Cortical Structure Predicts the Pattern of Corticocortical Connections

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Cortical areas are linked through pathways which originate and terminate in specific layers. The factors underlying which layers are involved in specific connections are not well understood. Here we tested whether cortical structure can predict the pattern as well as the relative distribution of projection neurons and axonal terminals in cortical layers, studied with retrograde and anterograde tracers. We used the prefrontal cortices in the rhesus monkey as a model system because their laminar organization varies systematically, ranging from areas that have only three identifiable layers, to those that have six layers. We rated each prefrontal area based on the number and definition of its cortical layers (level 1, lowest; level 5, highest). The structural model accurately predicted the laminar pattern of connections in ~80% of the cases. Thus, projection neurons from a higher-level cortex originated mostly in the upper layers and their axons terminated predominantly in the deep layers (4–6) of a lower-level cortex. Conversely, most projection neurons from a lower-level area originated in the deep layers and their axons terminated predominantly in the upper layers (1–3) of a higher-level area. In addition, the structural model accurately predicted that the proportion of projection neurons or axonal terminals in the upper to the deep layers would vary as a function of the number of levels between the connected cortices. The power of this structural model lies in its potential to predict patterns of connections in the human cortex, where invasive procedures are precluded.

Introduction

Corticocortical connections in primates form a massive communication system which is likely to mediate elementary sensory processes as well as complex cognitive processes such as learning and memory. It is, therefore, important to identify specific neuronal populations involved in this massive communication system, and to determine whether there are general rules that govern its organization.

Projection neurons in sensory cortices originate and terminate in specific layers depending on their destination (Rockland and Pandya, 1979; Wong-Riley, 1979; Fitzpatrick and Imig, 1980; Tigges *et al.*, 1981; Galaburda and Pandya, 1983; Maunsell and Van Essen, 1983). In addressing whether general rules govern the connections of association cortices, we previously examined the laminar origin of projection neurons directed to frontal cortices (Barbas, 1986). We showed that the laminar organization of a given area is the best indicator of the laminar origin of its projections to the frontal cortex. Thus, projection neurons from those sensory association areas that have the sharpest laminar borders arise mostly from the upper layers. In contrast, in areas with blurred laminar borders, such as the limbic areas, projection neurons originate primarily from the deep layers. Between the above extremes, laminar definition decreases in a graded fashion in a direction from elementary processing sensory cortices towards the limbic areas, and the distribution of projection neurons shifts gradually from the upper to the deep layers. This rule applies to all cortical systems, transcending the type of modality, functional specialization or distance between the connected areas (Barbas, 1986).

Here we addressed several additional questions on the relationship of cortical structure to corticocortical connections. Do projection neurons from one area originate and terminate in different layers when they project to two structurally disparate areas? For example, do limbic areas issue projections from neurons in their deep layers when they project to eulaminate areas as well as when they communicate with each other? What is the pattern of connection between eulaminate areas with different laminar organization? What is the relative distribution of projection neurons or axonal terminals in different layers when structurally distinct cortices, in general, are connected?

The present study addresses these questions by focusing on connections between prefrontal cortices in the rhesus monkey. The prefrontal region is composed of structurally heterogeneous areas, ranging from agranular type areas, which have only three identifiable layers, to eulaminate areas which have six distinct layers. Thus, the prefrontal region is ideal for addressing the relationship of structure to connectional patterns.

Structural analysis as defined in this study classifies areas into a few cortical types, determined by the number of identifiable layers in each area and by how distinct the layers are from each other. By contrast, cytoarchitectonic analysis is a more detailed process, which identifies cortical type, and further subdivides each cortical type into individual cytoarchitectonic areas on the basis of the unique cellular features of each area. The rationale for focusing on structure is based on our previous findings suggesting that cortical type, rather than cellular morphology, appears to underlie the pattern of corticocortical connections (Barbas, 1986). For example, whereas limbic cortices in the orbitofrontal, cingulate and temporal regions differ on the basis of their cellular features, they are structurally similar, belonging either to the agranular or the dysgranular type, and all issue projections to eulaminate areas through their deep layers (Barbas, 1986). Thus, even though there are many cyto-architectonic areas in the cortex, there are only a few types of cortex, and only a few patterns of connection.

In the present study we focused exclusively on laminar organization to test the hypothesis that cortical structure underlies the pattern of corticocortical connections. We used a previous parcelling system of the prefrontal region (Barbas and Pandya, 1989) to assign a numerical rating (1–5) to prefrontal cortices on the basis of the number and definition of their layers. We then used quantitative procedures to test whether cortical structure predicts the pattern and relative laminar distribution of corticocortical connections. To the extent that the structural model succeeds, it can be used to infer the pattern of connections in the human cortex, where invasive procedures are precluded.

Materials and Methods

Surgical Procedures and Tissue Processing

Experiments were conducted on 18 adult rhesus monkeys (*Macaca mulatta*) according to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 80-22, 1987). The animals were anesthetized with ketamine hydrochloride (10 mg/kg, i.m.) followed by sodium pentobarbital administered i.v. until a surgical level of anesthesia was achieved. Surgery for injection of neural tracers was performed under aseptic conditions as described previously (Barbas, 1993). In each case the injectate was delivered 1.5 mm below the pial surface over a 30 min period in the following quantities and concentrations: 0.05-0.1 µl, 8% HRP-WGA; 0.4 µl, 3% diamidino yellow or fast blue; 0.4-1 µl of [^{3}H]leucine and [^{3}H]proline, sp. act. 40-80 Ci/mmol.

Injections of HRP-WGA (Sigma) were placed in prefrontal cortices in seven animals, fluorescent dyes were injected in five animals and [^{3}H]amino acids were injected in six animals. In cases with fluorescent dyes two different sites were injected in each animal. Injections were placed in the following architectonic areas in the number of sites indicated in parentheses: areas PAll and Pro ($n = 5$); area 13 ($n = 3$); area 32 ($n = 3$); area 11 ($n = 1$); orbital area 12 ($n = 3$); lateral area 12 ($n = 1$); medial area 14 ($n = 2$); medial area 9 ($n = 1$); area 46 ($n = 4$). The total number of prefrontal sites examined was 23. After a post-surgical survival period of 40-48 h for animals injected with HRP, or 10 days for animals injected with fluorescent dyes or [^{3}H]amino acids, the monkeys were anesthetized deeply and perfused through the heart with saline followed by a fixative, as described previously (Barbas and Pandya, 1989; Barbas, 1993). The brain was then removed from the skull and photographed. In the HRP-WGA or fluorescent dye experiments the brains were frozen in –75°C isopentane, transferred to a freezing microtome and cut in the coronal plane at 40 µm in 10 series. Brain sections were processed histochemically to visualize HRP-WGA label (Mesulam *et al.*, 1980), or directly mounted on glass slides to visualize neurons labeled with fluorescent dyes, as described previously (Barbas, 1993). The brains of animals injected with [^{3}H]amino acids were embedded in paraffin, cut in the coronal plane at 10 µm and processed for autoradiography according to the procedure described by Cowan *et al.* (1972). Exposure time of radiolabeled material was 3-6 months. In all experiments, series of sections adjacent to those prepared to visualize retrograde tracer labeling were stained for Nissl bodies, and acetylcholinesterase (AChE), or myelin (or both) to aid in delineating architectonic borders.

Data Analysis

Retrogradely Labeled Neurons

Brain sections were viewed microscopically under bright-field illumination for HRP cases, and fluorescence illumination in experiments with dyes. Drawings of prefrontal sections and the location of labeled neurons ipsilateral to the injection site were transferred from the slides onto paper by means of a digital plotter (Hewlett Packard 7475A) electronically coupled to the stage of the microscope and to a computer (Austin 486), as described previously (Barbas and De Olmos, 1990). This procedure allows accurate topographic presentation of labeled neurons with respect to anatomic landmarks. Software developed for this purpose ensured that each labeled neuron noted by the experimenter was recorded only once. Labeled neurons within each architectonic area were counted by encircling the area by moving the x and y axes of the stage of the microscope. The number of labeled neurons within the perimeter was calculated automatically using an algorithm written in our program. This method provided the number of labeled neurons in the upper layers (2-3) and in the deep layers (5-6), as well as the total number (sum of labeled neurons in upper and deep layers) for each section and for each area. We did not see labeled neurons in layers 4 or 1.

In cases injected with fluorescent dyes, brain outlines and labeled neurons were drawn and plotted from unstained sections and the data were stored on computer disk. The brain sections were then stained with thionin, coverslipped and returned to the microscope to count the labeled neurons in the upper (2-3) and deep (5-6) layers and determine cytoarchitectonic borders. The architectonic borders were superimposed on prerecorded drawings of the brain sections with the labeled neurons.

Anterograde Label

Anterograde label in the HRP-WGA or autoradiographic experiments was examined microscopically from coronal sections under dark-field illumination. The density of anterograde grain was initially evaluated on a scale of 1-6, with ratings of 1 and 2 assigned for light, 3 and 4 for moderate, and 5 and 6 for dense label for the upper (1-3) and deep (4-6) layers for each area in each section. If more than one site contained label within a single architectonic area, then each site was rated separately. In addition, in some cases density values were obtained with an image analysis system (MetaMorph, Universal Imaging). This high-resolution system uses a CCD camera mounted on the microscope and captures images directly from brain sections. Measurements were made under dark-field illumination at a magnification of 100×. An initial density measure in each section was taken in an area with no anterograde label to determine the level of background. The background density was subtracted from subsequent density measures in the areas of interest to determine the density of anterograde label. Measurements of density of anterograde label represented the mean density taken from sample squares distributed throughout each area to avoid retrogradely labeled neurons. The cumulative density of label within the entire extent of each architectonic area was calculated from serial coronal sections. Thus for each area we obtained the density of anterograde label in the upper (1-3) and the deep (4-6) layers, and the total density (total density = density in upper layers + density in deep layers).

In all experiments, the number of labeled neurons or density of anterograde label throughout an architectonic area was obtained from serial coronal sections and entered into a database processing system for further analysis. References to architectonic areas of the prefrontal cortex are according to a previous study (Barbas and Pandya, 1989).

Normalization of Data

After injection of tracers in the areas listed above, labeled neurons or axonal terminals were observed in many prefrontal cortices, which included a large enough sample in each of the five levels to conduct statistical analyses. The charting and initial evaluation of the density of labeled neurons and terminals established which areas were consistently interconnected. Data from each architectonic area where the number of labeled neurons in each animal exceeded 100 labeled neurons after a single injection were included for further analysis. For anterograde label, the criterion for inclusion in the analyses was a cumulative score of 8 or greater in each architectonic area, based on the density scale of 1-6 (described above). Areas with lower densities of label than the above were not included in the statistical analyses. Also excluded from the analyses were labeled neurons or terminals found in the same architectonic area as the injection site.

The total number of labeled neurons in the cases and areas examined for each injection site ranged from 118 to 2480. For anterograde grain the cumulative density for individual areas was 10-165, based on the rating scale of 1-6. It was thus necessary to normalize the data so that comparisons could be made across areas. To this end, for each individual projection area mapped after a single injection, we expressed the number of labeled neurons in the upper layers (2-3) and in the deep layers (5-6) as a percentage of the total number for that area. Following the same approach, the density of anterograde label in the upper (1-3) and in the deep (4-6) layers was expressed as a percentage of the total of each individual area in each animal. Thus, the data were converted into percentages so that for each area mapped after each injection, the sum of the density of label in the upper and in the deep layers totaled 100%.

Anterograde label in some cases was analyzed using the imaging system (see above), as well as the rating method (1-6). The reliability estimate of the normalized density obtained using the two independent methods of measurement was 0.99 (Pearson r).

Construction of Structural Levels

The next step was to use a common method to classify connected cortices according to their structure. Classic architectonic methods for parcelling the cortex are based on detailed descriptions of the cellular characteristics of an area (e.g. Walker, 1940). A variation of the classic approach takes into account the degree of laminar definition of an area, as well as its specific cellular characteristics (Barbas and Pandya, 1989). This
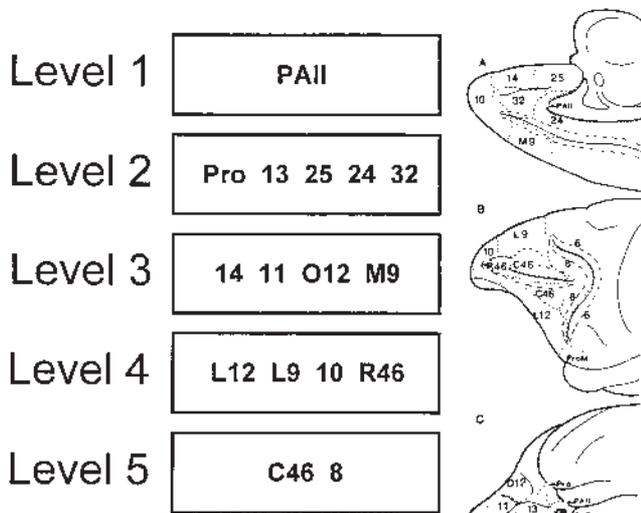
Figure 1. Prefrontal areas were classified into five levels based on the number of identifiable layers and the degree of their laminar definition. Level 1 includes agranular limbic areas, level 2 dysgranular limbic areas, and levels 3–5 eulaminate areas with increasing laminar definition. The maps on the right show the architectonic areas on (*A*) the medial, (*B*) lateral and (*C*) basal surfaces. The maps were adapted from Barbas and Pandya (1989). Abbreviations: PAll, periallocortex (agranular); Pro, proisocortex (dysgranular); Letters appearing before architectonic areas stand for: C, caudal; L, lateral; M, medial; O, orbital; R, rostral.

method relies on the principle that laminar definition changes gradually and systematically within the cortex (Sanides, 1970). Using this method, areas are classified into a few cortical types based on the number and definition of the layers in each area, and each cortical type is further subdivided into individual cytoarchitectonic areas on the basis of the unique cellular features of each area.

We used the above method to assign injection sites and projection zones into specific architectonic areas. We then focused exclusively on the broad laminar features of areas for the purpose of testing the structural model. This allowed us to group areas which are architectonically distinct but similar in cortical type. For example, areas 25, 13 and 32 in the prefrontal cortex are architectonically distinct, yet they all belong to the dysgranular cortical type on account of their poorly developed layer 4.

To test the relationship of structure to patterns of connection we classified injection sites as well as projection areas into five structural levels. Each level included all structurally similar areas and was given a numerical rating (1–5), as shown in Figure 1. Using the criterion of laminar definition for grouping areas, the agranular limbic areas, which have only three distinguishable layers, were placed in level 1. Adjacent dysgranular limbic areas, which have four distinguishable layers, occupied level 2. Eulaminate areas, which have six layers, were placed in levels 3–5 according to the degree of their laminar definition. Eulaminate areas with the most distinct lamination, including a wide granular layer 4, were placed in level 5. Eulaminate areas found adjacent to dysgranular areas have six layers, but the width of layer 4 and the distinction between the layers is lower than in other eulaminate areas; these were placed in level 3. Eulaminate cortices with an intermediate laminar definition were placed in level 4. When an injection of tracer or a projection zone straddled the border of two areas, it was given a rating in between the two. For example, an injection site in area PAll (level 1) and in the adjacent area Pro (level 2) was given a rating of 1.5.

Areas with label were grouped according to their structural level in order to address specific connectional relationships. For each group of cortices we computed the mean percentage of labeled neurons or density of axonal terminals in the upper layers and in the deep layers and the standard error. Because the composition of the groups depended on the number and type of areas with label after the injections, the number of areas in each group varied.

All statistical analyses compare the percentage of labeled neurons or terminals in the deep layers between two groups of cortices. Because the comparisons were based on normalized data, the same results would be obtained by comparing the percentage of label in the upper layers (% labeled cells in upper layers = 100 – % cells in deep layers). Unless otherwise stated, differences between groups of areas were confirmed using Student's *t*-test.

Results

Injection Sites

The injection sites included areas from all three surfaces of the frontal lobe: 12 sites were sampled from the orbital surface, six from the medial and five from the lateral surfaces. Most cases in this study had been used previously to map thalamic, amygdaloid, hippocampal or corticocortical connections. The previous studies focused on the topography of connections between the above structures and prefrontal cortices, but did not address the questions investigated here. In recent years, cases have been designated consistently by the same codes indicated below in parentheses. These include all cases injected with WGA-HRP or fluorescent dyes in this study. For reference to the maps of these injection sites see Barbas (1993, 1995), Dermon and Barbas (1994) and Barbas and Blatt (1995). The HRP injections were located in the following areas: area PAll/Pro (case AG); area Pro (case AF); area 11 (case AM); orbital area 12 (case MBY); area 9 (case AO; Fig. 2*B*); ventral area 46 (cases AA; MAV; Fig. 2*A*). Case AA was designated as case 5 in Barbas and De Olmos (1990). The following areas were injected with fluorescent dyes: orbital area Pro (case ALy); area 13 (cases AJb; ALy); area 32 (cases DLy; AKy); orbital area 12 (cases AJy; ANb); area 14 (cases DLb; AKb); lateral area 12 (case ANy). The following areas were injected with [³H]amino acids: orbital area Pro (2 cases); area 13; area 32 (Fig. 2*C*); ventral area 46 (caudal part); area 46 (rostral part).

All injection sites encompassed the entire cortical depth and the needle marks were confined to the cortical mantle (Fig. 2). This is a critical feature, because all analyses assume that every cortical layer at the injection site has the opportunity to transport the tracer. Thus, the distribution of labeled neurons or axonal terminals in cortical layers provides an accurate indication of the laminar pattern of the connections between areas.

The Relationship of Structure to Cortical Connections

Labeled neurons or axonal terminals were observed in all prefrontal areas, covering the entire range of structural levels. The areas which contained labeled neurons or terminals were grouped according to their structural level as shown in Figure 1. The standard error of the normalized labeling data for each group was relatively small regardless of the number of areas in each (see Figs 3–5, 8–9). This suggests that areas in each group were similar in their connectional pattern. Moreover, the laminar pattern of connections did not fluctuate significantly on the basis of the overall density of label in individual areas.

The above grouping of areas by laminar definition made it possible to compare the laminar distribution of connections between structurally distinct groups of cortices. The structural model was tested against a large body of quantitative data, and the details of the main analyses are shown in Tables 1 and 2. For each comparison, connections are described in terms of the difference between the levels of origin and termination of the groups of cortices compared. This relationship was expressed as delta, Δ (Δ = level of origin – level of termination). A negative Δ
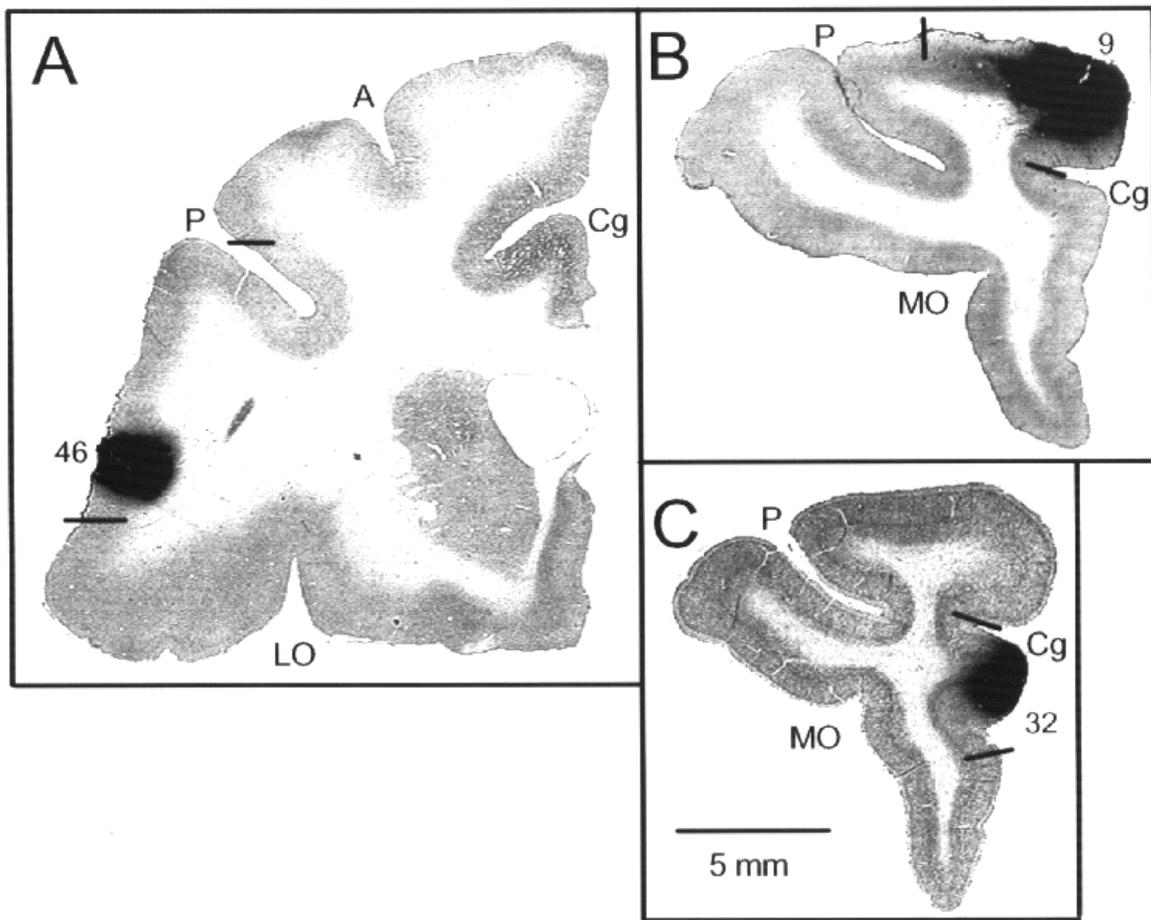
Figure 2. Bright-field photomicrographs showing the injection sites (black areas) in three different prefrontal cortices: (*A*) an injection of HRP-WGA in the caudal part of area 46, a level 5 cortex; (*B*) an injection of HRP-WGA in area 9, a level 3 cortex; (*C*) an injection of [³H]amino acids in area 32, a level 2 cortex. The images were captured directly from tissue slides with a CCD camera. Abbreviations: A, arcuate sulcus; Cg, cingulate sulcus; LO, lateral orbital sulcus; MO, medial orbital sulcus; P, principal sulcus. Numbers indicate the architectonic areas with the injection sites.

denotes a projection originating from a cortex at a lower structural level and terminating in a cortex of a higher structural level. Connections proceeding in the reverse direction are indicated by positive Δ.

In our first analysis we tested for global differences in the laminar distribution of connections between limbic areas (agranular, level 1, and dysgranular, level 2) and eulaminate areas (≥level 3). We found that limbic areas issued projections to eulaminate areas primarily from the deep layers when Δ was –1 (Table 1, A), whereas the reciprocal projections arose predominantly from neurons in the upper layers of level 3–4 eulaminate areas when Δ was +1 to +2 ($P < 0.005$; Table 1, B). This result confirms our previous findings (Barbas, 1986). Efferent fibers from limbic cortices terminated mostly in layers 1–3 of eulaminate areas when Δ was –1 to –3 (Fig. 3*A*; Table 2, A), whereas efferent fibers from eulaminate cortices terminated primarily in the deep layers of limbic cortices when Δ was +1 to +3.5 ($P < 0.0005$; Fig. 3*B*; Table 2, B).

In our second analysis we compared the bidirectional connections of two types of limbic cortices whose structural relationship is clear. The comparison was between the agranular (level 1) and dysgranular (level 2) limbic cortices. This analysis revealed that when level 1 (or 1.5) areas projected to level 2 areas, and Δ was thus negative, most projection neurons were
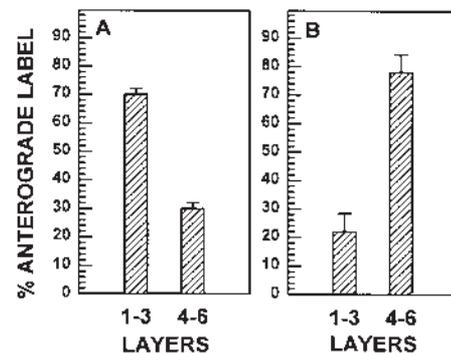

Figure 3. The laminar distribution of axonal terminations linking agranular/dysgranular (limbic) and eulaminate cortices. (*A*) Axons originating from agranular and dysgranular cortices (levels 1 and 2) terminated mostly in the upper layers of eulaminate cortices (levels 3–5). (*B*) In the reciprocal relationship, axons originating in eulaminate areas terminated mostly in the deep layers of agranular and dysgranular cortices. See Table 2, A, B. In all bar graphs (Figs 3–5 and 8–9) vertical lines on bars indicate the standard error, and the differences in the distribution of connections in the deep layers between groups of cortices are statistically significant.

found in the deep layers (Fig. 4*A*; Table 1, C). In the reciprocal relationship, when Δ was positive, projection neurons were found mostly in the upper layers ($P < 0.025$; Fig. 4*B*; Table 1, D).
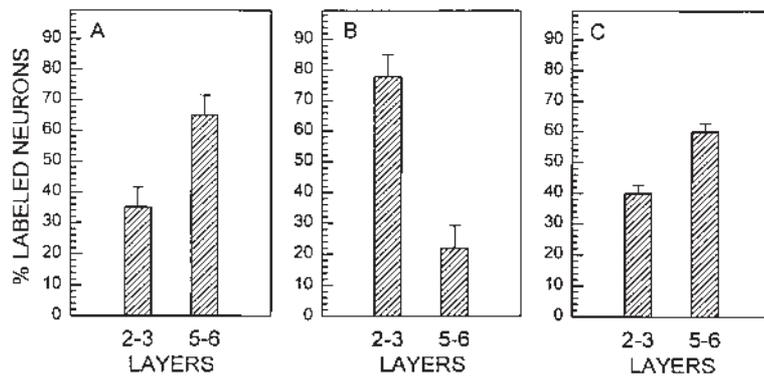
Figure 4. The laminar distribution of projection neurons linking agranular (level 1) and dysgranular (level 2) cortices. (*A*) Labeled neurons found mostly in the deep layers of level 1 and 1.5 cortices projected to level 2 cortices. (*B*) In the reciprocal relationship, labeled neurons found mostly in the upper layers of level 2 cortices (area 13) projected to level 1.5 cortices. (*C*) Labeled neurons found mostly in the deep layers of area 13 (level 2) projected to eulaminate areas (level 3). See Table 1, C, D.

The above findings indicate that when the cortex of destination has a lower laminar definition than the origin, even limbic cortices issue projections from the upper layers.

All the examples of projections from dysgranular cortices to agranular cortices originated in area 13 (Fig. 4*B*; Table 1, D). In sharp contrast to its projection to agranular cortices, when area 13 (level 2) projected to eulaminate cortices (level 3) and Δ was –1, it issued projections mostly from its deep layers (*P* < 0.025; Fig. 4*C*; Table 1, E). This finding is consistent with a projection from agranular/dysgranular to eulaminate areas, in general. Efferent fibers from level 1 and 1.5 cortices terminated primarily in layers 1–3 of area 13 when Δ was –0.5 to –1 (Fig. 5*A*; Table 2, C), whereas efferent fibers from eulaminate cortices terminated primarily in the deep layers (4–6) of area 13 when Δ was +1 (Fig. 5*B*; *P* < 0.025; Table 2, D).

Examples of the above relationships are shown in photomicrographs of tissue sections in Figures 6 and 7. Thus, axonal fibers from dysgranular area 32 (level 2) terminated in the upper layers of eulaminate area 9 (level 3) when Δ was negative (Fig. 6*A*), but they terminated in the deep layers (5–6) of the agranular area PAll (level 1; Fig. 6*B*) when Δ was positive (Fig. 6*C*). Figure 7 illustrates the origin as well as terminations of projections simultaneously, obtained after injection of the bidirectional tracer HRP-WGA. A coronal section through area 13 (Fig. 7*A*) shows the connectional pattern of area 13 with area PAll/Pro (level 1.5) which had been injected with HRP-WGA (not shown). Labeled neurons found mostly in layers 2–3 of area 13 (Fig. 7*A*, arrowheads) projected to area PAll/Pro when Δ was positive, and axonal fibers from area PAll/Pro terminated primarily in layers 1 and 2 of area 13 (Fig. 7*A*, arrow) when Δ was negative, as seen in the same section. In another monkey, a coronal section through area 13 (Fig. 7*B*) shows its connectional pattern with eulaminate area 12 (level 3), which had been injected with HRP-WGA (not shown). Labeled neurons found mostly in the deep layers of area 13 (Fig. 7*B*, arrowheads) projected to orbital area 12 when Δ was negative, and axonal fibers from area 12 terminated primarily in the deep layers of area 13 (Fig. 7*B*, white grain) when Δ was positive, as seen in the same section. A comparison of the pattern of connections in Figure 7*A* and 7*B* illustrates the differences in the origin and terminations of projections when area 13 (level 2) was connected with two structurally disparate areas, a level 1.5 area (Fig. 7*A*) and a level 3 area (Fig. 7*B*).

The above findings support the hypothesis that the structural relationship between two cortices determines which layers
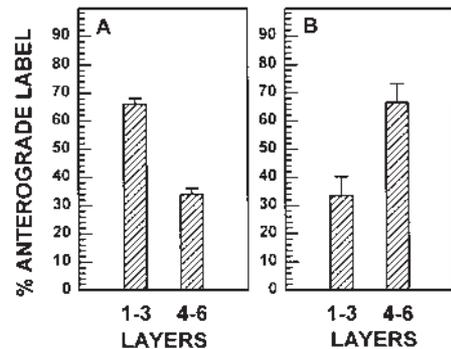

Figure 5. Axonal fibers from agranular and from eulaminate cortices terminated mostly in different layers of area 13. (*A*) Axonal fibers originating from level 1 and 1.5 cortices terminated predominantly in the upper layers of area 13 (level 2). (*B*) Axonal fibers originating from level 3 eulaminate areas terminated primarily in the deep layers of area 13. See Table 2, C, D.

participate when they are connected. In our next analysis we tested this hypothesis further by examining the pattern of connections between eulaminate cortices belonging to different structural levels. We found that when those eulaminate areas that have the most distinct layers (e.g. level 5) projected to eulaminate areas with comparatively less distinct laminar borders (e.g. level 3) and Δ was +1 to +2, the projection neurons were found primarily in the upper layers (Fig. 8*A*; Table 1, F), and axonal fibers terminated mostly in the deep layers (Fig. 8*B*; Table 2, E). The reciprocal connections arose primarily from the deep layers of level 3 cortex (not shown). Efferent fibers from levels 3 and 4 eulaminate areas terminated mostly in the upper layers of areas belonging to levels 4 or 5, for Δ = –1 to –2 (Fig. 8*C*; Table 2, F; comparison between 8*B* and 8*C*, *P* < 0.0005). These findings indicate that the laminar pattern of connections of eulaminate areas, like agranular and dysgranular areas, depends on the structural levels of the connected cortices. In all cases, the projection pattern showed a predominant, though not exclusive, distribution either in the upper or in the deep layers.

Predicting the Direction of a Connectional Pattern
The main findings thus far indicated that connections between prefrontal cortices are direction specific, where direction refers to whether Δ is less or greater than 0. We asked how reliable the structural model was in predicting the direction of connections, i.e. whether the majority (>50%) originated or terminated in the

Table 1

Density of retrogradely labeled neurons in the deep layers in relation to the structural levels of the connected cortices

Group of connected cortices	Level of areas compared		Δ	Normalized density in layers 5–6*	Figure
	Projection origin (retr. labeled neurons)	Projection destination (RT injection site)			
A	level 2	level 3	−1	54[a]	not shown
B	levels 3–4	level 2	+1 to +2	34[a]	not shown
C	levels 1 and 1.5	level 2	−0.5 to −1	65[b]	4A
D	level 2	level 1.5	+0.5	22[bc]	4B
E	level 2	level 3	−1	60[c]	4C
F	levels 4–5	levels 3–4	+1 to +2	28	8A

The levels of connected cortices are shown as the projection origins (retrogradely labeled neurons) and the areas of destination [retrograde tracer (RT) injection sites]. Delta, Δ = level of the projection origin − level of the projection destination. Statistical analyses compared the normalized density of retrogradely labeled neurons in the deep layers (5–6) between two groups of connected cortices (pairs compared are designated with the same letter, a–c). Significance levels for comparisons are: [a]$P < 0.005$; [b]$P < 0.025$; [c]$P < 0.025$.

*The normalized density was expressed as a percentage: mean % labeled neurons in the deep layers is 100 − (mean % labeled neurons in superficial layers). For each group, the number of areas, number of sections and total number of labeled neurons are: A, 10 areas, 67 sections, 7537 neurons; B, 9 areas, 54 sections, 6836 neurons; C, 2 areas, 11 sections, 1205 neurons; D, 3 areas, 14 sections, 2204 neurons; E, 2 areas, 13 sections, 1997 neurons; F, 7 areas, 52 sections, 2436 neurons.

Table 2

Density of anterograde label in the deep layers in relation to the structural levels of connected cortices

Group of connected cortices	Level of areas compared		Δ	Normalized density in layers 4–6*	Figure
	Projection origin (injection site)	Projection destination (anterograde label)			
A	levels 1–2	levels 3–5	−1 to −3	30[a]	3A
B	levels 3–5	levels 1–2	+1 to +3.5	78[a]	3B
C	levels 1 and 1.5	level 2	−0.5 and −1	34[b]	5A
D	level 3	level 2	+1	67[b]	5B
E	levels 4–5	level 3	+1 to +2	64[c]	8B
F	levels 3–4	levels 4–5	−1 to −2	25[c]	8C
G	levels 3–4	level 2	+1 to +2	64[d]	9A
H	level 5	levels 1.5–2	+3 to +3.5	92[d]	9B

The levels of connected cortices are shown as the projection origins (injection sites) and the areas of destination (anterograde label). Delta, Δ = level of the projection origin − level of the projection destination. Statistical analyses compared the normalized density of anterograde label in the deep layers (4–6) between groups of connected cortices (pairs compared are designated with the same letter, a–d). Significance levels for comparisons are: [a]$P < 0.0005$; [b]$P < 0.025$; [c]$P < 0.0005$; [d]$P < 0.01$. *The normalized density was expressed as a percentage: mean % density in the deep layers (4–6) is 100 − (mean % density in superficial layers). For each group, the number of areas, number of sections, number of sites and cumulative density of anterograde label are: A, 8 areas, 35 sections, 42 sites, density = 340; B, 8 areas, 51 sections, 76 sites, density = 430; C, 2 areas, 9 sections, 10 sites, density = 104; D, 2 areas, 10 sections, 14 sites, density = 184; E, 4 areas, 23 sections, 23 sites, density = 122; F, 5 areas, 19 sections, 20 sites, density = 199; G, 4 areas, 17 sections, 23 sites, density = 236; H, 4 areas, 18 sections, 19 sites, density = 104.

upper or in the deep layers for the above data. The structural model accurately predicted the direction of a connectional pattern in 75% of cases using retrograde data, and in >80% of the cases when based on anterograde data. Some of the deviations may be attributed to experimental variation, particularly for areas which are close in laminar definition. For example, our observations indicated that projection neurons in medial dysgranular areas (level 2) originated predominantly in the upper layers when their destination was an orbitofrontal dysgranular area, such as area Pro or area 13 (level 2). Moreover, the reciprocal connections from orbitofrontal to medial dysgranular areas involved a majority of projection neurons from the deep layers. We also noted that the pattern of projection from medial area 14 (level 3) was comparable with the pattern expected for dysgranular (level 2) cortices.

Predicting the Laminar Distribution of Connections

The structural model predicts that the proportion of projection neurons or axonal terminals in the upper or deep layers, will depend on the value of Δ, i.e. the number of levels between the connected cortices. We tested the sensitivity of the structural model to predict the laminar distribution of labeled neurons within the deep layers, using the set of retrograde data obtained after injection of fluorescent dyes. We found that the relative distribution of projection neurons differed according to the number of levels separating the connected cortices (Pearson r =

−0.72, $P < 0.001$). We then tested whether the relative density of anterograde label within cortical layers varied as a function of Δ as well. As described above, efferent fibers from eulaminate areas (levels 3–5) terminated mostly in layers 4–6 of limbic areas (levels 1 and 2; Fig. 3B). We subdivided the data from areas depicted in Figure 3B into those originating in a lower (levels 3 and 4) and a higher (level 5) structural group (Fig. 9). Analysis of the subdivided data showed that efferent fibers from eulaminate cortices one or two levels higher than the limbic terminated primarily in the deep layers of limbic cortices (Fig. 9A). As the model predicts, the proportion of terminations in the deep layers was higher when Δ was higher (Fig. 9B; $P < 0.01$; comparison between Fig. 9A and B; Table 2, G, H). The normalized density of axonal terminals in the deep layers was plotted as a function of the difference in the level of connected areas (value of Δ), and the correlation was significant (Pearson $r = 0.95$, $P < 0.001$; Fig. 10). In addition, when distinct architectonic cortices which are at the same structural level are connected (Δ = 0), the distribution of axonal terminals is approximately equal in the upper and deep layers.

Comparison of the Area Occupied by Layers 1–3 and 4–6 in Structurally Distinct Cortices

We measured the proportion of area under the upper (1–3) and deep (4–6) layers (in mm^2) for several prefrontal cortices in order to determine whether there are consistent differences in
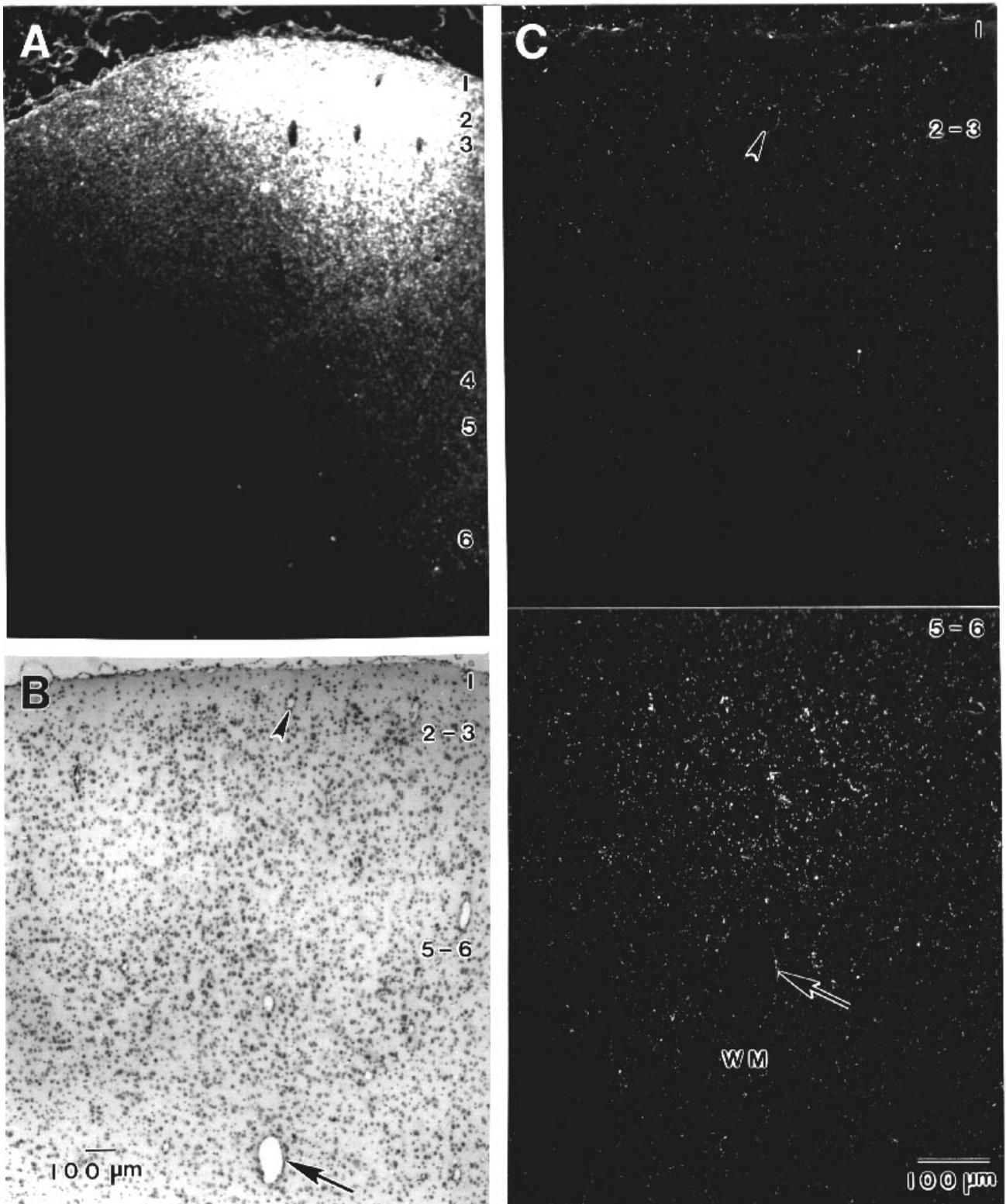
Figure 6. Axonal fibers originating from area 32 (level 2) terminated predominantly in different layers of two structurally disparate cortices, area 9 (level 3) and area PAll (level 1). (A) Dark-field photomicrograph of a coronal section showing the axonal terminations in the upper layers (1–3) of area 9 (white grain). (B) Bright-field photomicrograph showing the architecture of area PAll (level 1). (C) Dark-field photomicrograph of a coronal section showing the axonal terminations in the deep layers (5–6) of area PAll (level 1; white boutons and grain). Note the difference in the laminar distribution of anterograde label when Δ was negative (A) than when Δ was positive (C). Pairs of silhouette arrows and arrowheads in (B) and (C) point to the same blood vessels for landmarks. Scale in (B) applies to (A).
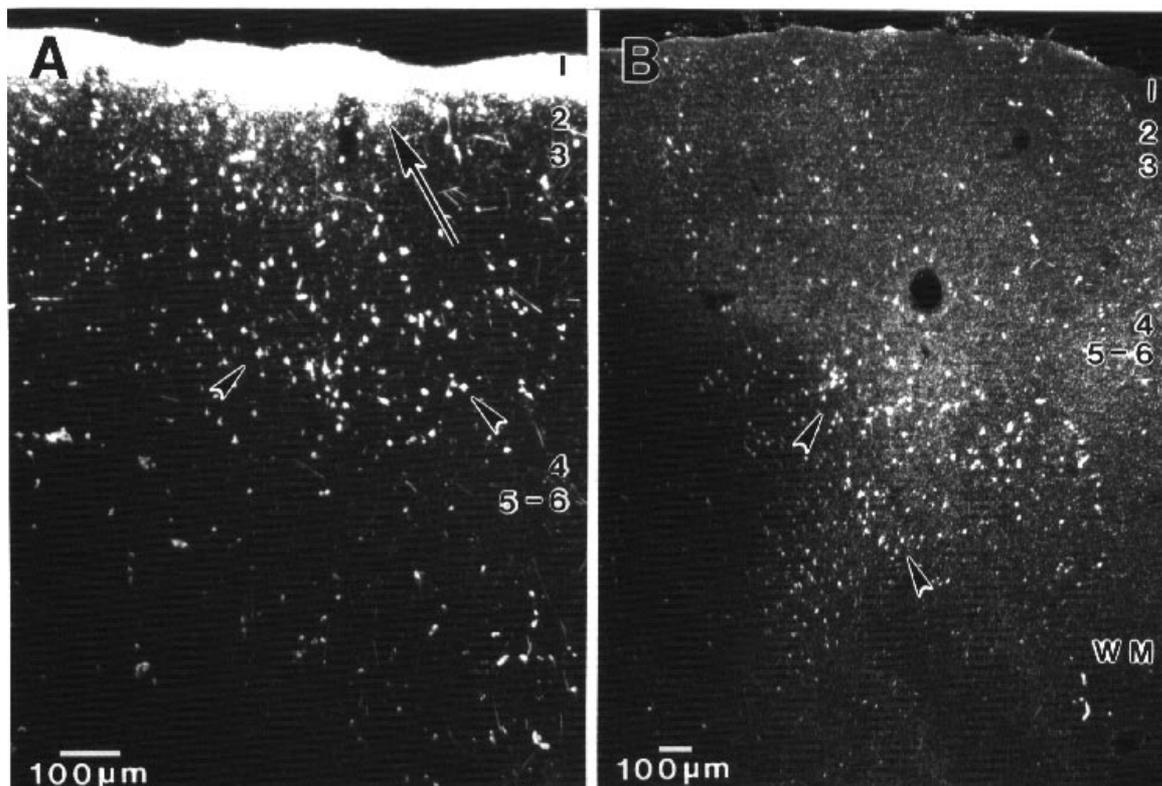
Figure 7. Differences in the origin and termination of projections when one area is connected with two structurally disparate areas. The laminar origin and termination of projection neurons from, and to, area 13, are shown simultaneously in dark-field photomicrographs of single coronal sections in two animals, after injection of the bidirectional tracer HRP-WGA in area PAll/Pro or orbital area 12 (O12). (*A*) HRP-labeled neurons seen mostly in layers 2–3 (arrowheads) of area 13 (level 2) projected to area PAll/Pro (level 1.5); labeled terminals seen in the upper layers, and predominantly in layer 1, of area 13 (white band, arrow) originated from axons in area PAll/Pro. (*B*) HRP-labeled neurons in area 13 seen mostly in layers 5–6 (arrowheads) projected to eulaminate area O12 (level 3); labeled terminals seen mostly in the deep layers of area 13 (white grain, arrowheads) originated from axons in area O12.
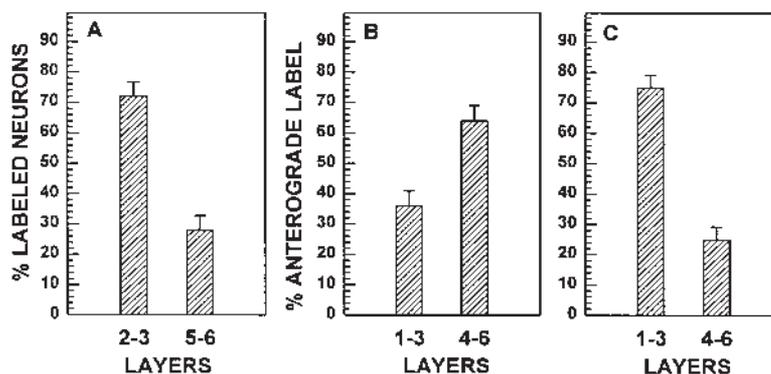

Figure 8. The laminar origin and termination of projections linking eulaminate areas of different levels. (*A*) Labeled neurons found mostly in the upper layers of levels 4 and 5 eulaminate areas projected to levels 3 or 4 eulaminate areas (Table 1, F). (*B*) Axons from levels 4–5 eulaminate areas terminated primarily in the deep layers of level 3 eulaminate areas. (*C*) In the reciprocal relationship, axons originating from levels 3–4 eulaminate areas terminated mostly in the upper layers of levels 4–5 eulaminate areas. For data in Figure 8*B* and 8*C* see Table 2, E, F.

cortices belonging to different levels. The deep layers occupied 52% of the total area for level 1 cortices, 44% for level 2 cortices and 41% for eulaminate cortices. There were no significant differences in the area occupied by the deep layers of eulaminate areas which belonged to different structural levels (3–5), even though their connectional pattern differed. This evidence suggests that the proportion of area occupied by the deep and upper layers cannot account for the observed differences in connections.

Discussion

Predicting Connections from Structure
The results demonstrate that the structural relationship of two cortices predicts which layers will be involved in their interconnections. These results extend our previous findings on the role of architecture to corticocortical connections (Barbas, 1986; Barbas and Pandya, 1989). Here we also present the first systematic and quantitative analysis indicating which specific
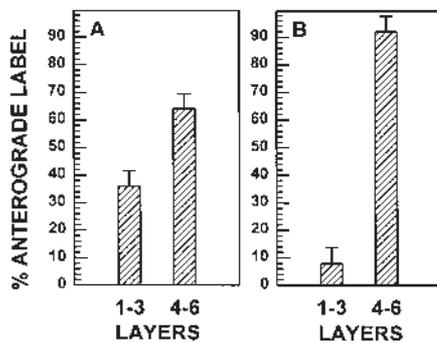
Figure 9. The sensitivity of the structural model to differences in the number of levels between connected areas. The data shown in Figure 3*B* were subdivided into those where axonal fibers originated in lower (levels 3–4, *A*, n = 4) and higher (level 5, *B*, n = 4) level eulaminate areas, and terminated in agranular and dysgranular cortices. (*A*) Axonal fibers from level 3–4 eulaminate areas terminated mostly in the deep layers of level 2 dysgranular cortices. (*B*) Axonal fibers from level 5 eulaminate areas terminated overwhelmingly in the deep layers of level 1.5 and level 2 cortices. Note the differences in the relative laminar distribution of anterograde label for different values of Δ. See Table 2, G, H.
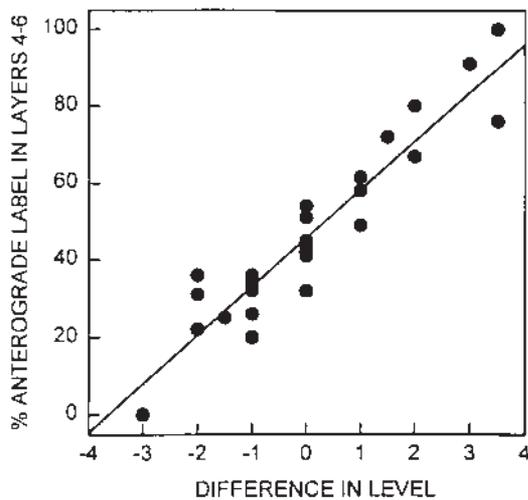

Figure 10. The normalized density of anterograde label in the deep layers differed as a function of the difference in levels (Δ) between pairs of connected areas (represented by dots; Pearson r = 0.95, P < 0.001). Difference in level was determined by subtracting the level of termination from the level of origin, as shown in Figure 1. Points −4 to −1 show the termination of efferent fibers in areas with comparatively higher laminar definition than the origin (−Δ); points 1 to 4 show the termination of efferent fibers in areas with comparatively lower laminar definition than the origin (+Δ). 0 point shows connections between distinct areas which are at the same structural level (Δ = 0).

neuronal populations are involved when a given pair of prefrontal cortices is connected. The present study of a major association system extends these analyses to neural interactions not previously addressed, including those between limbic cortices and between different types of eulaminate association cortices.

The structural model made two predictions successfully. First, it accurately predicted whether connections would originate or terminate predominantly in the upper or the deep layers for most pairs of connected cortices. Second, the structural model predicted the relative distribution of projection neurons or axonal terminals within cortical layers. A small number of comparisons consistently departed from predictions. One

possible explanation is that there are subtle differences in the structure of these cortices which were not detected using architectonic methods.

The Salient Features and Limitations of the Structural Model

The analyses that we have conducted rely on accurate parcelling of the prefrontal cortices according to their laminar characteristics. Architectonic parcelling of the cortex is not a trivial task, which may explain the differences in the maps of classic and recent studies (e.g. Brodmann, 1905; Vogt and Vogt, 1919; Walker, 1940; Von Bonin and Bailey, 1947; Preuss and Goldman-Rakic, 1991; Morecraft *et al.*, 1992; Carmichael and Price, 1994). In parcelling the prefrontal cortex we previously considered the degree of laminar definition of areas, in addition to their cellular features (Barbas and Pandya, 1989). This approach is based on a principle which formulates the method for parcelling the cortex. The principle is that laminar definition changes gradually and systematically within the cortex (Sanides, 1970). The method of parcelling the cortex is based on the number of identifiable layers in each area, and by how distinct the layers are. In this study we focused exclusively on the laminar organization of areas to test the hypothesis that cortical structure underlies the pattern of corticocortical connections.

In its present form the structural model does not address the question of whether the connections of different areas will be robust or weak, or whether any two cortices will be connected. In addition, the procedure of classifying areas into five cortical types is suitable for comparing structurally distinct cortices, but has to be refined in order to discriminate the pattern of intrinsic connections within one large architectonic area. For example, whereas as an entity area 13 is dysgranular in type, its laminar definition is not uniform, being lower at the border with level 1 cortex than at the border with level 3 cortices. This observation is consistent with the principle that changes in laminar definition within the cortex are gradual rather than punctuated (Sanides, 1970). Such 'focal' differences in laminar definition are not significant when considering the connections of one area with cortices that have an overall higher or lower laminar definition, and which thus belong to a different type or structural bracket. However, the structural model predicts that focal differences in laminar definition will be important, and finer areal subdivisions must be made, when considering the intrinsic connections of one large area.

The present study focused on the role of structure on corticocortical connections. It is likely that other areal features are important to the pattern of corticocortical connections as well. In recent years studies have shown that several molecular markers, such as GAP-43 and CAT-301, are not uniformly distributed in the cortex (Benowitz and Routtenberg, 1987; Hendry *et al.*, 1988; Benowitz *et al.*, 1989; McGuire *et al.*, 1989). An examination of the regional distribution of the above markers suggests that their expression is graded in a fashion that matches gradients in laminar definition. The present findings suggest that features which are likely to be important to the pattern of connections must vary systematically among cortices in order to account for the observed differences in connections. This is based on findings from this and previous studies indicating that each area can issue predominantly upper or deep layer projections depending on destination (for review see Felleman and Van Essen, 1991).
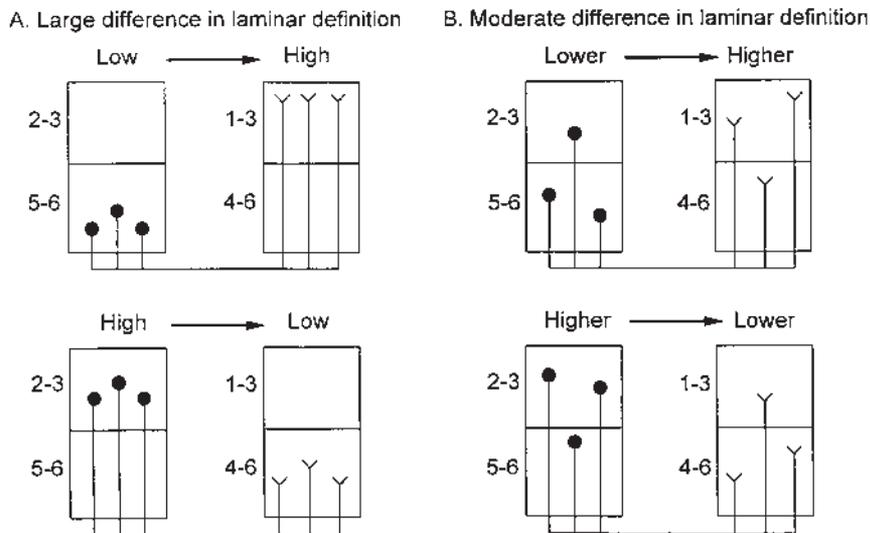
Figure 11. Summary of the pattern of connections predicted by the structural model. (*A*) Connections between cortices with large differences in laminar definition show a readily distinguishable pattern. (Top) Projection neurons originate predominantly in the deep layers of cortices with low laminar definition and their axons terminate predominantly in the upper layers of cortices with high laminar definition. (Bottom) The opposite pattern is seen for the reciprocal connections. (*B*) A less extreme version of the above pattern is predicted in the interconnections of cortices with moderate differences in laminar definition. (Top) Most neurons (though fewer than in *A*) originate in the deep layers of the cortex with comparatively lower laminar definition, and their axons terminate primarily in the upper layers of the cortex with comparatively higher laminar definition. (Bottom) The opposite pattern is predicted for the reciprocal connections.

Comparison with Sensory Cortices

In the sensory cortices connectional patterns have been described as forward and backward to denote their relationship to the primary cortices, or into ascending and descending, which suggests function (for review see Felleman and Van Essen, 1991). Forward connections proceed away from the primary cortices. In functional terms, they originate in cortices concerned with elementary sensory processing, and terminate in areas concerned with more complex aspects of sensory processing. Backward projections proceed towards the primary areas (Rockland and Pandya, 1979; Friedman *et al.*, 1986; Rockland and Van Hoesen, 1994).

Are the connectional patterns in the prefrontal and in the sensory cortices comparable? Projections originating from areas with lower laminar definition and terminating in areas with higher laminar definition in this study are comparable to the backward projections described in sensory cortices. In both cases, this type of projection originates primarily in the deep layers and terminates primarily in the upper layers, with layer I most heavily targeted. Projections originating in areas with higher laminar definition and terminating in areas with lower laminar definition in this study are comparable to forward projections described in sensory cortices. In both cases, this type of projection originates primarily in the upper layers (layers 2 and 3). In the sensory cortices, forward projections terminate around layer 4, and in the prefrontal cortices they terminated mostly in the deep layers (4–6). Thus, the prefrontal interconnections are comparable to those in the sensory cortices, though somewhat more diffuse (see also Friedman *et al.*, 1986; Saleem and Tanaka, 1996).

The structural model may be applied to the sensory and motor cortical systems as well because they too show systematic structural differences in primates (for review see Pandya *et al.*, 1988). For example, if we consider the entire ventral visual cortical system, laminar definition is highest in V1 and declines gradually along a direction leading to ventral temporal polar areas. A similar pattern is observed for the dorsal visual cortical

system, which extends from dorsal V1 to superior temporal visual cortices. Viewed within the framework of the structural model, ascending projections in visual areas always originate in areas with higher laminar definition in comparison with the site of termination, while the opposite is true for descending projections. Thus, whereas the pattern of corticocortical connections has previously been interpreted within a sensory specific directional framework (e.g. Rockland and Pandya, 1979; Maunsell and Van Essen, 1983), the present study demonstrated that it is the structural relationship of two cortices that ultimately permits one to predict the pattern of their interconnections.

The structural model differs from previous qualitative descriptions of patterns in corticocortical connections in several ways. First, the structural model predicts connectional relationships. Connections can then be used to test the model. In contrast, the establishment of ascending or descending relationships in sensory cortices relies on a study of the connections. Second, the structural relationship of two areas, described here by Δ, can be applied to all cortices. In contrast, the ascending and descending terms apply only to sensory cortices. Third, the structural model can discriminate among grades of connections within a direction, a relationship described here by the value of Δ. In contrast, most descriptions of connections in the sensory cortices have not taken into account relative differences in the pattern of connections within the ascending and descending directions. For example, the projections from area TE3 to TE1 and from TE2 to TE1 may be described simply as forward, or ascending. However, the structural model predicts that the proportion of projection neurons in the upper layers will be higher in TE3 than in TE2, because laminar definition is highest in TE3, intermediate in TE2 and lowest in TE1. Therefore, Δ is greater in the first than in the second pair of cortices. The structural model similarly predicts a graded pattern for connections proceeding in the opposite direction. In fact, there is evidence that as the distance from V1 increases so does the proportion of projection neurons in the

deep layers of visual association areas which project to V1 (Kennedy and Bullier, 1985). Longer distance in the latter case coincides with a higher absolute value of Δ within the framework of the structural model. Finally, the structural model does not assume that cortices are organized in strict hierarchies, and there is considerable evidence that connections are highly distributed (for discussion see Goldman-Rakic, 1988). However, the ascending and descending terminology implies a connectional and functional hierarchy. Hierarchical schemes in sensory cortices are based on the assumption that directionally specific corticocortical connections are qualitatively different. This idea is contrary to evidence suggesting that connections differ quantitatively but not qualitatively, as shown in previous studies and here (Schwartz and Goldman-Rakic, 1984; Barbas, 1986; De Lima *et al.*, 1990; Einstein, 1997). A qualitative scheme of connections has been used to place areas in hierarchies in the sensory cortical systems (Felleman and Van Essen, 1991) with limited success (see Hilgetag *et al.*, 1996). In assigning hierarchical position, models based on a qualitative pattern of connections succeed in cases where neurons originate overwhelmingly in the upper layers, or in the deep layers, but fail for patterns in between the above extremes (see Fig. 3 in Felleman and Van Essen, 1991).

What Are the Factors that Determine these Stereotyped Connections?

An intriguing question is what factors contribute to the establishment of these highly consistent connectional patterns. In the rhesus monkey, connections of prefrontal cortices occur prenatally (Schwartz *et al.*, 1991), and their establishment is likely to depend on a variety of developmental factors (for reviews see O'Leary *et al.*, 1994; Allendoerfer and Shatz, 1994; Sur and Cowey, 1995). Developmental abnormalities may disrupt the pattern of these highly ordered corticocortical connections. Human disorders which are thought to have their roots in development include dyslexia, learning disabilities, schizophrenia and certain forms of epilepsy (Akbarian *et al.*, 1993; for review see Mischel *et al.*, 1995). There is evidence that in dyslexia there are changes in the structure of cortices associated with auditory perception (Galaburda and Kemper, 1979). The present findings suggest that abnormal structure may lead to abnormal connections. Disruption of cortical pathways is also likely to account for the complex cognitive and mnemonic deficits observed in Alzheimer's disease, which appears to affect preferentially neurons participating in corticocortical connections (Hyman *et al.*, 1984; Pearson *et al.*, 1985; Lewis *et al.*, 1987; Morrison *et al.*, 1990; Arnold *et al.*, 1991; Van Hoesen *et al.*, 1991).

The present study demonstrated that the structural model predicts the pattern of connections within the prefrontal association cortices. Identification of the specific neurons involved in neural circuits is critical in understanding basic principles of neural communication. Further studies are necessary to determine whether these findings apply to other systems and in other species as well. The strength of the structural model lies in its potential to predict connectional relationships in the human cortex, which shows systematic changes in laminar definition as well (Sanides, 1969). The significance of the highly ordered pattern of corticocortical pathways ultimately lies in identification of their functional attributes.

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