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Intrinsic Circuit Organization of the Major Layers and Sublayers of the Dorsolateral Prefrontal Cortex in the Rhesus Monkey

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ABSTRACT

Intrinsic connections are likely to play important roles in cognitive information processing in the prefrontal association cortex. To gain insight into the organization of these circuits, intracortical connections of major laminar and sublaminar divisions were retrogradely labeled in Walker's area 9 and 46 in rhesus monkeys by using cholera toxin (B-subunit) conjugated to colloidal gold. Microinjections placed within particular cortical laminae produced unique patterns of retrograde labeling. Injections in layers II/III yielded labeling which was laterally widespread (2–7 mm) in supragranular layers, and more narrowly focused, i.e., conforming to a column, in layers IV–VI. In contrast, local circuits associated with layers IV and Vb displayed a regular, cylindrical organization, whereas intrinsic connections of layer Va were laterally extensive (3–5 mm) in layers III and Va. Finally, injections in layer VI gave rise to a narrow column of cell labeling traversing all layers, augmented by laterally extensive labeling (~ 7 mm) in layer VI. The intrinsic connections of the prefrontal cortex were arrayed within mediolaterally elongated stripes which were often distributed asymmetrically in either the medial or lateral direction. In addition, labeled cells within these mediolaterally oriented fields were frequently grouped within discrete clusters or narrow bands. The intrinsic connections identified in this study differ from the local circuits of corresponding layers reported for primary visual cortex; the unique intrinsic wiring diagram of the prefrontal cortex may be related to its specialized cognitive and mnemonic functions.

Indexing terms: local circuits, association cortex, non-human primates, working memory, retrograde tracing

The prefrontal cortex of human and non-human primates has a long-standing association with spatial memory and other types of learned and cognitive behaviors (e.g., Stuss and Benson, 1986; Goldman-Rakic, 1987; Fuster, 1989). Neurons recorded in the primate prefrontal cortex respond in a time-locked manner to sensory, motor, and/or mnemonic contingencies during the performance of delayed response tasks (Kubota and Niki, 1971; Fuster, 1973; Niki and Watanabe, 1976; Kojima and Goldman-Rakic, 1982). In the region of the principal sulcus, for example, neurons have "best directions" or "memory fields," i.e., they increase their firing during the delay period of oculomotor delayed response tasks only for targets located at a particular locus in the visual field (Funahashi et al., 1989, 1991). In contrast, cells in ventrolateral portions of the frontal lobe are relatively insensitive to target location, but respond preferentially to faces or objects used as stimuli or memoranda (Wilson et al., 1993). In addition, neighboring pyramidal and nonpyramidal prefrontal cortical neurons exhibit synergistic profiles of activation during performance of working memory tasks (Wilson et al., 1994).

In sensory regions such as the primary visual cortex, intrinsic cortical circuits have been shown to play important roles in sculpting such hallmark receptive field properties as direction and orientation selectivity (Gilbert and Wiesel, 1979; Gilbert, 1983; see Katz and Callaway, 1992). Intrinsic connections of the prefrontal cortex undoubtedly also figure prominently in the fine-tuning of the event-related patterns of neural activity which are recorded in these association regions, yet few studies have described the anatomical organization of these connections. Thus, while Golgi impregnation and immunocytochemical studies in human and non-human primates have suggested layer-specific and tangentially organized local wiring (Schwartz et al., 1988; Mrzjlak et al., 1990, 1992; Lund and Lewis, 1993), there has been only a single anterograde tracing study examining local circuits explicitly (Levitt et al., 1993).

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Using focal injections of biocytin, this earlier study focused on the local connections of pyramidal cells, and primarily those of the supragranular layers of Walker's areas 46 and 9 in macaque monkeys (*Macaca fascicularis*). Understanding the roles of local prefrontal circuits in the cognitive and mnemonic operations of the frontal lobe, however, will require additional exploration of the organization of intracortical connections. Accordingly, we have examined the intrinsic connections of the five cellular layers (II–VI) of the prefrontal cortex in rhesus monkeys (*Macaca mulatta*). Specifically, we have analyzed and compared the laminar and tangential distribution of pyramidal and non-pyramidal neurons retrogradely labeled following microinjections of gold-conjugated cholera toxin (B-subunit) into specific laminar compartments of Walker's area 9 and 46. Our results, which confirm and extend those of the previous anterograde tracing study, indicate that the intrinsic connections of particular laminar and sublaminar divisions of the dorsolateral prefrontal association cortex differ markedly from one another. Furthermore, the patterns of intrinsic connections in areas 46 and 9 differ significantly from those of corresponding layers of area 17 (e.g., Blasdel et al., 1985; Fitzpatrick et al., 1985; Kisvarday et al., 1989), indicating that the layer-specific intrinsic connections of the prefrontal cortex are specialized to meet its unique mnemonic, spatial, and cognitive functions.

METHODS AND ANALYSIS

Surgical procedures

Tissue from five macaque monkeys (*Macaca mulatta*), aged 5–15 years was used. Under surgical anesthesia, consisting of i.v. sodium pentobarbitone (40 mg/kg), animals were placed in a stereotaxic apparatus, and craniotomies were performed to expose the dorsolateral frontal cortex in one hemisphere. Some animals were given 0.5 ml dexamethasone (I.V.) at the outset of the procedure to guard against edema. Injections of 8 or 12 nl of cholera toxin (B subunit) conjugated to 7 nm colloidal gold (0.5% in sterile water, List Biologicals), were placed by visual inspection in the portion of Walker's areas 46 or 9 lying dorsal to the principal sulcus and extending along its length (Fig. 1). Horseradish peroxidase-conjugated, unconjugated, and gold-conjugated forms of the cholera toxin subunit have been used as mixed or retrograde tracers (see Llewellyn-Smith et al., 1990) in several mammalian species. In our study, the tracer was injected by pressure through calibrated glass capillaries (tip diameter 10–50 μm), with volumes precisely controlled by visual monitoring of the meniscus within the capillaries using an eyepiece reticule and an operating microscope. Injections were placed in a hexagonal array composed of two rows containing two to four injections each and lying roughly parallel to the midline (diagramed in Fig. 1). Spacing between the two rows of injections was at least 10 mm, and within rows spacing between adjacent sites was about 10 mm; injections within the two rows were offset relative to each other, with an anteroposterior distance separating neighboring sites across rows of not less than 7 mm. Injections were made at depths ranging from 0.2 to 2.5 mm below the pial surface, with depth measured on the vernier scaled electrode carrier which secured injection pipettes. Injections within one row targeted either deep *or* superficial layers to produce an interdigitation across the injection field of supra- or granular and infragranular injections; the row containing either deep vs. superficial sites, i.e., the more medial or lateral row, was alternated across animals. On average, six injections were made in each animal, with all injections being placed in the same hemisphere; from 33 injections, 20 sites were selected for evaluation based on the confinement of the injection to an identifiable laminar compartment (see Table 1). Four of the animals used in this study also received injections of either fluorescent tracers or *Phaseolus vulgaris*-leucoagglutinin (PHA-L) in the medial prefrontal and/or motor regions of the opposite hemisphere for use in unrelated tracing studies.

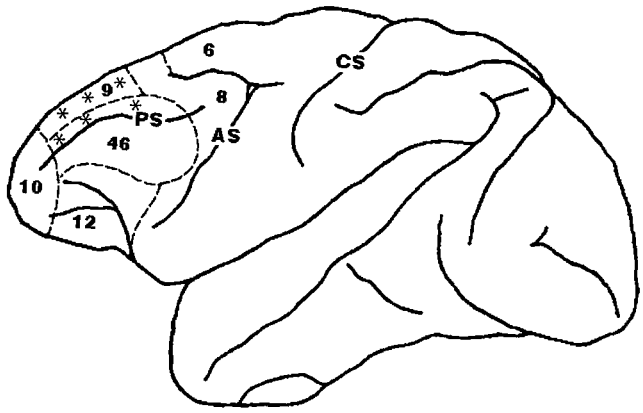

Fig. 1. A.E. Walker's (1940) cytoarchitectonic parcellation scheme of the prefrontal cortex superimposed on a diagram of the lateral aspect of the macaque cortex. Numbers correspond to Walker's designations of the fields that make up the lateral prefrontal region. Areas 46 and 9 dorsal to the principal sulcus (PS) represent the region of the prefrontal cortex injected in this study; on average, six injections, separated from one another by 7–10 mm, were made within this area in each animal. AS, arcuate sulcus; CS, central sulcus.

TABLE 1. Lateral Distance in Millimeters Measured From the Central Axis of the Injection of Neurons in Identified Layers of Dorsolateral Prefrontal Cortex Retrogradely Labeled Following Injection of Gold-Conjugated Cholera Toxin (8 or 12 nl) in Particular Cortical Layers[1]

Injections	LYR II	LYR. III	LYR. IV	LYR. V	LYR. VI
Upper II (n = 3)	1.25–1.50	0.5–1.0	0.4–0.5	0.3–0.5	0.25–0.3
Mid/lower III (n = 5)	1.25–1.50	5.0–7.0	0.4–0.5	0.5–0.75 (1.5–3.0)[2]	0.4–0.5
Layer IV (n = 3)	0.5–1.0	1.0–1.25	0.5–1.0	0.75–1.25	0.5–1.0
Layer Va (n = 4)	0.75–1.0	1.75–2.5	0.5–0.75	1.0–3.0	0.5–1.0
Layer Vb (n = 3)	0.4–0.5	0.25–0.75	0.2–0.4	0.4–0.5	0.2–0.3
Layer VI (n = 2)	1.3–2.0	1.0–3.0	0.4–0.5	2.5–3.0	5.0–7.0

[1]The range of values given for each group of injections includes *all* values of lateral distance obtained for sites evaluated in that group.
[2]Denotes lateral distance of small Nos. of outlying neurons.

Tissue preparation

Following a 10-day survival period, animals were deeply anesthetized (sodium pentobarbitone) and transcardially perfused with buffered saline (500 ml), followed by 2–31 of 4% paraformaldehyde in 0.1 M phosphate buffer (PB); for one animal, a fixative of 4% paraformaldehyde containing 0.1% glutaraldehyde was used. Following perfusion, injected regions of the dorsolateral prefrontal cortex were dissected out and cryoprotected in phosphate buffer (0.1 M) containing 10, 20, and finally 30% sucrose prior to sectioning at a thickness of 40 μm on a freezing microtome in the coronal or horizontal plane. For horizontally sectioned material, the cortex was flattened gently between two gel-subbed glass slides prior to mounting on the freezing microtome chuck. The flattened block was placed on the

chuck with the top slide kept in place and used as a guide to align the microtome blade with the cortical surface; the slide was removed from the frozen tissue block prior to sectioning by gentle warming. For both horizontally and coronally sectioned material, every section was saved through the injected zone, and every other sections was reacted to visualize injected tracer.

Tracer visualization

Colloidal gold conjugated to the cholera toxin subunit was revealed by a silver intensification protocol. Sections were washed for more than 1 hour in several changes of 0.25 M citrate-acetate buffer, pH 5.5, prior to being developed for 40 minutes to 1 hour in a solution composed of equal volumes of Initiator and Enhancer solutions (InsenSE BL, Amersham). Sections were then rinsed briefly in the citrate-acetate buffer, fixed for 10 minutes in 3.5% sodium thiosulphate, and rinsed in several changes of 0.1 M PB. Sections were then slide-mounted, counterstained with 1% cresyl violet, dehydrated, and placed under coverslips.

Three-dimensional reconstructions

Under darkfield illumination injection sites were identified as dense accumulations of gold particles, and retrogradely labeled neurons were identified by the presence of silver-enhanced gold particles in their somata and proximal processes. For every other section through a given injection site, camera lucida drawings were made charting the location of injection sites and/or retrogradely labeled neurons in relation to cortical layering identified in the cresyl-counterstained material; for each site, drawings began at the level of the injection itself and proceeded systematically anteriorly and then posteriorly for coronal sections, or dorsally and then ventrally for horizontal sections, from the parent site. Labeling was assigned to a particular injection site based on the proximity of retrogradely labeled neurons to the parent site. In nearly every case, labeling from individual sites occupied separate, non-overlapping series of coronal sections. In sections which contained labeling from more than one site, retrogradely labeled neurons were assigned to the field of labeling associated with one injection by a clear separation from labeling surrounding a second site by distances which exceeded those separating any clusters of cells produced from a single site by at least two- to threefold. The reliability of these criteria to resolve labeling associated with a specific injection site was illustrated in one animal where only three sites were broadly spaced across the entire dorsolateral extent of areas 46 and 9; the layer-specific patterns of labeling produced from these three sites were qualitatively and quantitatively indistinguishable from reconstructions obtained for injections made in corresponding layers of animals receiving six injections per hemisphere (the maximal number of sites made in a single animal in this study). The injection sites were also deliberately alternated between upper vs. middle and deeper cortical layers (see "Surgical Procedures") to maximize separation of projection fields of individual injections. Nevertheless, even as we consider the possibility remote, it is nonetheless important to include a cautionary note to indicate that a fraction of the lateral-most retrogradely labeling reported in this study may have been missed or mis-assigned. Camera lucida drawings were from a given site digitized and aligned on the basis of landmarks of vasculature and surface contours using MacStereology Software (Runturly Microsystems, U.K.). Cells located in supragranular, granular and infragranular cortical layers, as well as identified cell subpopulations, e.g., layer IIIc pyramids, were digitized as separate populations to allow their visualization both in combination and isolation. The Mac Stereology software also allowed aligned digitized images to be rotated through any angle; in this study, this feature was used to examine retrograde labeling from both coronal and tangential perspectives.

RESULTS

Tracer uptake and selectivity

Injection sites were identified under darkfield (Fig. 2A,C,E) and brightfield (Fig. 2B,D,F) illumination as blackened accumulations of silver-enhanced gold particles within which cell labeling was completely obscured. These opaque injection cores, assumed to be the zones of effective tracer uptake, were surrounded by dense but distinctly cellular labeling. The tracer volumes used in this study (8 or 12 nl) produced injection sites which appeared to be 100–500 μm in diameter. Appropriately placed sites thus lay well within the bounds of most prefrontal cortical layers and sublayers. However, it must be emphasized that the dimensions of these sites were influenced by the duration of the silver intensification procedure used to visualize the cholera-linked colloidal gold. The disparate patterns of retrograde labeling produced by injections separated by vertical distances of less than 50 μm (compare Fig. 6A,B) argue that the zone of effective tracer uptake is in fact smaller than observed dimensions.

Outside of the injection site cores, silver-enhanced gold particles were mainly found within neuronal and glial cell somata and proximal processes. However, whereas labeled glial cells were found only in proximity to injections, labeled neurons were numerous and were found up to several millimeters from injection sites in all directions. Labeled neurons were not distributed evenly, however, but rather displayed prominent anisotropies in both inter- and intralaminar distribution. Further, considerable variation was observed in the somatic density of silver-enhanced gold particles among labeled neurons which in some cases was clearly independent of the distance of the cells from injection sites. These findings were consistent with axonal uptake and retrograde transport of the tracer, and suggested that somatic gold particle density provided an index of the number or proportion of terminals a given neuron maintained within the zone of the injection site.

Neuronal labeling

Labeling of both pyramidal (Fig. 3A,B) and non-pyramidal cells (Figs. 3C,D), as evinced from the morphology of more strongly labeled neurons, was observed in every case. However, whereas labeling located within 1 or 2 mm of injection cores always included both major cell subtypes, neurons located further laterally appeared to correspond exclusively to pyramidal cells. Because labeling did not permit all cells to be unambiguously identified, the data are generally presented without specific reference to neuronal type except for neuronal subpopulations such as the giant pyramids of layers IIIc and Va, which were clearly identifiable.

Three-dimensional patterns of retrograde labeling

A total of 20 injection sites were evaluated for this study. Of these, 13 were located in area 9, and seven sites were in

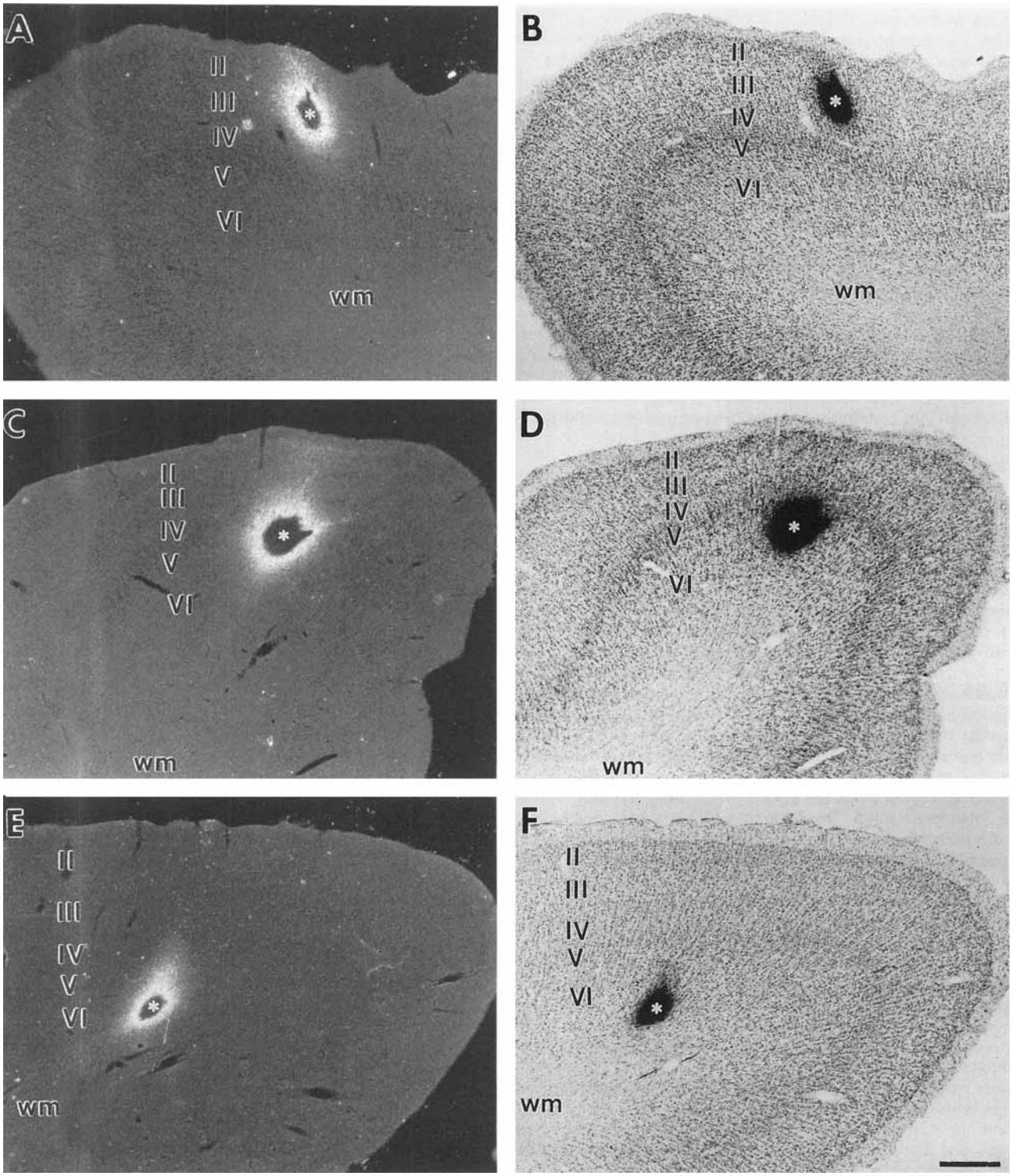

Fig. 2. Photomicrographs of representative injections (8 or 12 nl) of gold-conjugated cholera toxin imaged under darkfield (**A,C,E**) and brightfield (**B,D,F**) illumination. The injection procedures used in this study yielded circumscribed injection sites (blackened cores of silver product, asterisks) of typically less than 300 μm diameter, surrounded by a halo of dense cellular labeling, and accompanied by minimal efflux of tracer along the injection tract. These procedures thus allowed tracer to be deposited selectively within particular cortical layers and sublayers; the injections shown here are centered within layer III (A,B), layer Va (C,D), and layer VI (E,F). Each of these sites produced unique three-dimensional patterns of retrograde labeling (see text). wm, white matter. Scale bar = 500 μm.

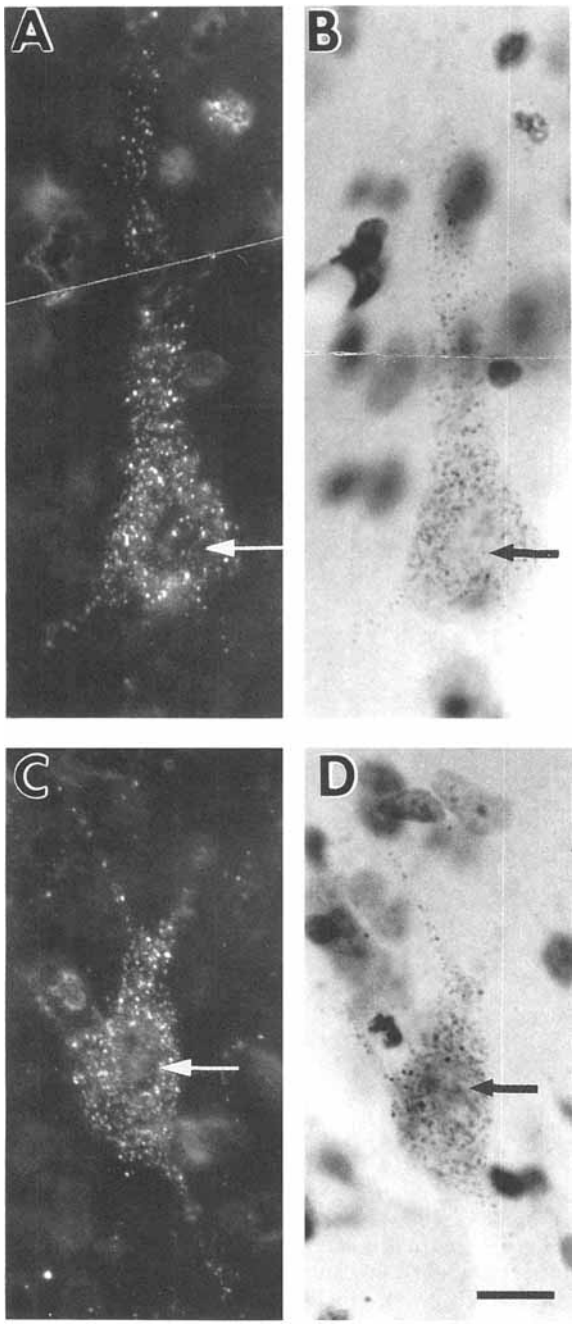

Fig. 3. Photomicrographs of representative pyramidal (**A,B**) and non-pyramidal (**C,D**) neurons retrogradely labeled following injection of 0.5% gold-conjugated cholera toxin and photographed under dark-field (A,C) and brightfield (B,D) illumination. Colloidal gold is sequestered within the cytoplasm of the soma and proximal dendrites of retrogradely labeled neurons, but is excluded from the cell nucleus (arrows). Densely intracellularly labeled neurons such as these display typical pyramidal and non-pyramidal morphology and indicate that the cholera toxin label is taken up by both of these major neuronal subclasses in primate prefrontal cortex. The patterns of labeling described in the text are thus presumed to be inclusive of both pyramidal and non-pyramidal neurons. Scale bar = 10 μm.

area 46; none of the injections selected for evaluation traversed the border region separating areas 9 and 46. The laminar location of the injection sites alone predicted distinct three-dimensional patterns of retrograde labeling, with both the configurations and dimensions of retrograde labeling being remarkably similar and consistent for injections in corresponding layers of areas 9 and 46 from animal to animal. Injections placed in supragranular, granular, or infragranular cortical layers and sublayers of these two areas have been combined for purposes of exposition. For each class of injection, e.g., supragranular or granular, qualitative and quantitative aspects of the coronal, i.e., laminar, and the tangential organization of labeling are presented. It should be emphasized, however, that the reported lateral spread of labeling may represent an underestimate of the true breadth of intracortical connections since the sparse terminal branches of axons may not provide uptake of the tracer sufficient for detection at the cell body. This potential underestimation is minimized, however, by the resolution of even lightly labeled neurons afforded by the sensitivity of the cholera toxin tracer and the very low background labeling associated with the silver enhancement procedure.

Supragranular injections: layers II/IIIa and IIIb/IIIc

Coronal reconstructions. Injections in supragranular cortical layers produced a basic pattern in which the most laterally widespread labeling occupied layers II/III, and much more narrowly focused fields of retrograde labeling characterized layers IV–VI (Fig. 4). Differences in the lateral spread and clustering of retrogradely labeled neurons in upper cortical layers, however, distinguished superficially vs. more deeply placed supragranular injections. Injections placed in layers II/IIIa, for example (Fig. 4A,A′), produced supragranular labeling which was arrayed within 1.0–1.5 mm of the center of the injection. At the lateral margins of labeling, neurons coalesced into a few loosely organized clusters which were separated from the main body of labeling by only a few hundred microns. In contrast, lower layer III injections (Fig. 4B,B′) produced more laterally extensive and patchy supragranular labeling. Thus, retrogradely labeled neurons were found 5–7 mm from the injection site. Within the first millimeter or so from the axis of the injection, labeling was homogeneous and dense and included cells in layers II and III. Further from the parent injection, however, retrogradely labeled cells formed discrete, discontinuous patches or columns. These cell clusters comprised exclusively layer III neurons, and often included two or three of the giant pyramids of layer IIIc (large spheres, Fig. 4B,B′), a cell type not prominently labeled by any other class of injection. Further, although in most cases the somatic gold-particle density of cell labeling decreased with lateral distance from injection centers, some of the most heavily labeled neurons associated with lower layer III injections were found among the cell clusters marking the lateral-most margins of labeling.

In contrast to layers II and III, the pattern of retrograde labeling in layers IV–VI appeared insensitive to the sublaminar positioning of supragranular injections. For all layer II/III sites, the transition from layer III to granular and infragranular layers was marked by an abrupt diminution in the number and intralaminar spread of retrogradely labeled cells. Thus, throughout the middle and deep cortical layers, labeled neurons were confined within single narrow columns lying directly beneath injection sites. In layer IV, labeled neurons were densely packed within 0.25–0.4 mm of the axis of the injection, whereas labeling in layers V and

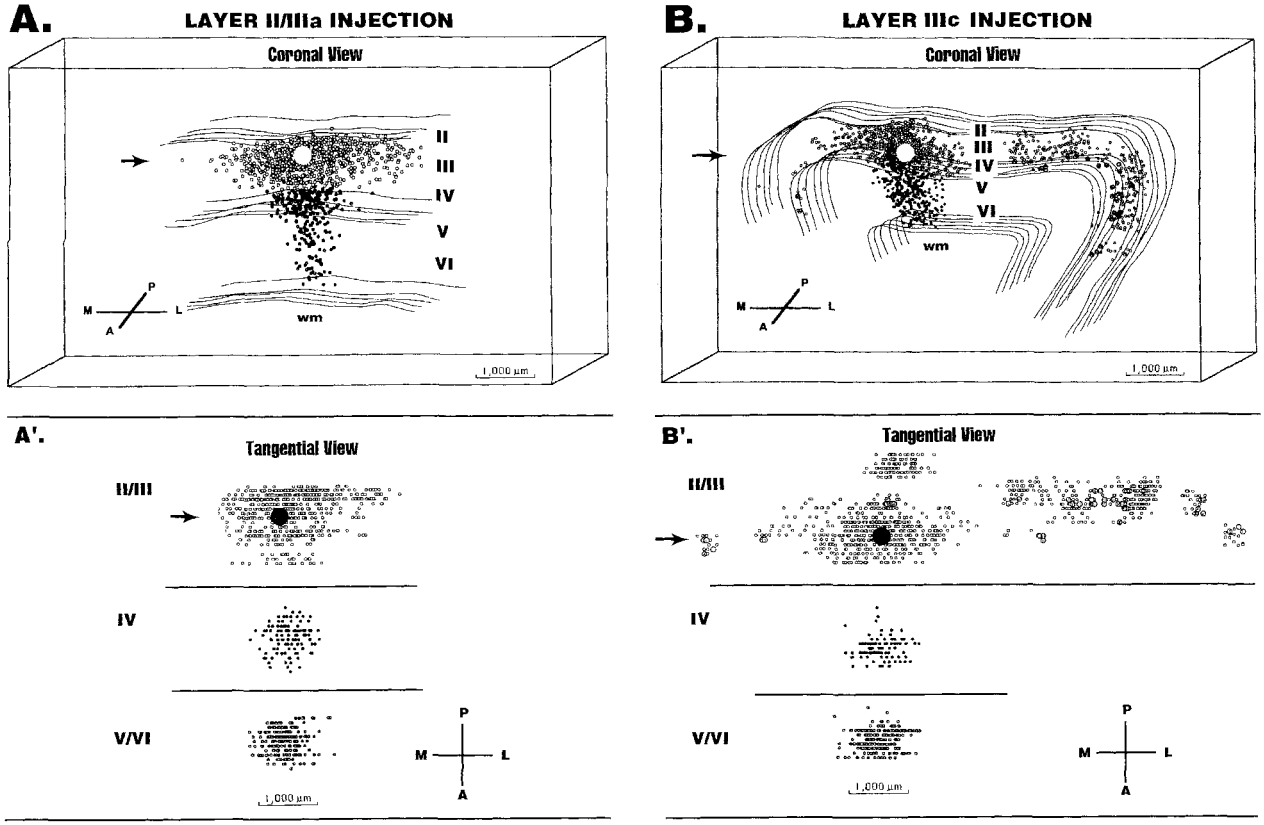

Fig. 4. Computerized, three-dimensional reconstructions of retrograde labeling produced by representative injections of gold-conjugated cholera toxin in layers II/IIIa (**A**) and IIIc (**B**) of Walker's areas 9 and 46; each injection site is presented in the coronal (A,B) and tangential (**A',B'**) perspective. In this and all computerized images small dots correspond to individual retrogradely labeled neurons: open dots are layer II/III cells, blackened dots represent layer IV cells, and gray dots depict layer V/VI cells. Larger dots depict specific cell subpopulations; in this figure, large light gray dots (B,B') mark the positions of giant layer IIIc pyramids. Injection sites are marked by large black or white dots (at the level of the arrows). Injections in layers II and upper III, and in lower layer III produce lateral labeling in supragranular layers, and a narrow column of label in granular and infragranular strata. The supragranular labeling associated with layer IIIc injections, however, is laterally widespread (up to 7 mm), patchy and includes labeling of layer IIIc pyramids (large dots); these features distinguish layer IIIc injections from those made more superficially in supragranular layers. The characteristic stripe-like array of intralaminar labeling is well illustrated in the tangential view of supragranular labeling associated with the deep layer III injection.

VI mainly involved small and medium-sized neurons which were more loosely distributed within similarly sized fields. The only exceptions to this columnar labeling came from a small number of layer V cells which were occasionally found in radial alignment with the laterally situated columns of supragranular cell labeling following deep layer III injections.

Tangential reconstructions. Horizontal reconstructions of supragranular injections revealed the clear preference, particularly for the laterally extensive fields of labeling of upper cortical layers, for the mediolateral vs. anteroposterior axis (e.g., Fig. 4B'). For deep layer III injections, for example, it was not uncommon for the spread of labeling mediolaterally to exceed that along the A-P axis by nearly an order of magnitude (e.g., 7 mm M-L vs 700 μm A-P). Labeling within the mediolateral domain was also typically offset with respect to injection site location, i.e., labeled cells extended further from the injection site in either the medial or the lateral direction. These differences were typically on the order of a few hundred microns.

The lateral extent of supragranular labeling provided an obvious contrast for superficial vs. more deeply placed layer III injections. Tangential reconstructions also confirmed the modest degree of patchiness of retrograde labeling produced by injections in layers II/IIIa, and emphasized the discontinuous organization of labeling associated with more deeply placed sites. The sizes of these latter clusters (on average 200–600 μm in diameter) and their linear separation from other patches of labeling (500–800 μm) were both considerably larger than corresponding values of cell labeling observed following more superficially placed supragranular injections, where clusters were never larger than 100–200 μm and always lay within 200 μm of the main body of labeling.

In contrast to the prominent tangential organization of supragranular labeling, retrogradely labeled neurons in granular and infragranular cortical layers showed a more homogeneous intralaminar distribution. No obvious or repeating pattern was observed in the horizontal distribution of labeled neurons in layers IV, V, or VI following any supragranular injections. Labeling in granular and infragranular layers was also distributed symmetrically about the axis of the injection.

Granular injections: layer IV

Coronal reconstructions. The prominent layer-by-layer differences in the lateral spread of labeling produced by supragranular (and infragranular) injections stood in sharp contrast to the more uniform labeling produced by injec-

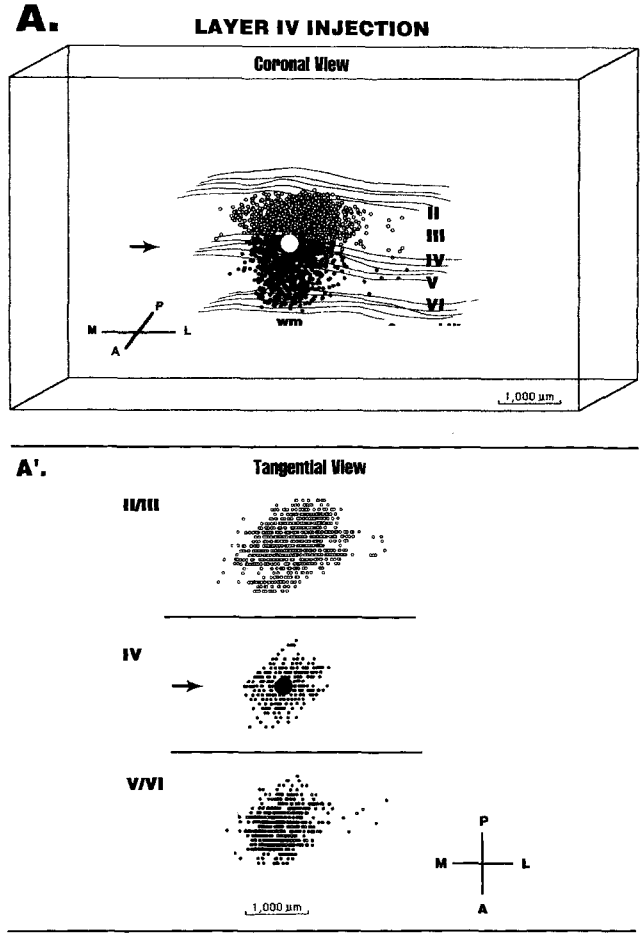

Fig. 5. Computerized, three-dimensional reconstructions of retrograde labeling produced by a representative injection of gold-conjugated cholera toxin in layer IV of Walker's areas 9 and 46, presented from the coronal (**A**) and tangential (**A'**) perspective (diagrammatic conventions as described for Fig. 4). Layer IV injections yield labeling which conforms to narrow columns (~1.0–1.5 mm in diameter) passing through all cortical layers (A). In each layer, labeling is distributed symmetrically with respect to parent injection location, and occupies similarly sized fields (see A').

tions of layer IV. Thus, following injections in the middle granular layer, retrograde labeling conformed to what was in essence a column some 1–2 mm in diameter, which spanned all cortical layers (Figs. 5A,A'). Labeling was most dense and tended to occupy the full 2 mm diameter in supragranular layers, and diminished slightly in density and diameter in granular and infragranular laminae. Labeling in lower layer VI was the most sparse and occupied a width only about half of that observed in layers II/III.

Tangential reconstructions. The columns of labeling produced by injections in layer IV were composed of intralaminar fields of retrogradely labeled cells in all layers which were distributed more or less symmetrically about the axis of the injections. No obvious clustering or tangential organization among labeled cells was evident within any of the cortical layers (see Fig. 5A').

Infragranular injections: layer Va

Coronal reconstructions. Injections placed in upper layer V (layer Va) gave rise to a unique, bistratified pattern of labeling in which retrogradely labeled cells stretched 3–5 mm from the axis of the injection in layers II/III and Va (Fig. 6A,A'). However, whereas lateral labeling was present in both supra- and infragranular layers, their intralaminar patterns were markedly different. In layers II/III, for example, laterally labeled neurons coalesced within one or two relatively broad yet distinct cell columns (0.6–0.8 mm in diameter), whereas labeling in layer Va involved a specific subpopulation of medium-sized pyramids which were more evenly distributed along the top of layer V (cells represented as large spheres in Fig. 6A,A').

Labeling in remaining layers, i.e., layers IV, Vb, and VI, lay within 1–2 mm of the axis of the injection. The relatively widespread labeling in layer III thus provided a sharp contrast to the restricted pattern of cell labeling which marked the transition with layer IV, where few neurons were labeled, and those that were confined to a radius of about 0.5 mm. Similarly, the lateral distribution of retrogradely labeled neurons in layer Va stood out against the labeling in layers Vb and VI where very few labeled cells strayed beyond 1–2 mm from the axis of the injection.

Tangential reconstructions. The laterally extensive fields of labeling produced by injections in layer Va were arrayed within mediolaterally oriented stripe-like fields. Although these fields were somewhat foreshortened (e.g., compare with Figs. 4B,B', 7A,A'), a striking feature was their prominent asymmetry along the medial or lateral axis. Thus, whereas in some cases retrograde labeling was distributed more or less equidistantly along medial and lateral domains, more typically injections in layer Va produced neuronal labeling which extended several millimeters in one direction, and on the order of hundreds of microns along remaining axes (Fig. 6A').

Tangential reconstructions underscored layer-specific differences in the tangential organization of labeled neurons in layers II/III vs. Va. Thus, in layer III, labeled neurons located beyond the central focus of labeling formed discrete clusters, some 0.6–0.8 mm in diameter, which were clearly separated from one another by regions of similar breadth containing little or no labeling. In marked contrast, labeled neurons in layer V showed no obvious columnar organization, but rather were grouped into a series of narrow, discrete stripes (200–400 µm across) oriented along the mediolateral axis and separated from one another by intervening narrow strips (~200 µm wide) containing few or no labeled neurons.

Infragranular injections: layer Vb

Coronal reconstructions. Injections in layer Vb produced labeling which was similar in some respects to the columnar pattern of labeling observed following injections in layer IV (Fig. 6B,B'). However, injections in layer Vb gave rise to columns of cell labeling which were generally less than half the diameter of that associated with injections in layer IV, i.e., only 0.5 mm in diameter. In addition, layer Vb injections also revealed uniquely robust vertical links between layers V and II/III. Layer II in particular contained a band of unusually strongly labeled neurons lying directly above the infragranular injection sites. Labeling in granular and infragranular layers, however, was much more sparse, involving very few neurons even in the immediate vicinity of the injection sites. However, layer Vb injection sites were unique in labeling the giant layer Va pyramids; these cells, although few in number, were very heavily labeled and were arrayed in close proximity to the axis of the injections (cells represented as large spheres in Fig. 6B,B').

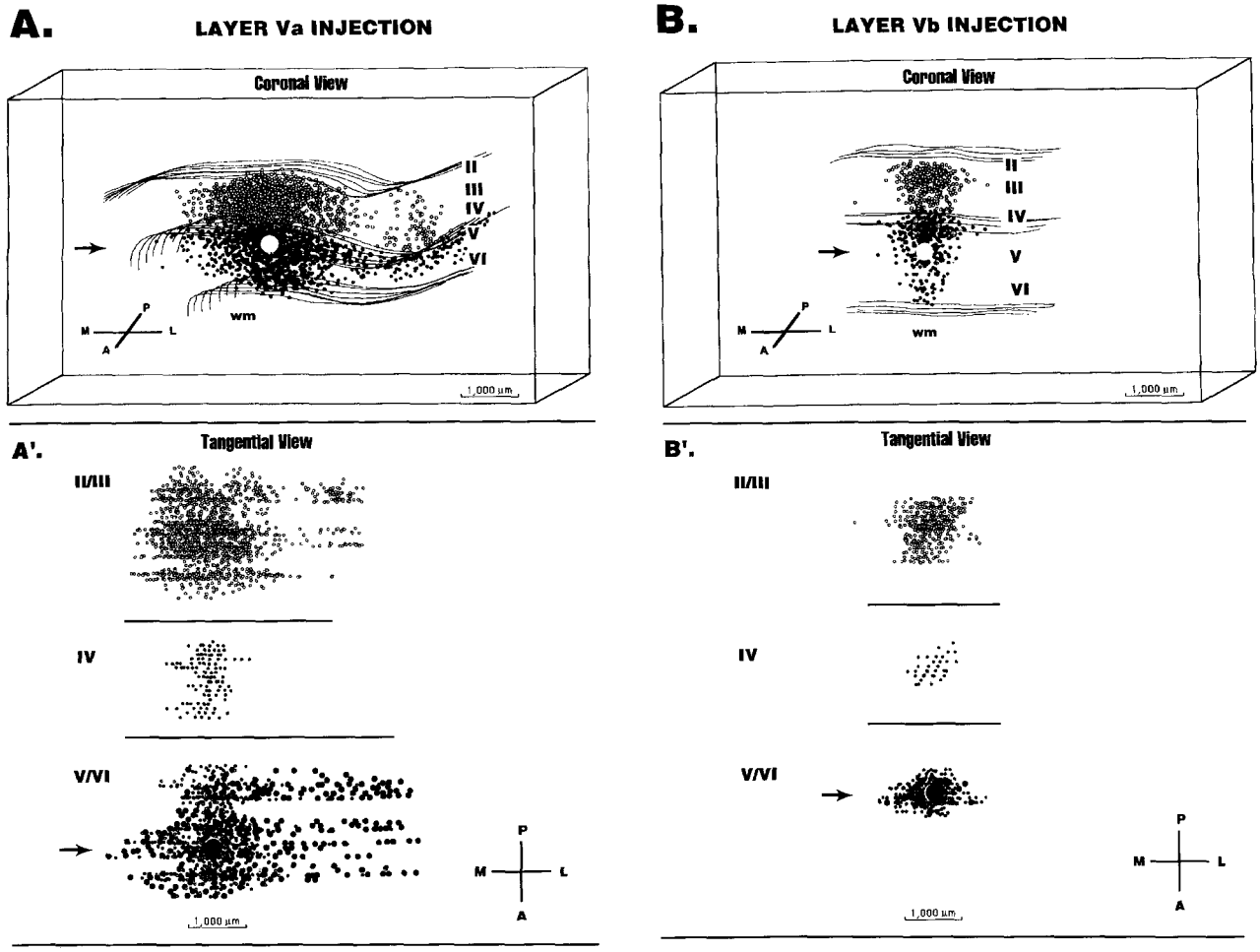

Fig. 6. Computerized, three-dimensional reconstructions of retrograde labeling produced by representative injections of gold-conjugated cholera toxin in layers Va (**A**) and Vb (**B**) of Walker's areas 9 and 46; each injection site is presented in the coronal (A,B) and tangential (**A'**,**B'**) perspective (diagrammatic conventions as per Fig. 4). Injections placed in upper layer V (layer Va) produce laterally widespread labeling in layers II/III and Va superimposed upon a narrow columnar labeling which traverses all layers (A). Lateral labeling in layers II/III is patchy, whereas that of layer Va is distinctly stripe-like, and composed mainly of a subpopulation of medium-sized pyramids located just beneath layer IV (large dark dots). In both layers, labeling is also elongated along the mediolateral axis (A'); the preference of label for the lateral vs. medial axis typical of layer Va injections is well illustrated (A'). Injections made in layer Vb, on the other hand, yield highly circumscribed columns of labeling, often less than 1 mm in diameter, which pass through all cortical layers (B). Labeling is particularly sparse in layer IV, whereas layer V is distinguished by labeling of a small number of giant layer Va pyramids (large dots, B,B').

Tangential reconstructions. The intralaminar fields of labeling associated with layer Vb injections, like those of layer IV sites, were essentially circular and symmetrically centered about the axis of the injection in all layers. The typically small numbers of cells occupying the narrow intralaminar fields did not display any obvious or repeating tangential organization.

Infragranular injections: layer VI

Coronal reconstructions. Injections in layer VI produced arrays of retrograde labeling which were in some respects mirror images of those produced by injections in supragranular layers. Thus, retrograde labeling was widespread in infragranular layers and more narrowly focused in granular and supragranular layers (Fig. 7A,A').

Labeling was generally sparse in layers I–IV, with only small numbers of modestly labeled cells scattered within a single distinct column (0.25–0.5 mm radius) surrounding the axis of the injection. Labeling was particularly diffuse in layer IV. The density and lateral distribution of retrograde labeling increased dramatically, however, in infragranular layers. In layer V, for example, labeled somata were scattered up to 1–3 mm from the axis of the injection, and an even greater lateral spread of labeling was found in layer VI where retrogradely labeled cells were distributed more densely and uniformly up to 7 mm from injection sites.

Tangential reconstructions. The laterally extensive fields of labeling associated with layer VI injections conformed to distinctly stripe-like intralaminar fields which stretched preferentially along the mediolateral axis (Fig. 7A'). As in layer Va injections, these fields were often sharply skewed with respect to the medial or lateral direction. Thus, it was not uncommon for the lateral spread of label to differ by an order of magnitude, e.g., 0.5 mm vs. 5 mm, among these directions.

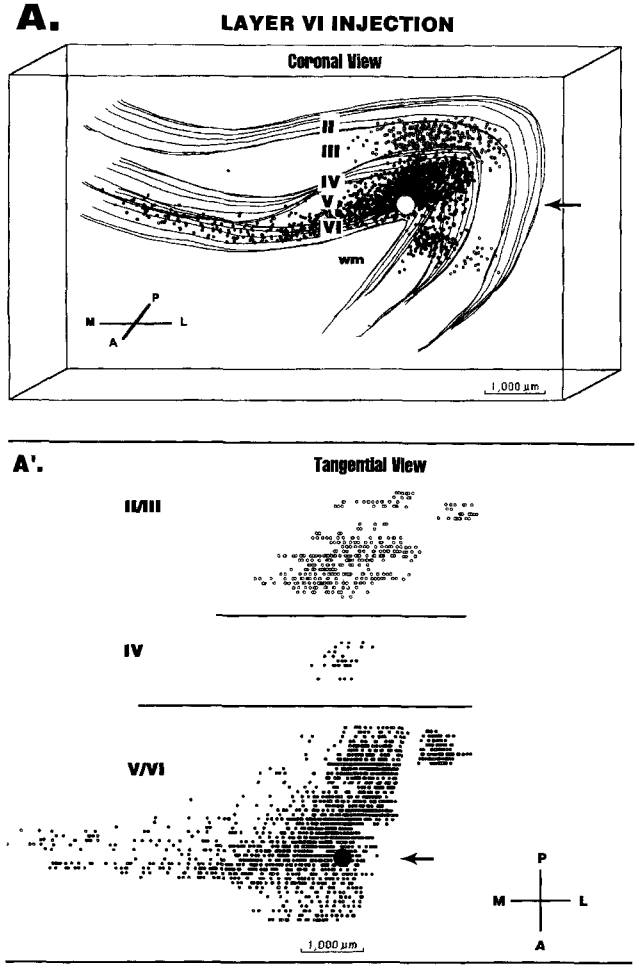

Fig. 7. Computerized, three-dimensional reconstructions of retrograde labeling produced by a representative injection of gold-conjugated cholera toxin in layer VI of Walker's areas 9 and 46 presented from the coronal (**A**) and tangential (**A'**) perspective. Injections in layer VI give rise to prominent lateral labeling in layers V and VI, and narrow, radial labeling in remaining layers (A); in the injection shown, an additional small column of label located laterally to the main body of labeling is also apparent (A,A'). The infragranular labeling associated with layer VI injections is arrayed within mediolaterally elongated fields, composed of more or less homogeneously distributed cells, and often shows a marked preference for the medial or lateral direction; in the site illustrated, labeling stretches further in the medial vs. the lateral direction by nearly an order of magnitude.

Intralaminar labeling associated with layer VI injections was unique for the largely homogeneous distribution of its composite neurons. In sharp contrast to the highly patterned lateral labeling observed in layers II, III and V (observed in conjunction with injections in other layers), the laterally dispersed intralaminar cell labeling in layer VI showed no obvious discontinuities in tangential organization. In some cases, however, medial or lateral sectors left empty of cell labeling by the anisotropic pattern of infragranular labeling were punctuated by small clusters of labeled cells which traversed layers II, III, V, and VI (as shown in representative injection site in Fig. 7).

A note about layer I. Retrograde labeling observed in this study in layer I was inconsistent; although some weak cell labeling in this layer was sometimes observed, no systematic relationship between the distribution of labeling and the location of injection sites could be discerned. It is uncertain whether this sparse labeling reflects the true connectivity of layer I cells, i.e., indicates that the axons of layer I cells are largely confined to this uppermost layer, which was not targeted by any of our injections, or whether layer I cells do not express GM1 gangliosides on their surface and are thus unable to take up the cholera toxin label (Ledeen and Mellanby, 1977). Because of this uncertainty, we were unable to interpret findings in layer I.

DISCUSSION

The present study was undertaken to provide insight into the layer-by-layer organization of intrinsic connections of the primate dorsolateral prefrontal cortex. Our findings indicate that each layer of the prefrontal cortex demonstrates a signature pattern of intrinsic connectivity, which falls into one of three broad organizational schemes: 1) a simple, narrow interlaminar column of connections, as is observed in layers IV and Vb; 2) columnar connections augmented by patchy horizontal connections, such as seen in layers II and III and 3) columnar labeling accompanied by diffuse horizontal connections, which exemplifies the local circuits of layers Va and VI. Importantly, these patterns reflect layer-specific differences in local circuit organization rather than, for example, a greater or lesser lateral transport of tracer in larger or smaller constituent neurons of the various cortical layers. The giant pyramids of layer Va, for example, are associated with some of the most narrowly restricted local lateral connections in the prefrontal cortex, whereas the much smaller pyramids of layer VI give rise to lateral connections which course over several millimeters. Finally, the injection sites analyzed in this study were surrounded by circumscribed, cohesive patterns of labeled cells which were clearly separated from the projection fields of other sites. Nevertheless, because multiple injections of the same tracer were made in single animal subjects, the possibility must be borne in mind that a portion of the lateral-most label associated with some sites may have been mis-assigned. This possible mis-assignment, however, can only be expected to apply to a small number of sites since for most of the injections analyzed, labeling associated with one or another site occupied non-overlapping sets of coronal sections (see "Methods and Analysis"). Below, the intrinsic radial, *inter*laminar and horizontal, *intra*lamaninar connections revealed within the specific layers of the dorsolateral prefrontal cortex are discussed in relation to known aspects of its modular organization.

Intrinsic interlaminar connections and columnar cortical organization

Virtually every injection made in our study produced a distinct column of cell labeling 1–2 mm in diameter centered on the axis of the injection, and spanning all cortical layers. Nonetheless, patterns of interlaminar connectivity associated with specific cortical layers were distinguished by features including the diameter of retrograde cell labeling, the relative weighting of inputs from particular layers, and, less frequently, the participation of specific subpopulations of neurons in local radial connections. Layer Vb, for example, was unique in receiving a strong and focused input from layer II and from the giant pyramidal cells of layer Va. Injections in layers Va and VI, on the other hand, had characteristically sparse interconnections with layer

IV, and with layers II, III, and IV, respectively. The observed layer-specific organization of intrinsic radial links suggests that particular classes of afferents, e.g., thalamocortical, corticocortical, which terminate in specific strata may in turn be relayed differentially among cortical layers. Such a scenario has been established in the primary visual cortex where parvi- and magnocellular streams of visual information terminate in sublayers IVCa and IVCb, respectively, and from there are conveyed to different and largely non-overlapping layers of area 17 via the specific sets of intrinsic connections which arise from these two granular sublayers (e.g., Valverde, 1971; Lund and Boothe, 1975; Blasdel et al., 1985; Fitzpatrick et al., 1985).

In addition to their laminar specificity, afferent terminations within the primate prefrontal cortex also exhibit precise tangential organization. Projections arising from the mediodorsal nucleus of the thalamus, and contra- and ipsilaterally located cortices, for example, project to disjunctive, often interdigitating cortical columns, some 0.3–0.8 mm in width (Goldman-Rakic and Schwartz, 1982; Giguere and Goldman-Rakic, 1988). Likewise, efferent neurons projecting back to these same areas (Arikuni et al., 1983; Schwartz and Goldman-Rakic, 1984; Giguere and Goldman-Rakic, 1988) are also grouped within clusters which range from 0.1 to 2 mm in diameter. The intrinsic interlaminar connections of the prefrontal cortex described in this study, however, are unlikely to be associated with the territory of any one particular efferent or afferent column. For example, the disparity between the size of cortical columns defined by afferent or efferent neurons (e.g., Arikuni et al., 1983; Schwartz and Goldman-Rakic, 1984; Giguere and Goldman-Rakic, 1988; Levitt et al., 1993) indicates that a close fit between these modules and intrinsic radial connections can only be expected for a subset of the latter, efferent stripes. Perhaps more importantly, however, the intrinsic interlaminar connections are invariably centered about the axis of the injection; if the local radial connections were systematically related to efferent, or indeed any extrinsic columnar system, the distribution of labeling would be expected to be skewed, at least part of the time, relative to parent injections. Rather, the radially directed intrinsic connections described here may represent elements which repeat across the cortex and contribute to the microstructure of larger modules of columns.

Although the precise relationship between local interlaminar connections and cortical columnar organization has yet to be determined, the role of these vertical links is likely to be analogous to that hypothesized for visual cortices, i.e., transmission of information from layer to layer in a manner which preserves the integrity of spatial maps (e.g., Lund and Boothe, 1975; Mitzdorf and Singer, 1979; Gilbert and Wiesel, 1979; Lund et al., 1981). Although the association cortices have not traditionally been thought of in terms of such orderly representations, recent findings of neurons in the dorsolateral prefrontal cortex encoding all points of visual space (e.g., Funahashi et al., 1989, 1991) suggest that visual, somatotopic, and auditory maps also exist within the prefrontal cortex. The narrow interlaminar links common to all layers of the dorsolateral prefrontal cortex may provide the vertical synthesis of specific types of information with respect to these representations.

Intrinsic intralaminar connections and horizontal cortical organization

Modern intra- and extracellular tracing methods have confirmed the complex and discontinuous patterns of local horizontal connections in primary motor and sensory cortices (Gilbert and Wiesel, 1979, 1983; Landry et al., 1980; Martin and Whitteridge, 1984) first described in degeneration studies (Szentagothai, 1965; Fisken et al., 1975; Creutzfeldt et al., 1977). Thus, in both visual and auditory regions, intracortical axons have been found which travel up to several millimeters laterally, and which emit clusters of terminal arbors at regular intervals along the trajectory (e.g., Kisvarday et al., 1986; Gabbott et al., 1987; McGuire et al., 1991; Ojima et al., 1991, 1992). Further, in primary visual cortex, local horizontal connections of layer III interconnect radial clusters of neurons with similar orientation tuning (e.g., Toyama et al., 1981; Michalski et al., 1983; Ts'o et al., 1986; Ts'o and Gilbert, 1988; Hata et al., 1988), whereas in the primary auditory cortex corresponding intralaminar connections are primarily made between groups of cells which respond best to similar tone frequencies (Matsubara and Phillips, 1987).

The intralaminar connections of supragranular layers of the prefrontal cortex also appear to be patchy. Thus, a recent anterograde tracing study in fascicularis monkeys using biocytin revealed a discontinuous distribution of horizontally directed supragranular axons following focal injections centered in layers II and III of Walker's areas 46 and 9 (Levitt et al., 1993), and in the present study in rhesus monkeys, we observed distinct patches in retrogradely labeled local intralaminar connections which were quantitatively and qualitatively similar. The question thus arises whether these patchy prefrontal circuits relate in some specific way to the modular organization of cortical efferents, afferents, and/or receptive field properties.

Reconstructions of most columnar systems of the cortex have shown that when viewed from a tangential perspective, these modular units coalesce across cortical space to form stripes, (e.g., Hubel and Wiesel, 1977). The disjunctive fields of afferent terminals and efferent neurons of the prefrontal cortex are no exception; columns defined by these fields correspond to a series of discrete, interdigitating stripes which are in most cases oriented roughly anteroposteriorly (see Goldman-Rakic, 1984). However, the horizontally directed intrinsic connections revealed both in the present investigation and in the earlier anterograde tracing study (Levitt et al., 1993) run mediolaterally and may thus be deployed orthogonally to efferent and afferent stripes. Horizontally extensive labeling in supragranular layers, however, corresponded mainly to discrete, patchy arrays of neurons some 0.4–0.8 mm in diameter and separated from one another by stretches of cortex of similar breadth containing few or no labeled cells. These dimensions are a good match to the stripe and interstripe widths of major corticocortical and thalamocortical projections (also see Levitt et al., 1993). It is thus possible that the lateral local connections which arise from neurons in layers II/III and project to layers II/III and V may function to conjoin or process the sensory, mnemonic, or limbic information relayed to the supragranular of the prefrontal cortex from various cortical and/or thalamic centers. In infragranular layers, however, lateral labeling fails to show any obvious tangential clustering which might bring local circuits into register with disjunctive efferent or afferent territories. Instead, a unique feature of the horizontally extensive labeling of infragranular layers is the marked anisotropy of labeling with respect to injection site location, i.e., the parent injection is found anywhere from the center of the field to the extreme medial or lateral margins. This selective positioning of the *field* of labeling itself could thus

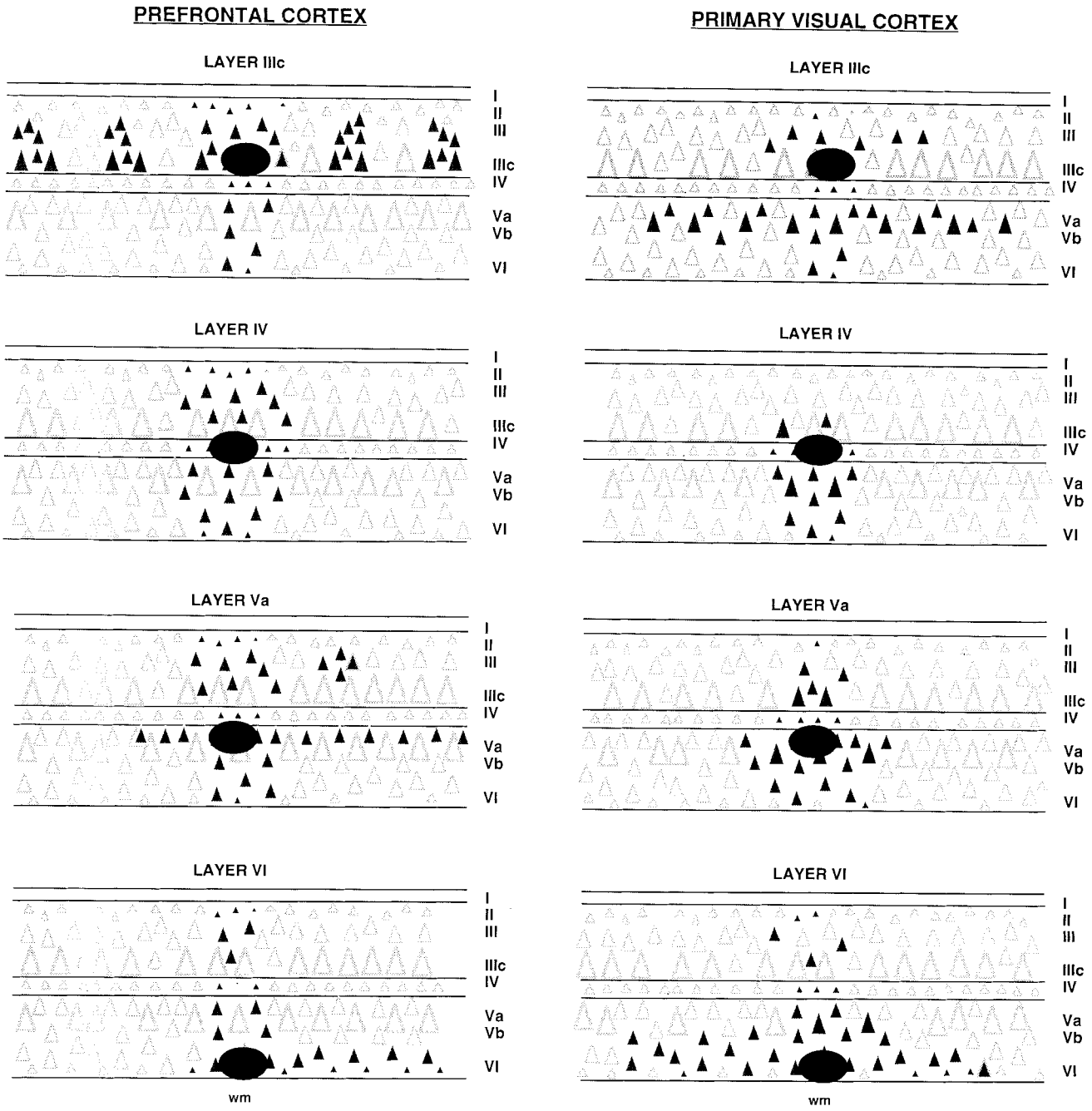

Fig. 8. Summary diagram illustrating the layer-specific patterns of intrinsic connections retrogradely labeled in Walker's areas 46 and 9 of the dorsolateral prefrontal cortex of the rhesus monkey (left-hand column). Patterns of retrograde labeling obtained in a previous analysis of macaque primary visual cortex, based on the study of Kisvarday et al. (1989), which utilized the laminar classification scheme for area 17 of Lund and Boothe (1975), is presented in the right-hand column for comparison. The patterns of labeling from the study of visual cortex were similar among all subdivisions of layer IV and for upper vs. lower aspects of layer V. Thus in this illustration, the sublaminae of layer IV have been collapsed and depicted as a single layer. The panel labeled Va for visual cortex represents the pattern of labeling illustrated for injections made in the upper one-half of layer V. In nearly every corresponding layer, qualitative and/or quantitative differences in local circuit organization distinguish the association cortex of the frontal lobe from visual cortex of the occipital lobe (see text for further detail).

serve to bring the lateral connections of deep cortical layers into alignment with contiguous sets of afferent or efferent stripes, and may reflect an end stage in the integration of corticocortical and/or thalamic information from the two hemispheres since callosal and associational efferent systems are contiguous (Schwartz and Goldman-Rakic, 1984).

Areal specialization of intrinsic cortical connections

Recent extracellular tracing (Yoshioka et al., 1992; Kritzer et al., 1992; Amir et al., 1993) studies in primary and visual association cortices of primates have uncovered subtle

differences in the organization of intracortical circuits even among regions devoted to a common modality. It is thus not surprising that the intrinsic connections of the prefrontal association cortex observed in this study show apparent qualitative differences from those previously described in primary visual cortex—the only cortical area where detailed, layer-by-layer information on intrinsic circuit organization is available in the primate (see Fig. 8). For example, injections of retrograde tracers such as horseradish peroxidase (HRP) and ^{3}H-D-aspartate, and anterograde tracers such as biocytin (Lund et al., 1993) in supragranular layers of the primary visual cortex produce relatively narrow columns of labeling in layers II, III, IV, and VI, and a single band of laterally widespread labeling (up to 2 mm from the axis of the injection) in layer V (Blasdel et al., 1985; Fitzpatrick et al., 1985; Kisvarday et al., 1989). In marked contrast, injections in layers II and III of the prefrontal cortex give rise to dense and laterally widespread labeling (up to about 7 mm) in supragranular layers, and radially restricted, columnar labeling in deep laminae including layer V. The intrinsic connections of layer IV also differ; although in both prefrontal and visual cortices cortices local connections are confined to narrow columns, the local inputs to *all* subdivisions of layer IV of the primary visual cortex are derived mainly from layers IV, V, and VI (Blasdel et al., 1985; Fitzpatrick et al., 1985; Kisvarday et al., 1989), whereas layer IV of the prefrontal cortex receives projections from all cortical laminae. Disparate intrinsic connectivity likewise marks layer V where no injections placed within layer V of area 17 (see Kisvarday et al., 1989) produced a pattern of label similar to either the bistratified pattern of label associated with prefrontal layer Va injections or the narrow, columnar organization of label typical of prefrontal layer Vb injections. The only layer which appears to have a similar laminar patterning of intrinsic connections in both areas is layer VI; in both sensory and association areas, layer VI receives an essentially columnar input from layers II–IV and more laterally widespread projections from layers V and VI (Kisvarday et al., 1989). The tangential organization of the intrinsic connections of layer VI, however, does distinguish the local circuits of visual and association areas, including those in layer VI. Thus, the essentially circular intralaminar distribution of labeling observed following focal injections of HRP (e.g., Rockland and Lund, 1983; Livingstone and Hubel, 1984) or biocytin (Yoshioka et al., 1992; Lund et al., 1993) in all layers of primary and visual association cortices contrasts with mediolaterally elongated fields labeled in the prefrontal cortex by both anterograde (Lund et al., 1993; Levitt et al., 1993) and retrograde tracers.

Importantly, although comparisons between prefrontal and visual cortex contrast studies using different techniques and neuroanatomical tracers, it is unlikely that methodological differences alone account for the differences in the laminar and tangential organization of local circuits in these two areas. First, studies in primary visual cortex using four different types of tracers, including the carbocyanine dye DiI in the human brain (Burkhalter and Bernardo, 1989), and retrograde and anterograde tracers in nonhuman primates all yield comparable patterns of local connections. Further, a recent study in the rat brainstem comparing the transport characteristics of gold-conjugated cholera toxin and wheat germ agglutinin-horseradish peroxidase (WGA-HRP) (Llewellyin-Smith et al., 1990) identified quantitative but no qualitative differences in labeling associated with these two tracers. Finally, studies in the same animals using the same tracers have uncovered differences in the intrinsic wiring cortical among visual, sensory, and motor areas (Kritzer et al., 1992; Lund et al., 1993), thus providing a precedent for varying connectional schemes among functionally specialized cortices. Thus, the qualitative differences noted between the intrinsic circuits of the prefrontal and primary visual cortices are likely to reflect biological differences, and may well have been anticipated since these two regions occupy essentially opposite ends in the cortical flow of sensory information (Jones and Powell, 1970; Pandya and Kuypers, 1969; Pandya and Seltzer, 1982) and have distinct functional specializations. Visual cortex is concerned with representation of sensory stimuli, whereas the prefrontal cortical neurons access these incoming representations and integrate them with information in long-term memory to guide behavior by current and past contingencies (Goldman-Rakic, 1987).

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