type per nucleus from CT22, CT30, or CT24 and shown for CT22 or CT30 as mean ± standard deviation from the number of cells indicated in parentheses). Estimated number of single-labeled CT⁺ cells was derived in the same manner with measurements of average large diameter (in CT22 and CT24, which as for parietal cortex, are not shown because they did not differ significantly from other CT⁺ cell groups). The percentage of CT⁺ cells that were double labeled with CBP (CBP⁺/CT⁺) was calculated by dividing the estimated total of each CBP⁺/CT⁺ cell type by the respective estimated total of CT⁺ cells in the corresponding series. +, Immunopositive; Avg, average; Calb, calbindin; Calret, calretinin; CBP, calcium binding protein; CT, cholera toxin; Est, estimated; Parv, parvalbumin. For other abbreviations, see Abbreviations list.

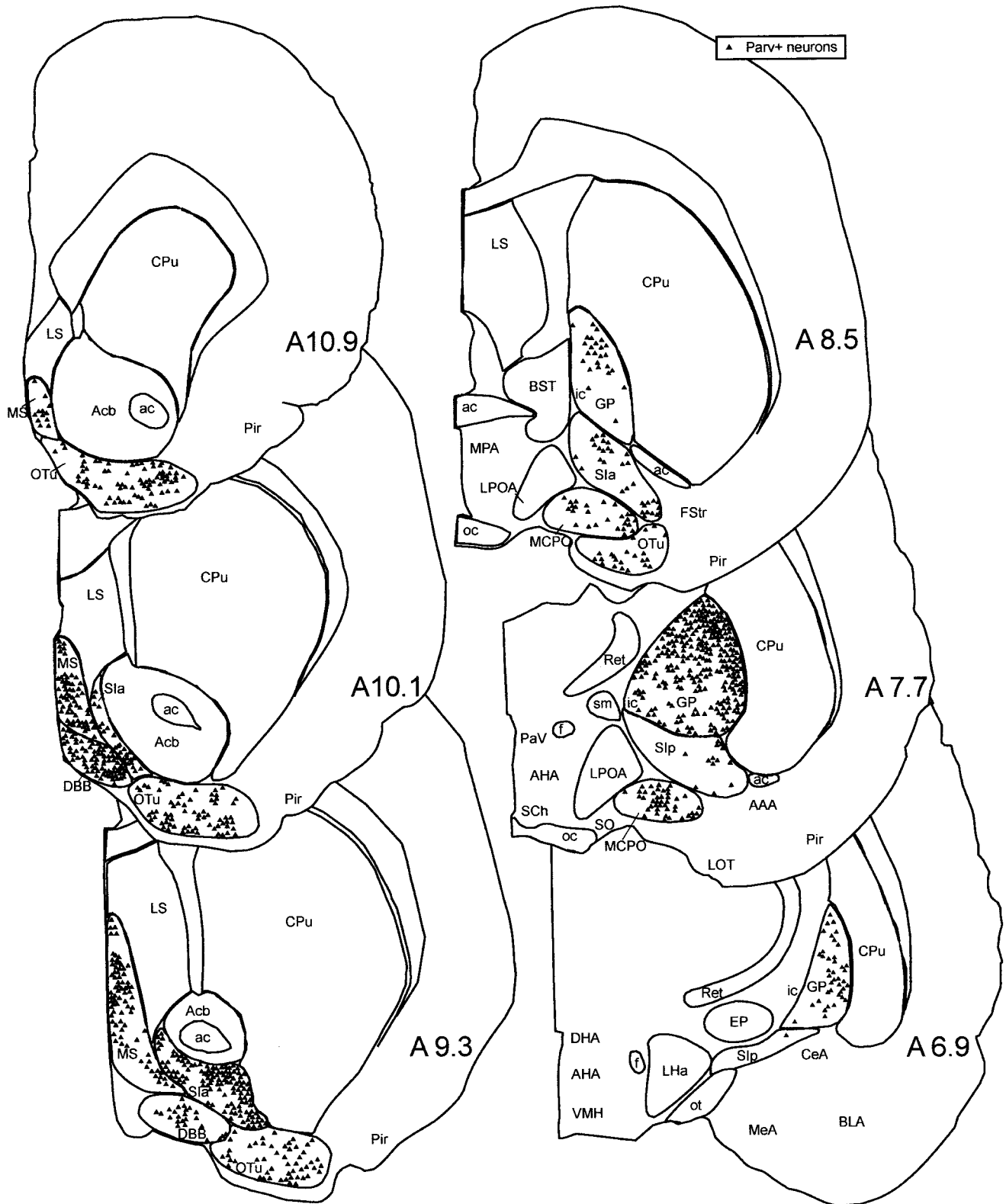


Fig. 2. Computer-based atlas figures of the basal forebrain cholinergic cell area (Gritti et al., 1993) in which parvalbumin-immunopositive neurons were plotted. Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

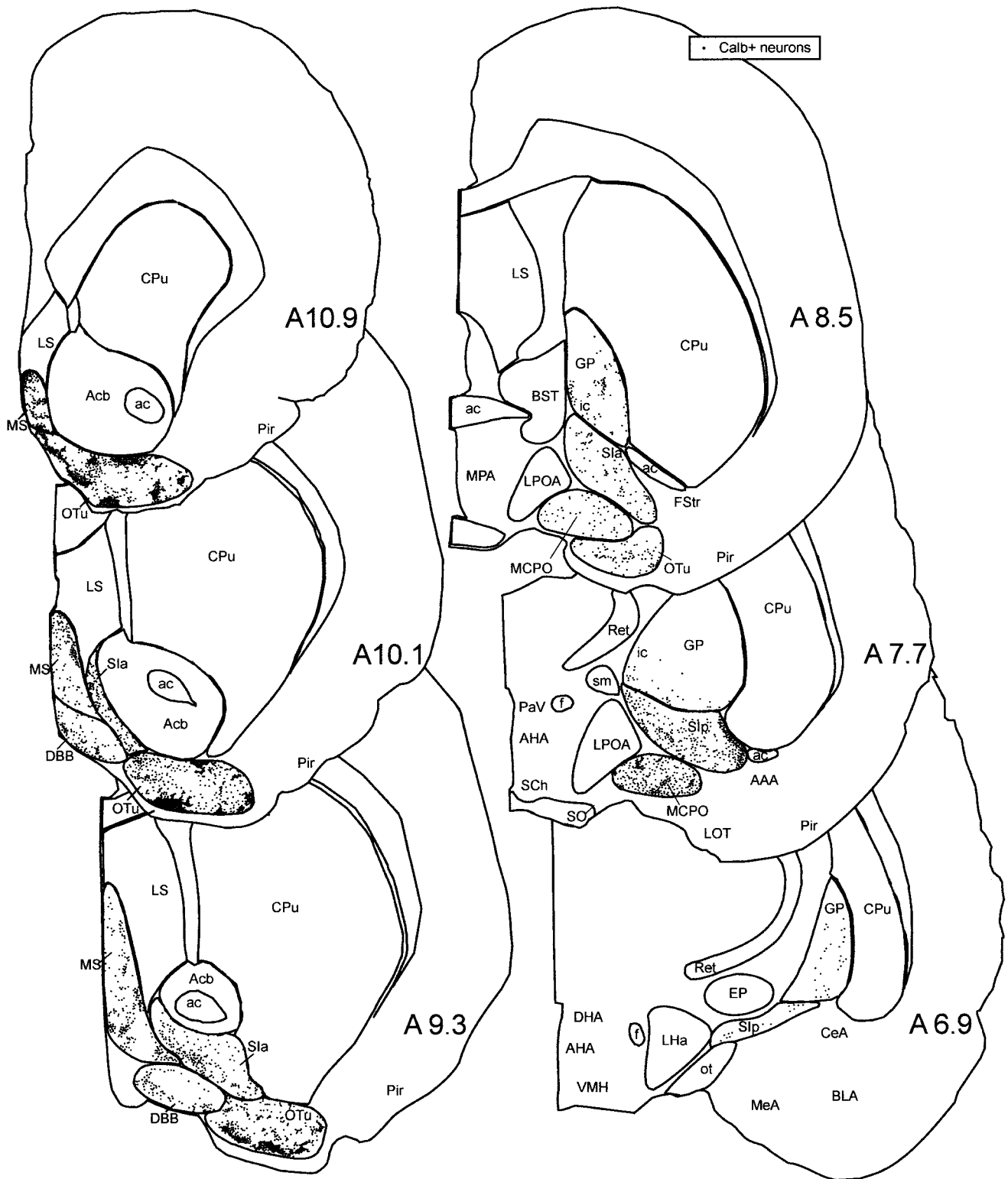


Fig. 3. Computer-based atlas figures of the basal forebrain cholinergic cell area in which calbindin-immunopositive neurons were plotted. Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

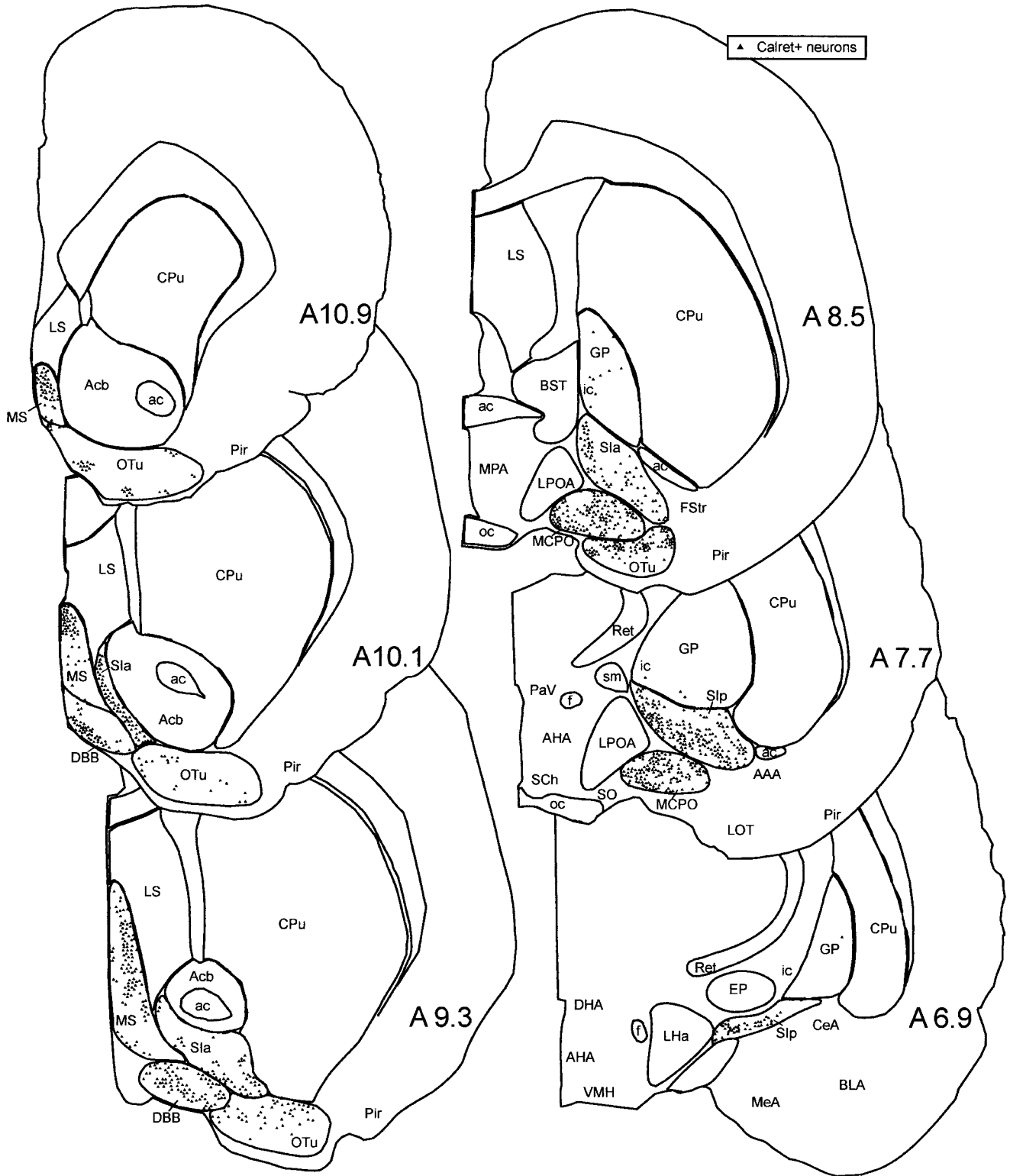


Fig. 4. Computer-based atlas figures of the basal forebrain cholinergic cell area in which calretinin-immunopositive neurons were plotted. Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

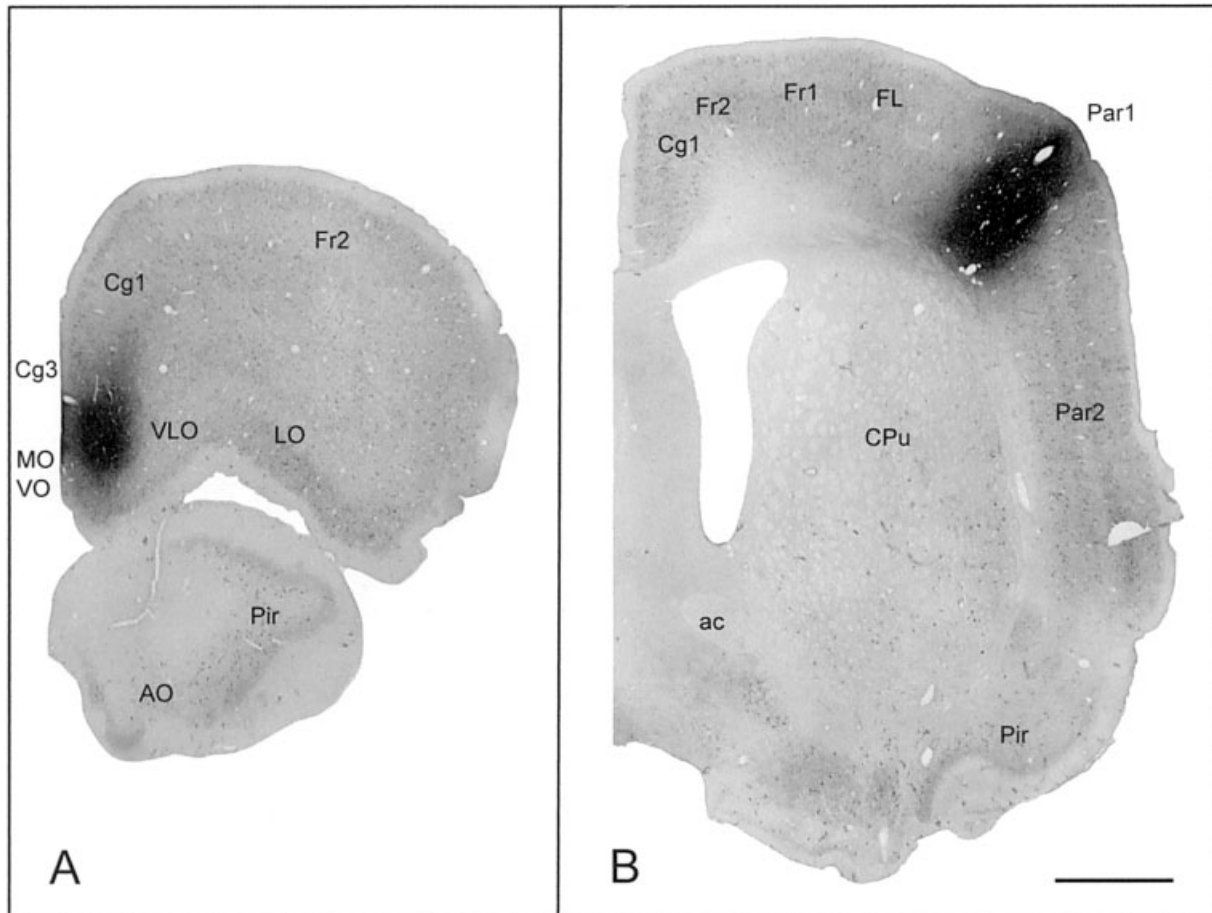


Fig. 5. Images of cholera toxin subunit b injection sites in the prefrontal cortex (A; ~A12.7) and parietal cortex (B; ~A9.7) taken from representative cases. The injection in the prefrontal cortex included the medial-ventral orbital cortex (MO/VO) and area 3 of the

cingulate cortex (Cg3). In the parietal cortex, the injection was situated in the dorsomedial region of area 1 (Par1) of the somatosensory cortex. For other abbreviations, see Abbreviations list. Scale bar = 1 mm.

ing, the Calret⁺/CT⁺ cells were not considered to represent a significant group of cortically projecting cells and thus were not systematically studied. Clearly representing significant numbers of retrogradely labeled cells, all Parv⁺/CT⁺ and Calb⁺/CT⁺ cells were mapped and tabulated in multiple brains with CT injections in the prefrontal or parietal cortex.

In the BF, Parv⁺ and Calb⁺ cells, which were retrogradely labeled from the prefrontal cortex or the parietal cortex, appeared similar in size. As for the total group of Parv⁺ cells, the Parv⁺/CT⁺ cells were often polygonal and relatively large (Fig. 1B; with average large diameter of $17.3 \pm 4.0 \mu\text{m}$ from prefrontal cortex; Table 1). Calb⁺/CT⁺ cells also were often polygonal and relatively large (Fig. 1D; with average large diameter of $16.5 \pm 3.1 \mu\text{m}$ from prefrontal cortex; Table 1). These cortically projecting CBP⁺ cells did not appear to differ significantly in shape or size from cortically projecting GAD⁺ or ChAT⁺ cells according to measurements in our previous studies (with mean large diameters of $17.7 \pm 3.9 \mu\text{m}$ and $19.1 \pm 3.9 \mu\text{m}$, respectively; Gritti et al., 1997). The BF CBP⁺/CT⁺ cells retrogradely labeled from the prefrontal (Figs. 6, 7) or parietal (Figs. 8, 9) cortex were intermingled with CBP⁻/

CT⁺ cells and collectively distributed throughout the BF in a manner similar to previously published maps of basal cortical projection neurons (Gritti et al., 1997). With CBP⁻/CT⁺ cells, the Parv⁺/CT⁺ and Calb⁺/CT⁺ cells comprised neurons that were lightly labeled (with ~5 to ~10–15 CT⁺ grains) in addition to those that were darkly labeled (with ~10–15 CT⁺, often prominent, grains; Figs. 6–9), which were tabulated together. The variation in the degree of retrograde labeling from dark to light was interpreted, as in our previous study, as reflecting the different densities of terminal innervation in the cortical target area by individual cells. Within the broader, overlapping distributions of retrogradely labeled neurons, darkly retrogradely labeled neurons from prefrontal as opposed to parietal cortex were clustered in different subregions of the BF, as previously noted (Gritti et al., 1997).

From the prefrontal and parietal cortexes, numerous BF Parv⁺/CT⁺, Calb⁺/CT⁺ as well as Parv⁻/CT⁺ and Calb⁻/CT⁺ neurons were retrogradely labeled (Figs. 6–9). The total number of retrogradely labeled cells and their apportionment across nuclei differed slightly across animals as a function of the variation in injection site placement. In addition, however, more retrogradely labeled cells were

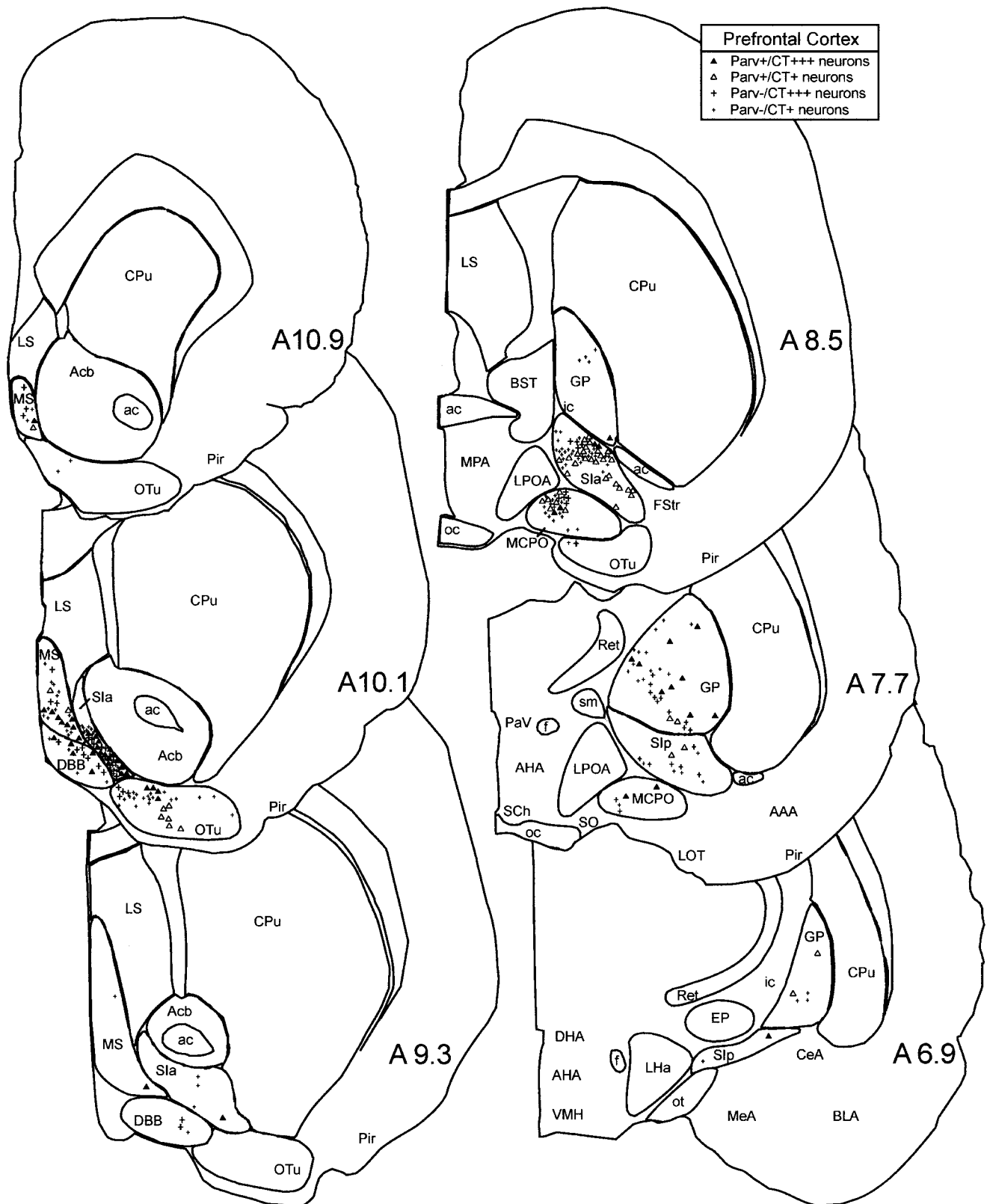


Fig. 6. Computer-based atlas figures of the basal forebrain (BF) cholinergic cell area on which parvalbumin-immunopositive (Parv⁺) and other neurons that were retrogradely labeled with cholera toxin subunit b (CT⁺) from the prefrontal cortex were mapped in a representative case. Parv⁺/CT⁺ neurons (triangles) and Parv⁻/CT⁺ neu-

rons (crosses) are codistributed throughout the BF. Darkly retrogradely labeled cells (CT⁺⁺⁺, solid symbols and thick crosses) are distinguished from lightly retrogradely labeled cells (CT⁺, open symbols and thin crosses). Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

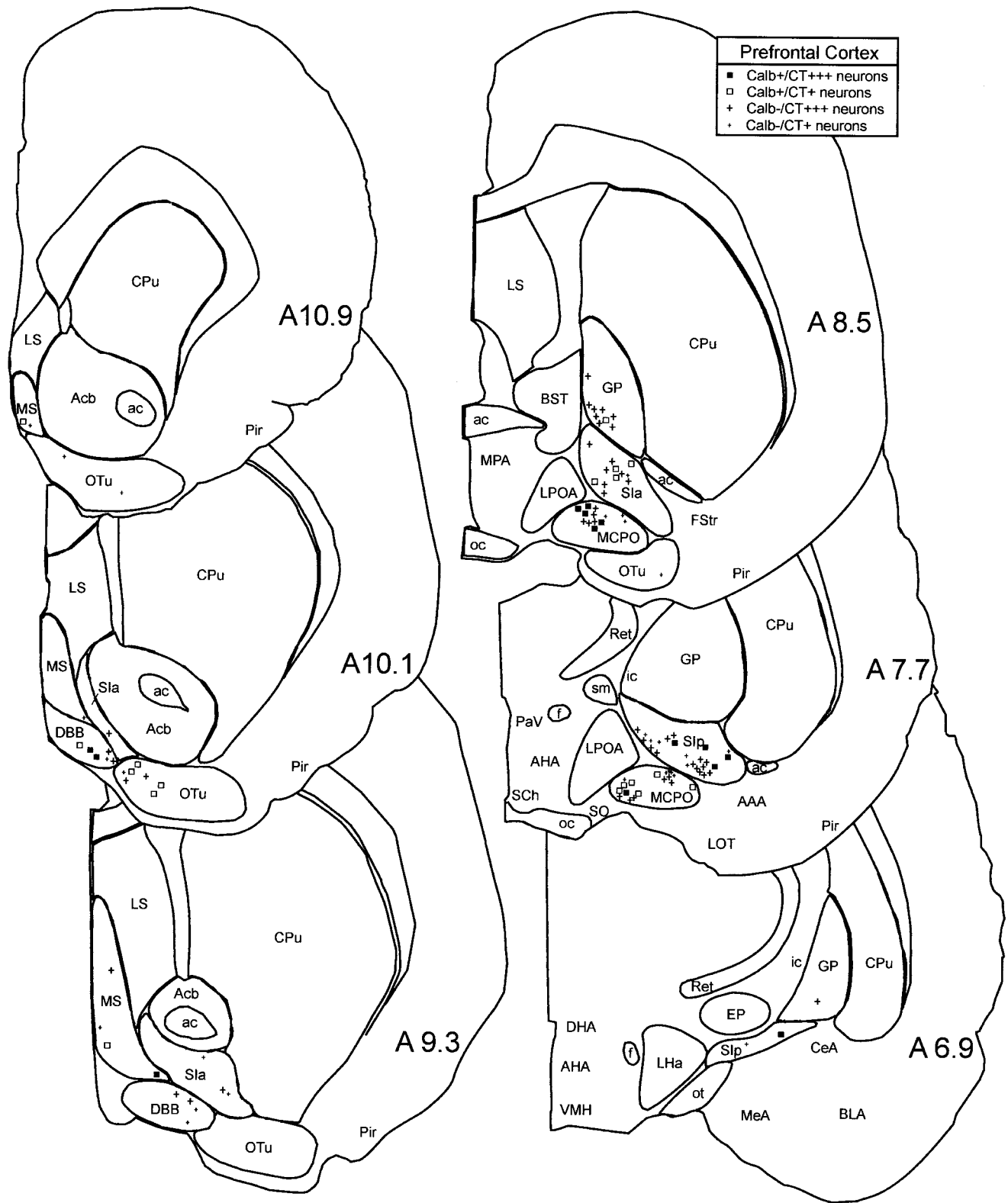


Fig. 7. Computer-based atlas figures of the basal forebrain (BF) cholinergic cell area on which calbindin-immunopositive (Calb⁺) and other neurons that were retrogradely labeled with cholera toxin subunit b (CT⁺) from the prefrontal cortex were mapped in a representative case. Calb⁺/CT⁺ neurons (squares) and Calb⁻/CT⁺ neurons

(crosses) are codistributed throughout the BF. Darkly retrogradely labeled cells (CT⁺⁺⁺, solid symbols and thick crosses) are distinguished from lightly retrogradely labeled cells (CT⁺, open symbols and thin crosses). Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

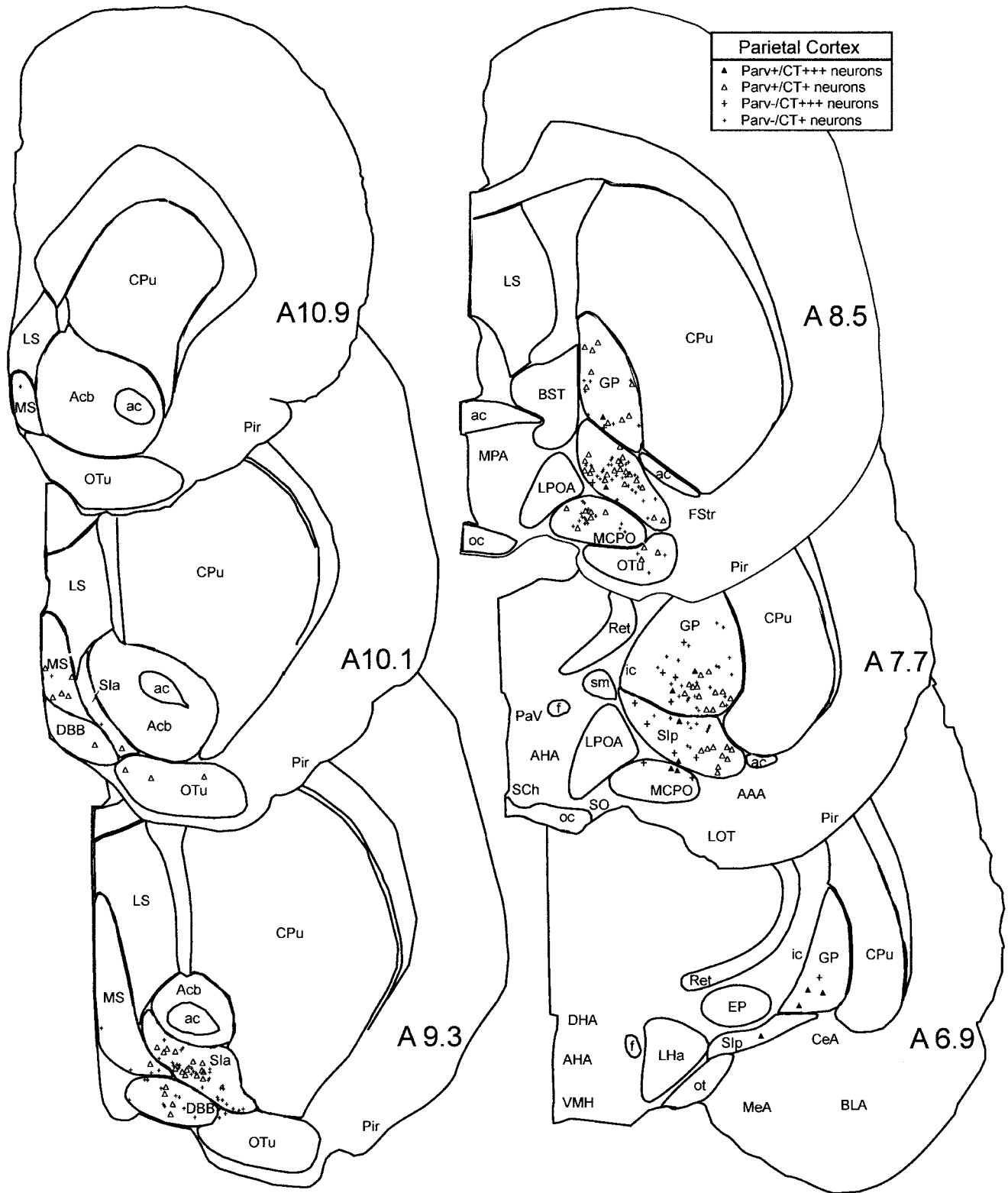


Fig. 8. Computer-based atlas figures of the basal forebrain (BF) cholinergic cell area on which parvalbumin-immunopositive (Parv⁺) and other neurons that were retrogradely labeled with cholera toxin subunit b (CT⁺) from the parietal cortex were mapped in a representative case. Parv⁺/CT⁺ neurons (triangles) and Parv⁻/CT⁺ neurons

(crosses) are codistributed throughout the BF. Darkly retrogradely labeled cells (CT⁺⁺⁺, solid symbols and thick crosses) are distinguished from lightly retrogradely labeled cells (CT⁺, open symbols and thin crosses). Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

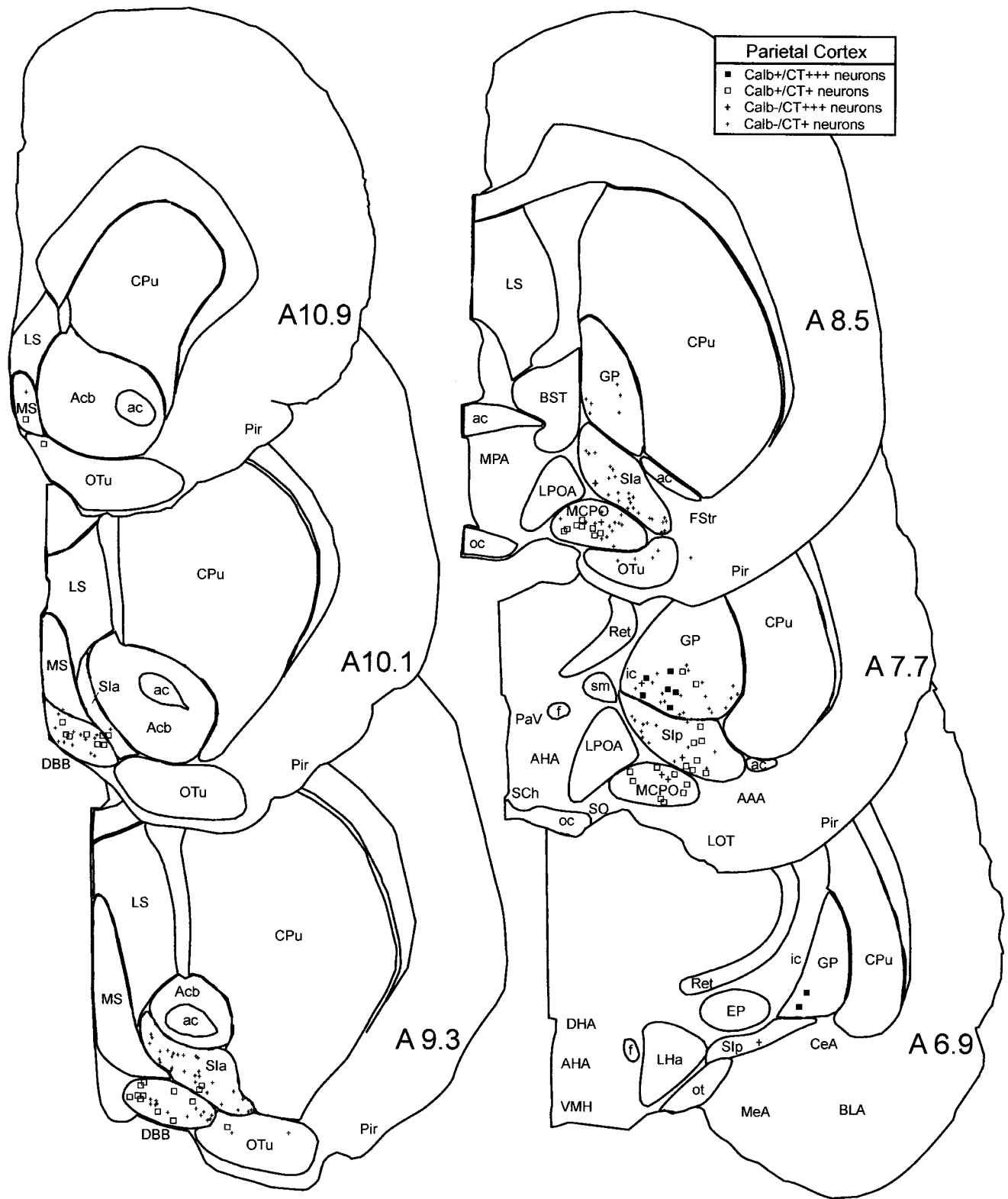


Fig. 9. Computer-based atlas figures of the basal forebrain (BF) cholinergic cell area on which calbindin-immunopositive (Calb⁺) and other neurons that were retrogradely labeled with cholera toxin subunit b (CT⁺) from the parietal cortex were mapped in a representative case. Calb⁺/CT⁺ neurons (squares) and Calb⁻/CT⁺ neurons (crosses)

are codistributed throughout the BF. Darkly retrogradely labeled cells (CT⁺⁺⁺, solid symbols and thick crosses) are distinguished from lightly retrogradely labeled cells (CT⁺, open symbols and thin crosses). Each symbol marks one labeled cell. For abbreviations, see Abbreviations list.

TABLE 2. Calcium Binding Protein Immunopositive Neurons in Immunofluorescent Material That Were Dual Immunopositive for GAD or PAG in the Basal Forebrain¹

Cell type	% CBP+			% CBP+		
	CBP+	CBP+/GAD+	(CBP+/GAD+)	CBP+	CBP+/PAG+	(CBP+/PAG+)
Parv+	98.7 ± 3.8	90.0 ± 5.0	91.2	58 ± 1.7	30.0 ± 1.0	51.7
Calb+	122.7 ± 16.9	5.3 ± 0.6	4.4	83.3 ± 1.5	37.3 ± 3.5	44.8
Calret+	158.8 ± 14.4	13.0 ± 1.0	8.2	110.0 ± 3.6	95.7 ± 5.8	86.9

¹The number of CBP⁺ neurons (mean ± standard deviation) examined across basal forebrain nuclei (except the olfactory tubercle) and the number of those that were double labeled for GAD (CBP⁺/GAD⁺) or PAG (CBP⁺/PAG⁺) are presented with the average percentage of each cell type that was double labeled (based on three dual-immunostained series per CBP from CT80, CT81, CT82, CT83, PP1 or PP2). +, Immunopositive; Calb, calbindin; Calret, calretinin; CBP, calcium binding protein; GAD, glutamic acid decarboxylase; PAG, phosphate activated glutaminase; Parv, parvalbumin.

detected in the Parv-CT than in the Calb-CT dual-immunostained series, possibly suggesting a different sensitivity in CT immunostaining depending on the antibodies used in the sequential dual immunostaining. Proportions of retrogradely labeled cells that were Parv⁺ or Calb⁺ were calculated for individual series (Table 1). Similar relative proportions of these cell groups were found when considering only the darkly retrogradely labeled cells in the BF. From the prefrontal cortex, Parv⁺/CT⁺ neurons represented on average ~38% and Calb⁺/CT⁺ neurons represented ~36% of the cortically projecting cells. Taken together, Parv⁺/CT⁺ and Calb⁺/CT⁺ cells did not account for 100% of BF CT⁺ cells that were retrogradely labeled from the prefrontal cortex (Table 1). From the parietal cortex, the average proportion of Parv⁺/CT⁺ cells was ~44% and that of the Calb⁺/CT⁺ cells was ~34% (Table 1). Altogether, they did not account for 100% of BF CT⁺ cells that were retrogradely labeled from the parietal cortex (Table 1). For the prefrontal and parietal cortexes, the combined numbers and proportions of Parv⁺/CT⁺ and Calb⁺/CT⁺ cells greatly exceeded those previously estimated for GAD⁺/CT⁺ cells, which represented ~31–37% of CT⁺ cells labeled from the same cortical areas (Gritti et al., 1997). They also somewhat exceeded the combined numbers and proportions of GAD⁺/CT⁺ and ChAT⁺/CT⁺ cells, which represented 20–27% (Gritti et al., 1997).

Colocalization of CBPs with GAD, ChAT, or PAG

To determine whether the CBPs are colocalized with the synthetic enzymes for the neurotransmitters GABA, acetylcholine, or possibly glutamate in BF neurons, dual immunostaining was performed with immunohistofluorescence for the CBPs and EPs. Dual immunostaining was assessed in individual CBP⁺ cells across the entire population through the BF nuclei, except cells in the olfactory tubercle (Table 2).

In sections dually immunostained for CBPs and GAD, a very large number (>90%) of Parv⁺ neurons was GAD⁺ (Fig. 10A,B; Table 2). In contrast, a very small number (<5%) of Calb⁺ neurons was GAD⁺ (Fig. 10E,F; Table 2). Similarly, a very small number (<10%) of Calret⁺ neurons was GAD⁺ (Fig. 10I,J; Table 2).

In sections dually immunostained for Calb and ChAT, no Calb⁺ neurons were ChAT⁺ (not shown).

In sections dually immunostained for CBPs and the synthetic enzyme for the transmitter glutamate, PAG, a proportion (~50%) of Parv⁺ cells was PAG⁺ (Fig. 10C,D; Table 2). Slightly less than half (~45%) of Calb⁺ neurons was PAG⁺ (Fig. 10G,H; Table 2). Most Calret⁺ (>85%) neurons were PAG⁺ (Fig. 10K,L; Table 2).

DISCUSSION

The present immunohistochemical results revealed that within the BF cholinergic cell area of the rat, neurons containing the CBPs Parv, Calb, and Calret are diverse in size and shape and distributed in a manner overlapping with the GABAergic neurons, but they are greater in number than the GABAergic cells therein. Parv⁺ and Calb⁺ neurons were retrogradely labeled from the cerebral cortex, like GABAergic and cholinergic neurons, but they were also larger in number than the GABAergic projection neurons. These results indicated that CBP⁺ neurons might comprise GABAergic and non-GABAergic neurons in the BF. Dual immunostaining for the CBPs and transmitter enzymes showed that, whereas the vast majority of Parv⁺ neurons contains GAD, the vast majority of Calb⁺ and Calret⁺ neurons does not. They appeared to contain PAG in significant proportions, which is the enzyme for the synthesis of transmitter glutamate. Accordingly, caudally or locally projecting neurons would include Calret⁺, possibly glutamatergic, neurons, and the basal cortical projection neurons would be comprised principally of Parv⁺, likely GABAergic, neurons and Calb⁺, possibly glutamatergic, neurons in addition to CBP⁻ cholinergic neurons.

Methodologic considerations

The initial aim of the present study was to determine whether subgroups of GABAergic neurons contain different CBPs that would allow their subsequent differentiation and identification. The findings in immunoperoxidase-stained material indicated that estimated numbers of CBP-containing neurons greatly exceeded previously estimated numbers of GAD⁺ cells. To permit this comparison, we plotted cell profiles with the aid of the same two-dimensional image analysis system and estimated cell numbers with the same method (Abercrombie, 1946) used in our original work for estimating GAD⁺ and ChAT⁺ cells in delineated nuclei of the BF (Gritti et al., 1993, 1994, 1997). Although such methods are increasingly being replaced by three-dimensional stereologic counting techniques (Coggeshall and Lekan, 1996), they nonetheless allow approximate estimates of cell numbers and are deemed appropriate when they are employed as in the present study for comparison with data obtained in the same manner (Guillery and Herrup, 1997). This comparison indicated that the combined numbers of the CBP⁺ cell groups greatly exceeded the number of GAD⁺ cells and that the combined proportions of cortically projecting CBP⁺ cell groups greatly exceeded that of the cortically projecting GAD⁺ cells. Given these results, we examined in dual-immunofluorescent stained material whether

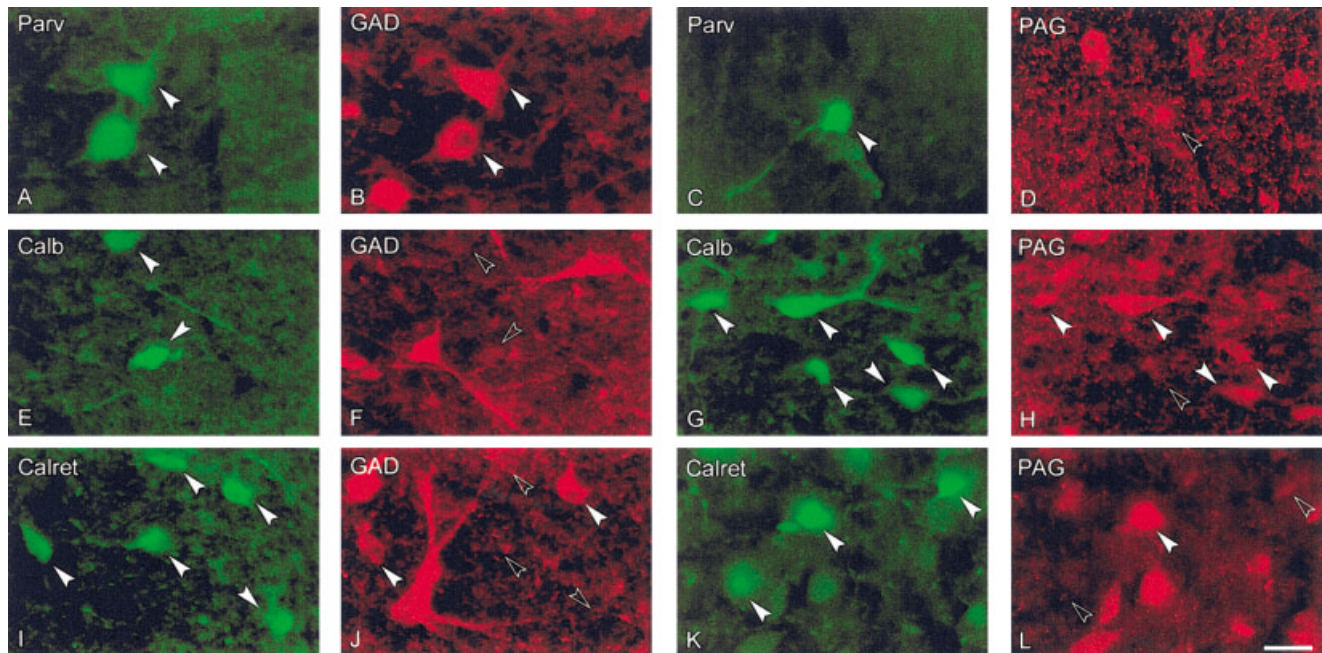


Fig. 10. Photomicrographs of sections through the basal forebrain (magnocellular preoptic nucleus) showing dual-immunofluorescent staining (using Cy2- and Cy3-conjugated secondary antibodies, in green and red, respectively) to reveal colocalization of the calcium binding proteins (CBPs; green) with enzyme proteins (EPs; red), glutamic acid decarboxylase (GAD; left panels) or phosphate activated glutaminase (PAG; right panels). CBP-immunostained cells are indicated by solid arrowheads, and EP immunostaining of the same neurons is indicated by solid arrowheads for EP⁺ neurons and open arrowheads for EP⁻ neurons. Dual immunostaining for parvalbumin (Parv) and GAD (A, B) or Parv-PAG (C, D) revealed that most Parv⁺

neurons (solid arrowheads in A) were GAD⁺ (solid arrowheads in B) and some Parv⁺ neurons (solid arrowhead in C) were PAG⁻ (open arrowhead in D). In sections dual immunostained for calbindin (Calb) and GAD (E, F) or Calb-PAG (G, H), most Calb⁺ neurons (solid arrowheads in E) were GAD⁻ (open arrowheads in F) and many Calb⁺ neurons (solid arrowheads in G) were PAG⁺ (solid arrowheads in H). In sections dual immunostained for calretinin (Calret) and GAD (I, J) or Calret-PAG (K, L), most Calret⁺ cells (solid arrowheads in I) were GAD⁻ (open arrowheads in J) and some Calret⁺ cells (solid arrowheads in K) were PAG⁺ (solid arrowhead in L). Scale bar = 20 μ m.

CBP⁺ cells were also immunopositive for GAD, ChAT, or PAG. In this examination by fluorescence microscopy, which did not allow processing by our image analysis system, we made no attempt to estimate cell numbers but simply calculated the proportions of double-labeled cell profiles, thereby permitting an estimation of the proportion of CBP⁺ cells with the capacity to synthesize GABA, acetylcholine, or glutamate, as discussed below.

Distribution of CBP-containing neurons

Parv⁺, Calb⁺, and Calret⁺ neurons varied in shape and size in the BF, as found by others in the medial septum–diagonal band nuclei (Kiss et al., 1990b, 1997), magnocellular basal nuclei (Celio, 1990; Chang and Kuo, 1991), and other forebrain structures (Freund and Buzsaki, 1996; DeFelipe, 1997; McDonald and Betette, 2001). Of the three groups of CPB-containing cells in the BF, the Parv⁺ neurons appeared to be the largest and most homogeneous in cell size, thus most closely resembling the prominent, magnocellular GAD⁺ cells in the BF (Gritti et al., 1993).

The CBP⁺ cell groups, like GAD⁺ cells, are distributed coextensively with cholinergic cells throughout the BF, thus substantiating reports by others of this distribution in the medial septum–diagonal band (Kiss et al., 1990b, 1997) and magnocellular basal nuclei of the rat (Celio, 1990; Chang and Kuo, 1991; Smith et al., 1994; Zaborszky et al., 1999). Whereas the distribution of the Parv⁺ cells

resembled that of the large GAD⁺ cells, that of Calb⁺ and Calret⁺ cells could not be simply likened to that of particular GAD⁺ cells.

The total number of CBP⁺ neurons (estimated at ~117,000) across the BF greatly exceeded that of the GAD⁺ neurons (~39,000; Gritti et al., 1993). A larger number of CBP⁺ cells than of GAD⁺ cells in the BF was noted by Zaborszky et al. (1999) and interpreted by them to mean that the number of GAD⁺ cells had been previously underestimated by us. It appeared more probable to us that neurons in addition to GAD⁺ neurons might be immunostained for CBPs, in particular for Calb and Calret. It was thus considered possible that Calb, contained in the largest number of cells, could also be contained in cholinergic neurons in the rat brain, as found in the monkey brain, despite claims to the contrary (Chang and Kuo, 1991; Ichitani et al., 1993). Given that the total number of CBP⁺ neurons actually exceeded the total number of GAD⁺ and ChAT⁺ neurons across the BF, it also appeared possible that other non-GABAergic and non-cholinergic neurons could contain CBPs.

Distribution and number of cortically projecting CBP-containing neurons

Parv⁺, Calb⁺, and Calret⁺ neurons were retrogradely labeled from the cerebral cortex, and Parv⁺ and Calb⁺ neurons were labeled in substantial numbers and thus

potentially representing the GABAergic cortically projecting cell group, in contrast to Calret⁺ neurons, which were retrogradely labeled in numbers considered too small to pursue systematically. It was also apparent in different dual-immunostained series that different numbers of retrogradely labeled cells were apparent, suggesting a differential sensitivity in the dual immunostaining for reasons of antibody competition and/or displacement. Accordingly, total numbers of retrogradely labeled neurons differed between the Parv-CT and Calb-CT series of sections, which were thus considered independently for the calculation of proportions of retrogradely labeled cells. However, we cannot be sure that these proportions were not biased by the different sensitivities and thus should be considered as estimates. We also noted, as described previously, that, of the total population of retrogradely labeled cells, many were lightly retrogradely labeled with CT (Gritti et al., 1997), which is a very sensitive retrograde tracer. To be sure that the proportions of retrogradely labeled cells calculated from the total numbers were not determined by a different density of retrograde labeling and thus projection, we also checked the proportions of the darkly retrogradely labeled cells. As established previously for the GAD⁺ and ChAT⁺ cells, the relative proportions did not change when comparing groups of darkly retrogradely labeled CBP⁺ cells. We interpreted these results to indicate that, like the GAD⁺ and ChAT⁺ cells, the Parv⁺ and Calb⁺ cell groups comprise substantial proportions of cortically projecting neurons that provide a dense terminal innervation to a focused cortical target area in addition to a potentially more dispersed innervation to surrounding areas.

Although they varied to some degree in size and shape, Parv⁺, Calb⁺, and even the Calret⁺ cortically projecting cells, like the GABAergic and cholinergic cortically projecting cells of the BF, were generally medium to large in size. Despite differences attributed to variations in injection site and sensitivity of dual-immunostaining, retrogradely labeled Parv⁺ cells and Calb⁺ cells were approximately similarly distributed through the nuclei of the BF from the medial septum to the globus pallidus and overlapped in their distribution with that of GABAergic and cholinergic neurons of the BF projecting to the prefrontal and parietal cortexes (Gritti et al., 1997).

Through the medial septum to the globus pallidus, Parv⁺ cortically projecting cells were present in substantial numbers, representing an average of ~40–45% of the cortically projecting cells across the BF. This proportion was similar to, albeit greater than, that of the GAD⁺ cortically projecting neurons (~30–37%), as calculated in our previous study (Gritti et al., 1997). The results indicated that the Parv⁺ cells could account for the GABAergic cortically projecting neurons of the BF. A similar conclusion was reached for the GABAergic neurons in the medial septum–diagonal band nuclei projecting to the hippocampus (Freund, 1989). Calb⁺ cortically projecting cells also were present throughout the BF and accounted for ~35% of cortically projecting neurons. These results were at odds with those of Smith et al. (1994) who did not find retrograde labeling of Calb⁺ cells in the BF. In view of our findings, such a negative result is surprising. It may be attributed to a lower sensitivity of the retrograde tracer (fluorogold) used or of the dual immunostaining for that tracer with Calb, which we also found to be less sensitive than that for CT-Parv. Given that the proportions of

Calb⁺ and Parv⁺ retrogradely labeled cells were ~30–45%, it appeared that both could not correspond to the cortically projecting GABAergic cells or that Calb and Parv could be colocalized in the same magnocellular GABAergic neurons. This possibility cannot be ruled out because such colocalization has been documented in the amygdala (McDonald and Betette, 2001; McDonald and Mascagni, 2001). The Calret seen in a small number of retrogradely labeled cells also could be colocalized in the same cells, given evidence for colocalization of the three CBPs in neurons within certain forebrain regions (Wouterlood et al., 2000). However, such colocalization appears less likely in the BF neurons because it is relatively rare in other forebrain structures (Gulyas et al., 1991; Kubota et al., 1993; DeFelipe, 1997) and not at all within the medial septum–diagonal band nuclei (Kiss et al., 1997). Thus, Parv⁺, Calb⁺, and Calret⁺ cortically projecting neurons likely corresponded predominantly to distinct cell subgroups and that those subgroups might not be all GABAergic.

Colocalization of CBP with ChAT, GAD, or PAG in the BF

Given the high degree of colocalization of Parv, Calb, and Calret with GAD or GABA in the neocortex, hippocampus, and the neostriatum (Kubota et al., 1993; Freund and Buzsaki, 1996; DeFelipe, 1997), we expected that such colocalization would also be frequent in the BF. Although this was the case for Parv, it was not for Calb or Calret. Of the Parv⁺ cells throughout the BF, more than 90% were dual immunostained for GAD in the present study, indicating that the vast majority of Parv⁺ cells has the capacity to synthesize GABA. In previous studies using dual immunostaining of anterogradely labeled septohippocampal fibers and terminals, it was believed that the Parv⁺ fibers emerging from the septum–diagonal band neurons contained GABA (Freund, 1989) and that the Parv⁺ neurons thus corresponded to the GABAergic septohippocampal projection neurons. Our present transmitter enzyme immunohistochemical and retrograde transport results also indicated that the Parv⁺ BF neurons have the capacity to synthesize GABA and appear to correspond in large part to the basal cortical GABAergic projection neurons. In contrast, few Calb⁺ cells (<5%) contained GAD. Such minimal colocalization of Calb with GAD had been noted in the magnocellular basal nucleus (Smith et al., 1994). We also confirmed that Calb⁺ neurons do not colocalize ChAT in the rat brain, although they had been found to do so in the primate brain (Chang and Kuo, 1991; Smith et al., 1994). We thus concluded from our retrograde and transmitter enzyme immunohistochemical results that Calb is predominantly contained within a distinct group of non-GABAergic, non-cholinergic basal cortical projection neurons. Like Calb⁺ cells, few Calret⁺ cells (<10%) contained GAD, leading us to conclude that Calret is contained predominantly in a distinct subgroup of non-cholinergic, non-GABAergic, caudally or locally projecting BF neurons.

Having recently established that the enzyme used in the synthesis of the transmitter glutamate, PAG, is localized in BF cortically projecting neurons (Manns et al., 2001), we sought to establish whether this enzyme was contained in the different CBP⁺ cell groups. In studies within the neocortex, where all CBP⁺ neurons also contained GAD, none contained PAG (Kaneko et al., 1992). The situation

was different in the present study. Approximately half of the Parv⁺ cells was also immunopositive for PAG, indicating that a proportion of these cells may have the potential to synthesize glutamate as a neurotransmitter. For GAD⁺ neurons, a similar proportion was found to contain PAG, indicating that, as in other areas of the brain, some BF neurons may have the capacity to synthesize GABA and glutamate (as discussed by Manns et al., 2001). Whether such cells use glutamate as a neurotransmitter can be determined only by immunostaining for the glutamate vesicular transporter proteins, which are evident in nerve terminals but not in cell bodies (Fremeau et al., 2001; B.E. Jones, unpublished observations). Among all Calb⁺ cells, slightly less than half was immunopositive for PAG, indicating that a significant proportion of these cells have the capacity to synthesize glutamate as a neurotransmitter. Because most cortically projecting neurons were found to be PAG⁺ (Manns et al., 2001), we concluded that the Calb⁺ cortically projecting neurons most probably contain this enzyme and are thus potentially glutamatergic. Such cells may parallel in their projections and actions the Calb⁺ glutamatergic neurons of the thalamus (Frassoni et al., 1997), which provide a widespread projection to the superficial layers of the cerebral cortex (Rausell and Jones, 1991; Jones, 1998). The vast majority of Calret⁺ neurons was immunopositive for PAG and not retrogradely labeled from the cortex, thus likely corresponding to glutamatergic neurons with caudally or locally directed projections. Such a possibility has been considered in the piriform and entorhinal cortex, where, in addition to containing glutamate, Calret⁺ cells were found to provide asymmetric, and thus excitatory, synapses to local cortical neurons (Frassoni et al., 1998; Wouterlood et al., 2000).

Functional significance of CBP content in BF cell groups

In the hippocampus and neocortex, neurons containing the CBPs, which are commonly GABAergic interneurons, have been distinguished in their electrophysiologic properties from those lacking CBPs, i.e., the pyramidal neurons, by their frequently continuous and fast spiking discharge (Celio, 1986; Kawaguchi et al., 1987; Kawaguchi and Hama, 1988; Kawaguchi and Kubota, 1993; Freund and Buzsaki, 1996). Indeed, the CBPs serve to buffer Ca²⁺ and accordingly prevent Ca²⁺-activated K⁺ currents from hyperpolarizing the membrane and inhibiting spike generation (Baimbridge et al., 1992). CBPs accordingly can also shape discharge patterns and in their absence or paucity permit phasic discharge by neurons (Li et al., 1995). Throughout the forebrain, Parv⁺ neurons are commonly fast spiking cells, as evident from *in vitro* and *in vivo* recording studies of identified neurons (Celio, 1986; Kawaguchi et al., 1987; Kawaguchi, 1993; Kawaguchi and Kubota, 1993; Sik et al., 1995). In the medial septum-diagonal band, Parv⁺ cells have been found to be fast spiking by *in vitro* recordings (Morris et al., 1999) and in the BF (in one cell) by *in vivo* recordings (Duque et al., 2000). In our *in vivo* recording studies, we identified a large number of cortically projecting GAD⁺ neurons that discharge tonically at relatively high rates (average > 30 Hz) in association with cortical activation (Manns et al., 2000a) and thus could correspond to the cortically projecting, Parv⁺ BF neurons identified here. ChAT⁺ cells that do not contain the CBPs in the rat brain (Kiss et al., 1990b, 1997; Smith et al., 1994) discharge in a slow tonic

manner and in a phasic bursting manner in association with cortical activation (Manns et al., 2000b). Other cortically projecting BF neurons that were GAD⁻ and presumed noncholinergic cells discharged tonically at a moderate rate and may correspond to Calb⁺ neurons (Manns et al., 2000a), which in other forebrain areas have been shown to discharge tonically (Li et al., 1995). Conversely, in the neocortex and hippocampus, Calb⁺ neurons displayed phasic discharge properties in addition to fast spiking (Kawaguchi and Kubota, 1993; Sik et al., 1995; Freund and Buzsaki, 1996). Thus, recently discovered GAD⁻, ChAT⁻, PAG⁺ cortically projecting BF neurons that were found to discharge at a moderate rate (average ~ 20 Hz) and to discharge in a phasic manner in association with cortical activation (Manns et al., 2000c) could also be Calb⁺ cells.

CBPs also have the capacity to prevent or attenuate damage to cells due to excessive entry of Ca²⁺ during intense activity (Scharfman and Schwartzkroin, 1989). Such protection has been thought to underlie the selective survival and, conversely, selective vulnerability of neurons containing or lacking the different CBPs (for review, see Morrison et al., 1998). Given that Parv⁺ and Calret⁺ neurons appear the most resistant and Calb⁺ neurons the least among CBP-containing neurons in the cortex of patients with Alzheimer's disease, it is also possible that similarly different degrees of resistance would apply to BF neurons. Thus, Parv⁺ GABAergic cortically projecting and Calret⁺ caudally or locally projecting neurons, documented here in the rat brain, might be expected to be more resistant than the Calb-containing cholinergic neurons in the human brain (Ichimiya et al., 1989) and other Calb⁺ potentially glutamatergic cortically projecting neurons therein.

Before gleaning more precise knowledge concerning the discharge properties and vulnerability to damage of the specific CBP-containing BF cell groups, the present results provide important information concerning the existence of noncholinergic, non-GABAergic basal cortical projection neurons and an immunohistochemical means of identifying them. From these results, it can be concluded that the basal cortical projection comprises, in addition to cholinergic neurons, likely GABAergic Parv⁺ and possibly glutamatergic Calb⁺ neurons. The existence of these noncholinergic basal cortical projection neurons may explain the finding that selective lesions of cholinergic neurons alone do not produce the serious attention and memory, as well as electroencephalographic, deficits of nonselective lesions of the entire BF cell population do in the rat (Dunnett et al., 1991; Lee et al., 1994; Voytko et al., 1994; Baxter et al., 1995). Accordingly, in addition to cholinergic and likely GABAergic Parv⁺ neurons, possibly glutamatergic Calb⁺ basal cortical projection neurons may play a critical role in modulating cortical activity and facilitating arousal, attention, and memory, functions that are greatly compromised with the degenerative changes occurring in the BF with Alzheimer's disease.

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