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Synaptic Termination of Thalamic and Callosal Afferents in Cingulate Cortex of the Rat

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ABSTRACT The distribution of degenerating thalamic and callosal afferents to cingulate cortex in the rat is analyzed. Both light microscopic silver impregnation and quantitative electron microscopic techniques demonstrate differences in the form, number, and laminar distribution of these two afferents in anterior and posterior cingulate cortices.

Afferents from the mediodorsal thalamic nucleus terminate in area 24. Most terminals are in layer IIIb, fewer in layer Ia–b, and least in layers V and VI. In contrast, callosal afferents terminate mainly in layers Ib–c, II, IIIa, V, and VI. Thus, thalamic and callosal afferents terminate in a complementary pattern except in layers Ib and IIIb where they overlap. Quantitative analysis of degenerating axon terminals in area 24 indicates that there may be as many as seven times more callosal than mediodorsal thalamic terminals in this cortex.

Projections of the anterior thalamic nuclei terminate in areas 29b and 29c, primarily in layer Ia, with fewer in layers Ib–IV and least in layers V and VI. Callosal afferents end mainly in layers V and VI and less densely in layers I–IV, which results in some overlap of thalamic and callosal afferents in layers Ic, IV, and V. In addition, patterns of termination of callosal afferents in posterior cingulate cortex change at borders between previously defined cytoarchitectural areas.

Anterior thalamic terminals in area 29c differ from other thalamocortical afferents described previously in that they form two types of terminals. One is large (2–4 μm in diameter) and occurs mainly in layer Ia, whereas the second type is smaller and is present in layers Ib–V. Both types of terminals form asymmetric synapses mainly with dendritic spines.

Functional studies of cingulate cortex in rodents indicate that this cortical region is involved in motor responses and some learning processes. Thus, Lende and Woolsey ('54) applied focal electrical stimuli to cingulate cortex and evoked somatic and autonomic motor responses, while behavioral studies have shown that learning tasks which involve alternation (Barker and Thomas, '65, '66) or aversively cued stimulus recognition (Peretz, '60; Thomas and Slotnick, '63) are disrupted following removal of cingulate cortex.

Recent single and multiple unit electrical recording studies in rats and rabbits have demonstrated changes in neuronal activity in cingulate cortex and the anterior thalamus during the acquisition of a conditioned response (Segal, '73; Vinogradova, '75; Gabriel et al., '80). The structural basis for interpreting these response sequences is provided by extensive neuroanatomical investigations of cingulate connections with the thalamus (Clark and Boggon, '33; Domesick, '72; Krettek and Price, '77), the ipsilateral cortex (White, '59; Swanson and Cowan, '77; Beckstead, '79), and contralateral cingulate cortex (Jacobson, '70; Jacobson and Trojanowski, '74). Nevertheless, there remain a number of anatomical questions regarding the termination of both thalamic and especially callosal afferents.

Light microscopic analyses indicate that thalamic afferents to cingulate cortex terminate in layers I and III of anterior (Krettek and Price, '77) and posterior (Domesick, '72) cingulate cortex. However, the presence or extent of termination in layers V and VI has not been established with light microscopy due to confounding of this termination with fibers of

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passage also contained in these layers. One reason to suspect deep layer termination is that Hunt and Schmidt ('78) have identified binding of the irreversible acetylcholine receptor ligand α-bungarotoxin in layers I, V, and VI. If the thalamic afferents to cingulate cortex are cholinergic as suggested by Lewis and Shute ('67), then this binding study suggests that they do terminate in the deeper layers. We have addressed this issue with an electron microscopic analysis of terminal axonal degeneration to resolve whether or not the axons of mediodorsal and anterior thalamic neurons terminate in layers V and VI.

The density and termination pattern of contralateral connections in cingulate cortex have not yet been established. Jacobson ('70) has reported that areas 25 and 32 of the rat cingulate cortex have no callosal connections and that areas 24 and 29c both receive heavy callosal termination in the deep layers V and VI. While agreeing that area 25 has no callosal connections in the mouse, Yorke and Caviness ('75) have reported that area 24 receives only light callosal termination in the superficial layers (I–III) and that the posterior divisions of area 29 receive a light callosal projection mainly in the deeper layers. Our observations suggest that variations in cytoarchitectural boundaries and experimental procedures rather than species differences could account for these diverse results.

The present investigation has been undertaken to evaluate the following issues. First, in light of our recent cytoarchitectural analysis of cingulate cortex (Vogt and Peters, '81), what is the areal and laminar distribution of callosal afferent termination in the rat and what neurons give rise to these axons? Second, what are the differences or similarities in the laminar distribution of thalamic and callosal afferents in anterior and posterior cingulate cortices? Finally, what types of axonal terminals degenerate following thalamic or callosal lesions and does their distribution correspond to that observed in light microscopic preparations?

MATERIALS AND METHODS

Degeneration: Light and electron microscopic

Adult, hooded (Long-Evans) rats were used for analyzing the distribution of degenerating afferents following either callosal or thalamic lesions. The animals were anesthetized with either 36% chloral hydrate (0.1 cc/100 gm body weight) or Chloropent (0.32 cc/100 gm body weight, Fort Dodge Labs, Fort Dodge, IA) and placed in a Kopf Small Animal Stereotaxic Instrument. For callosal transections, a longitudinal opening was made in the skull, 1-mm wide, parallel to and 0.75–1.0 mm lateral to the midsagittal suture and extending from 2 mm anterior to bregma to 1 mm posterior to lambda. Then a scalpel blade, held parallel to the midline, was inserted 3–4 mm deep into the brain and drawn along the length of the cranial incision. Lesions were placed in the thalamus with an angled, stainless steel electrode through which 1 mA of current (DC+) was passed for 8 seconds using a constant current lesion maker (Grass Instruments Co., Quincy, MA).

For light microscopy a survival period of 3–4 days was employed, at which time the rats were again anesthetized and perfused through the heart with approximately 100 ml of 0.9% saline followed by 200 ml of 10% formalin. The brains were then removed and postfixed in 10% formalin for at least 1 week before being embedded in albumin-gelatin and cut into 25-μm-thick coronal sections with a freezing microtome. In most cases, two series of sections were stained for degeneration according to a modified Fink-Heimer procedure (Fink and Heimer, '67). One of these series was differentiated with a 1% potassium ferricyanide/2% sodium borate solution and then placed on gelatin-chrome-alum-coated slides (Heimer, '70) and pressed to the surface with filter paper. Once dry these sections were counterstained with cresyl violet, allowing for delineation of degeneration patterns within distinct cytoarchitectural areas and layers. A third series was mounted without previous processing and stained with cresyl violet.

A second series of lesions was made similar to those already described. Following a 2 day postoperative survival period the animals were anesthetized and perfused for electron microscopy. During artificial respiration with 95% O_2/5% CO_2, the thorax was opened, 0.5 ml of 1% sodium nitrite was injected into the heart, and then 100 ml of a fixative was perfused through the heart. This first fixative contained 1% paraformaldehyde, 1.25% glutaraldehyde, and 0.015% calcium chloride in a 0.08 M cacodylate buffer at pH 7.2 and 30–40°C. This was followed with a similar volume of a second fixative which contained 2% paraformaldehyde and 2% glutaraldehyde in a 0.08 M cacodylate buffer (see Peters, '70, for further details). The heads of these perfused animals were left in

the refrigerator overnight and then the brains were removed and stored in the concentrated fixative. Blocks of cingulate cortex 2–4 mm thick were postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1½ hours, dehydrated, and embedded in Epon-Araldite. Thick sections (1–2 μm) were cut perpendicular to the cortical surface and stained with toluidine blue for light microscopy. Then adjacent thin sections were cut, poststained with uranyl acetate followed by lead citrate, and examined with an AEI-6 electron microscope. The remainder of each of these brains which was not used for electron microscopy was embedded in celloidin, cut at a 25-μm thickness, and stained for light microscopy with cresyl violet. Reconstruction of the thalami and extent of the lesion sites was based on the stereotaxic atlas of Albe-Fessard et al. ('71).

An estimate of the mean number of degenerating axonal terminals per unit area of cortex following either thalamic or contralateral lesions was made by placing thin sections of cingulate cortex on 300-mesh grids and staining with uranyl acetate followed by lead citrate. Degenerating axon terminals were then counted in three rows of grid squares extending between the pia and white matter. The term "degenerating axon terminal" refers to darkened processes which appear after a lesion. Such terminals contain the remains of synaptic vesicles and mitochondria and may or may not be seen in any one section to form a synaptic junction. Degenerating axon terminals which formed two synaptic contacts were counted twice to reflect more accurately the density of synaptic termination. The mean number of degenerating terminals present in each horizontal row of three grid squares (i.e., mean number per bin) was then calculated and plotted by bin number and approximate layer which each bin contained (Fig. 12). Since each 300-mesh hole is 2,916 μm^2, the mean number of degenerating terminals per bin represents the number in an area of 2,916 μm^2.

Horseradish peroxidase retrograde labeling

To identify the laminar and areal distribution of cingulate cortex neurons that project through the corpus callosum, discrete injections of horseradish peroxidase (HRP) were made in the contralateral cortex. The subjects were adult, hooded rats which were anesthetized with Chloropent. Single injections of 0.05 μl of a 20% solution of HRP (Miles or Worthington) were placed in different loci in the cingulate cortex by means of a 5-μl Hamilton Syringe with either a 32- or 33-gauge needle. The syringe was mounted in a microinjector device in which the syringe plunger was positively controlled by a microdrive.

Two days after the injection the rats were perfused with a dilute aldehyde fixative (1% paraformaldehyde, 1.25% glutaraldehyde, and 0.015% calcium chloride in 0.1 M cacodylate buffer at pH 7.2) following the perfusion procedure of Rosene and Mesulam ('78). The brains were removed from the skull and cut into 40-μm-thick sections on a freezing microtome and then treated according to the procedure of Mesulam ('78) with tetramethyl benzidine (TMB) as the chromogen. The sections were counterstained with neutral red and examined with the light microscope using both brightfield and darkfield illumination. Cases in which darkfield illumination revealed a homogeneous granular reaction product in layer I of cingulate cortex contralateral to the site of the HRP injection were excluded from analysis, since this indicated possible passive diffusion of the injected HRP across the midsagittal fissure into the opposite hemisphere. It should be noted that evidence of such potential spread could only be observed in sections treated with the TMB procedure but not with less sensitive HRP methods (Mesulam and Rosene, '79).

RESULTS

The cytoarchitectural subdivisions of the rat cingulate cortex and their cytological composition have been described by Vogt and Peters ('81). Figure 1 presents the conclusions of this study as to the positions of Brodmann's ('09) areas 25, 32, 24, and 29 on the medial surface of the rat brain. The essential cytoarchitectural differences between anterior (areas 25, 32, and 24) and posterior (area 29) divisions of cingulate cortex are that the anterior areas lack a layer IV and are characterized as agranular, whereas posterior areas 29a–c have the granular layers II–IV.

Another difference between anterior and posterior areas is the composition of layer I. In coronal, thick plastic sections layer I can be subdivided into outer, middle, and inner parts, or layer Ia immediately below the glia limitans, Ib, and Ic, respectively, as early recognized by Vogt and Vogt ('19). These subdivisions are most conspicuous in area 29 (Fig. 2) in which layer Ia primarily contains the ter-

Abbreviations

ahp	Anterior hypothalamus
As	Astrocyte
at	Axon terminal
CCr	Rostrum of corpus callosum
CCs	Splenium of corpus callosum
D	Dendrite
dt	Degenerating axon terminal
Fx	Fornix
H	Hippocampus
Hb	Habenula
m	Stria medullaris
OC	Optic chiasm
P	Pial surface
Ps	Postsubiculum
s	Spine
t	Stria terminalis
tt	Taenia tecta

(Thalamic nuclei)

AM	Anterormedial
CL	Centrolateral
L	Lateral
MD	Mediodorsal
MV	Medioventral
Pt	Parataenial
PV	Paraventricular
R	Reticular
Re	Reuniens
VA	Ventral anterior
VL	Ventral lateral
VM	Ventral medial
VP	Ventral posterior

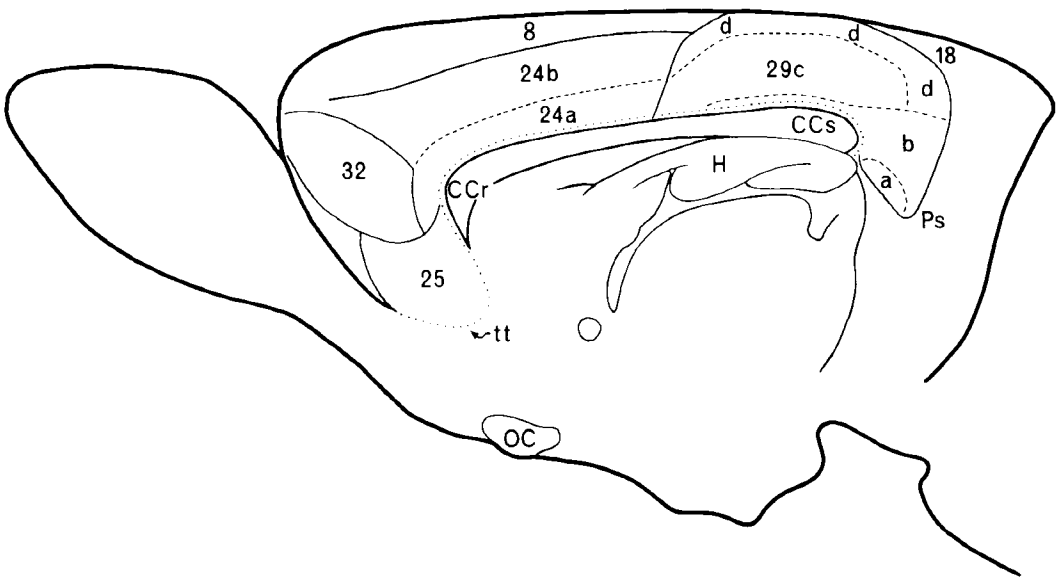

Fig. 1. Topographical positions of the cytoarchitectural areas of cingulate cortex in the rat.

minal tufts of small and fusiform pyramidal neurons of layer II–III and the tufts of layers V and VI pyramids (Vogt and Peters, '81). Layer Ia also has virtually no myelinated axons and, as described below, is the primary site of anterior thalamic termination. Layer Ib contains numerous large, rostrocaudally oriented, myelinated axons, thick primary and secondary apical branches of neurons in layer II–III and the apical tufts of layers IV, V, and VI pyramids. Layer Ic is similar to layer Ib except that it contains substantially fewer myelinated axons. These same layer I subdivisions are present in area 24 but, since there is a reduction in the number of myelinated axons, the distinctions among layers Ia, Ib, and Ic are less pronounced.

Cytoarchitectural subdivisions within areas 24 and 29 are recognized by the following criteria. Area 24a has a relatively even distribution of neurons in layers II–III, while in area 24b layer III is less cell dense, so that there is

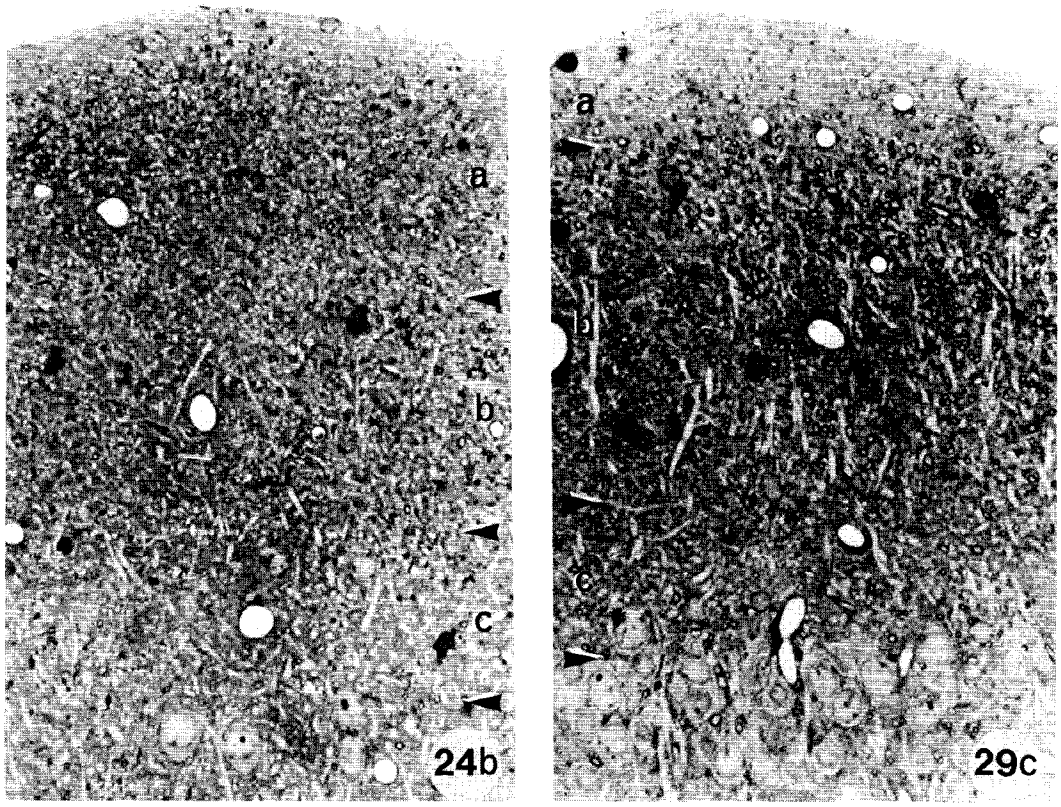

Fig. 2. Subdivisions of layer I in anterior (area 24b) and posterior (area 29c) cingulate cortex. Toluidine blue stain, × 460.

a clearer demarcation of layers II, III, and V. In posterior area 29a the somata of neurons in layers II–IV are homogeneously distributed, while in areas 29b and 29c there is both a cell-dense layer II–III and a less cell-dense layer IV. Furthermore, in area 29b the large pyramids of layer V directly abut the granular layer IV, while in area 29c these large pyramids are separated from layer IV by a layer of medium-sized pyramids and a thin lamina dissecans (clear zone). Finally, "agranular" retrosplenial cortex or area 29d has an incipient layer IV which lies below a typical layer III (i.e., like neocortex) and so is actually "dysgranular."

Origin and distribution of callosal afferents

Neuronal origin of callosal afferents. Injections of horseradish peroxidase (HRP) into cingulate cortex result in extensive retrograde labeling of neurons in the contralateral cingulate cortex. These cells are located primarily in layers II–V of areas 24 and 29 with very few present in layer VI (Fig. 3). To prevent spread of the HRP between the two hemispheres, injections were often placed lateral to cingulate cortex in area 8. In instances in which the HRP from these lateral injections diffused mainly into the deeper layers of cingulate cortex contralateral neurons were labeled primarily in layer V (Fig. 3, level A2), while contralateral neurons in layers II and III as well as layer V were labeled when the HRP injection involved all layers (Fig. 3, level A1). Therefore, in area 24 layer V neurons project primarily to layer V contralaterally, while layers II and III neurons project mainly to layers I–III.

Although areas 24a and 24b appear to have essentially the same laminar distribution of HRP-labeled neurons, in area 29 there are some distinctions among the subdivisions. The granular retrosplenial areas 29a–c contain a slightly higher density of HRP-positive neu-

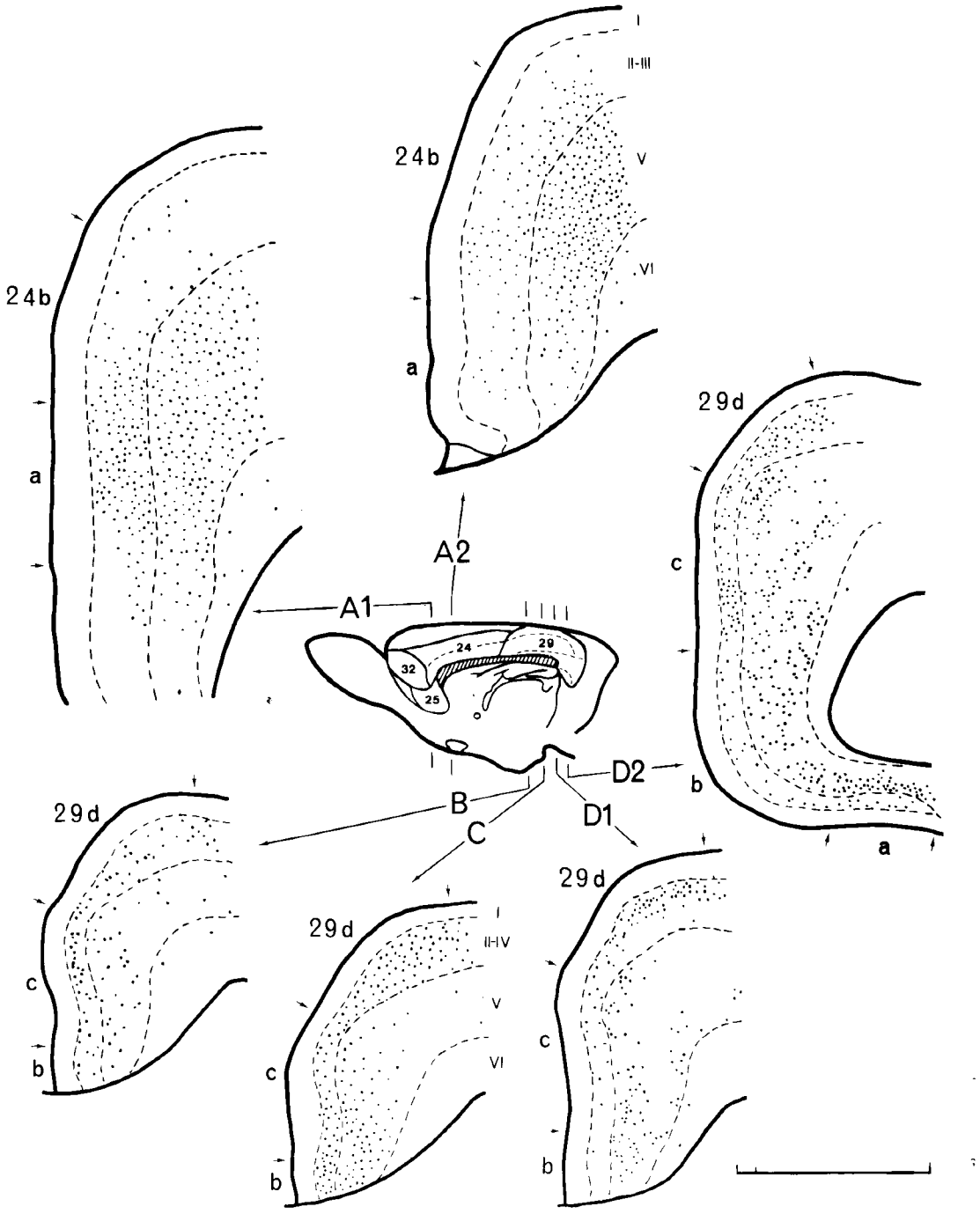

Fig. 3. Distribution of HRP-positive neurons in cingulate cortex following contralateral HRP injections. Sections A1 and A2 are from area 24, while sections B, C, and D are from area 29 of different experimental animals. Corpus callosum is hatched. Calibration = 1 mm with a 100-μm division.

rons in layer V than in layers II–IV. Although this may reflect differential injection sites or neuronal uptake, the anterograde degeneration experiments *(vide infra)* indicate that layer V in areas 29a–c also receives the strongest callosal termination. In contrast, area 29d has more labeled neurons in layers II–III than in layer V, although there are some patches of moderate labeling in layer V (Fig. 3B,C, D1, D2). Area 29d also has a generally more limited labeling of small neurons in layers II–IV than in these same layers of areas 29b and 29c.

It is often difficult to identify the types of neurons which have axons projecting across the corpus callosum because of the limited retrograde labeling of their dendritic tree or of the histological sectioning of the dendritic tree. In area 24, neurons with relatively complete filling of the dendritic tree are primarily medium and large pyramidal cells in layers II, III, and V, while in areas 29a–c small pyramids in layers II–III and IV, as well as medium to large pyramids in layer V, project contralaterally (Fig. 4). Although most of the callosally projecting neurons appear to be pyramidal cells, there are instances in which the shapes of the somata and dendritic tree do not conform to those characteristics of a pyramidal cell. One of these instances is illustrated in Figure 4, which shows an HRP-labeled neuron in layer II of area 29d with an oval soma and a bitufted distribution of dendrites. It is possible, therefore, that in addition to pyramidal cells, the bitufted nonpyramidal neurons in layer II of area 29d (Vogt and Peters, '81) may project across the corpus callosum.

Laminar distribution of callosal afferents. The laminar distribution of callosal afferents was evaluated in cases with contralateral lesions. These lesions involved the entire rostrocaudal extent of the corpus callosum and cingulum bundle and effectively deafferented one cingulate cortex from the other. In addition to the cingulum bundle, callosum, and overlying cortex, these lesions usually involved deeper structures including the septum, hippocampus, dorsal hippocampal commissure, dorsal thalamus, and superior colliculus.

Following these lesions, all subdivisions of cingulate cortex contain degeneration when stained by the Fink-Heimer procedure (Fig. 5). The density of degeneration within individual cases ranges from extremely dense degeneration in superficial layers Ib–III of areas 32, 24a, and 24b, through moderate degeneration in layers V and VI of areas 32, 24, and 29, and light or no degeneration in layer I of areas 29a–c, to no degeneration in layers V and VI of area 25.

Comparison of the distribution and density of degeneration in the superficial and deep layers of cingulate cortex indicates that most of the differences among areas are due to variation in the quantity found in layers I–IV, while degeneration in layers V and VI is moderate and relatively constant among cingulate areas (Fig. 5). Layers Ib–III of areas 32, 24a, and 24b are the main recipients of contralateral afferents, while layers I–III of area 25 and layers I–IV of area 29d contain less degeneration and layers I–IV of areas 29b and 29c have the least amount of degeneration.

Though layers V and VI of all subdivisions of area 29 (a–d) contain essentially uniform and moderate amounts of degeneration, degeneration in the more superficial layers varies (Figs. 5, 6, and 7). Overall, area 29d contains the most degeneration, but only light to moderate amounts are present in layers I–IV. In some transverse sections of area 29d very little degeneration is present in the superficial layers, but no consistent topography of light and moderately dense termination zones can be identified among these cases. In areas 29b and 29c, which have cytoarchitecturally distinct granular layers II–III and IV, layers Ia and Ib and both granular layers appear to be essentially free of degeneration, although electron microscopy indicates the presence of some synapse-forming terminals in layers II–IV *(vide infra)*, while layer Ic has a little degeneration with occasional places that are clear. In contrast, area 29a, which has a homogeneous granular layer II–IV, contains much degeneration among the densely packed somata of neurons in the granular layer whereas the granular layer in areas 29b and 29c is almost free of degeneration (Fig. 6). In addition, area 29a has slightly more degeneration in layer I than do areas 29b or 29c.

In summary, anterior agranular and posterior granular divisions of cingulate cortex receive different laminar distributions of callosal terminals, with area 24 receiving callosal terminals mainly in the superficial layers and area 29 receiving the projection mainly in the deeper layers. Although in areas 24a and 24b the layers containing efferent callosal neurons (shown by HRP) as well as the layers of callosal termination (shown by degeneration) are essentially the same, the differential distribution of callosal terminals in the posterior areas of cingulate cortex coincides with many of the

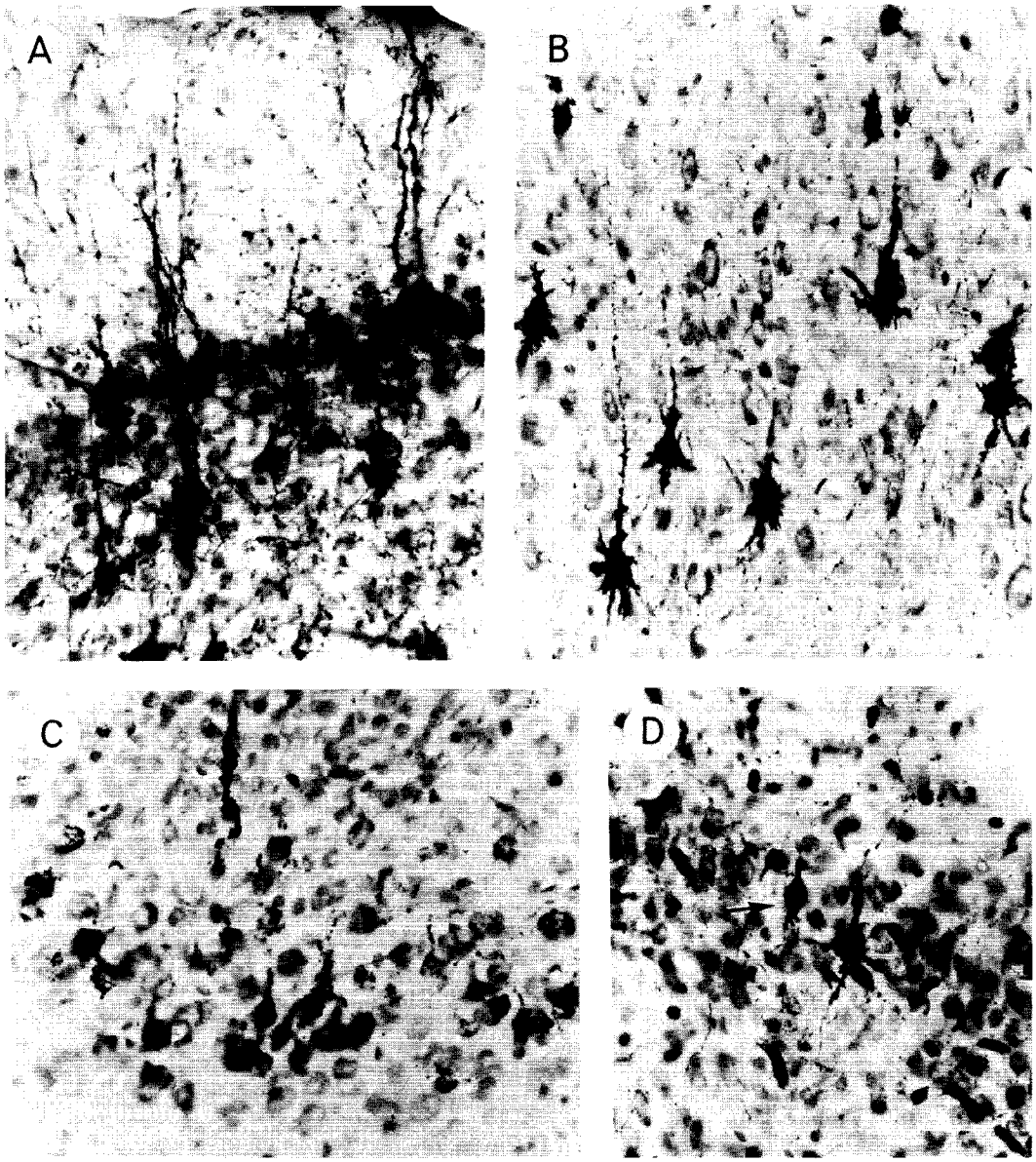

Fig. 4. HRP-positive neurons in area 29. A. Small pyramids in the granular layers II–IV of area 29c. B. Medium-sized to large pyramids of layer V in area 29c. C. Layer V pyramids in area 29a. D. "Bitufted" (arrow) and pyramidal neurons in layer II of area 29d. × 344.

cytoarchitectural subdivisions previously recognized. Area 29a receives a projection in the granular layer, while areas 29b and 29c receive so few degenerating terminals in these layers that they can only be seen with electron microscopy. Area 29d receives a moderate amount of termination in layers I–IV, while layers I–IV of areas 29b and 29c are almost free of degeneration.

Laminar segregation of thalamic and callosal afferents

Comparison of the distribution of callosal degeneration with previous reports of the distribution of thalamic termination suggested the possibility of a laminar segregation of the terminations of these systems. To examine this issue, thalamic lesions were placed in either

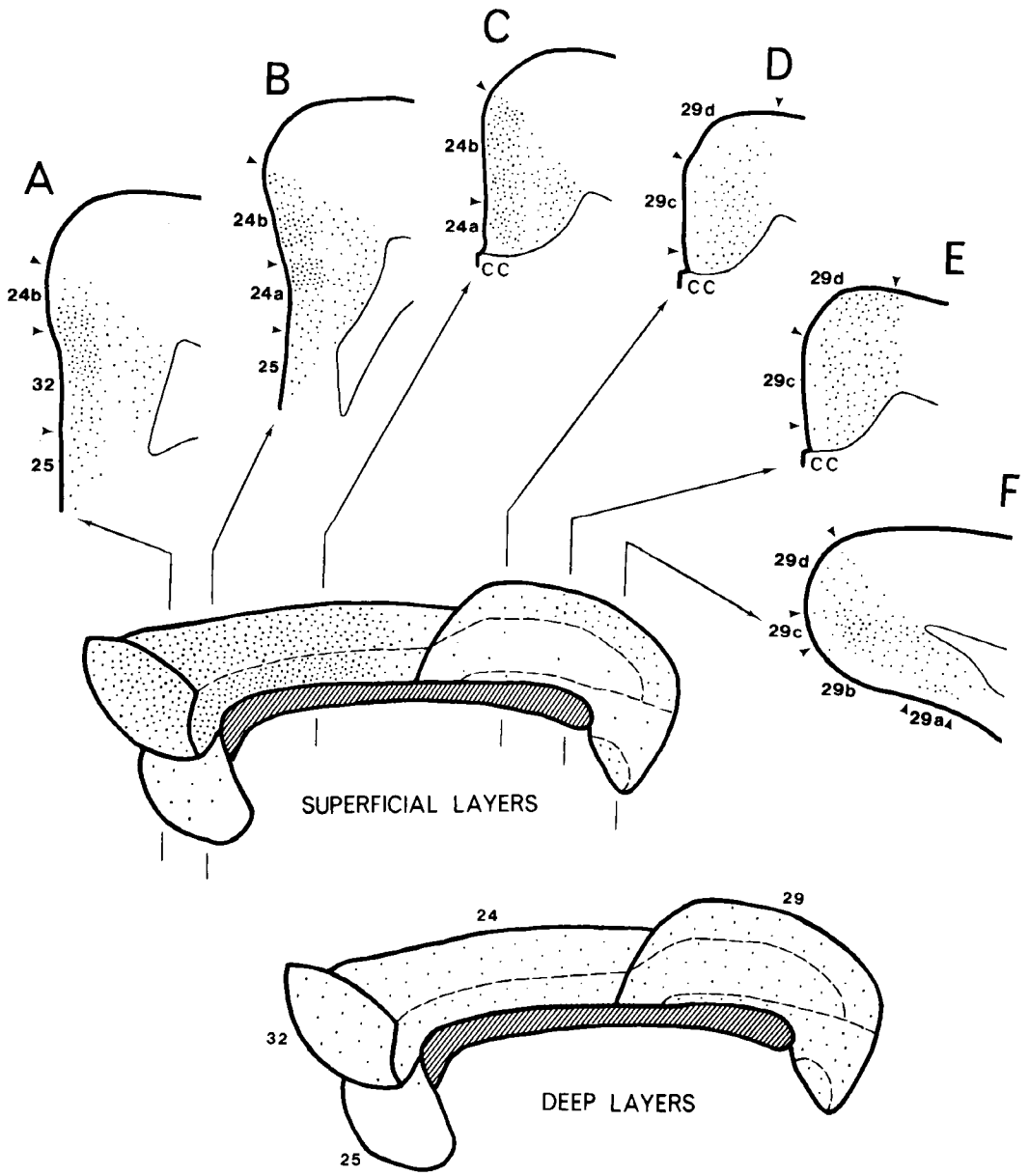

Fig. 5. Distribution of degeneration in cingulate cortex following a contralateral lesion. Coronal sections A–F demonstrate the laminar distribution of degeneration, while the density of degeneration in the superficial and deep layers is presented on surface drawings of the medial surface. The cytoarchitectural areas are outlined and the corpus callosum is hatched.

the mediodorsal or anterior thalamic nuclei for analysis of the distribution of these afferents in anterior or posterior cingulate cortex respectively.

The mediodorsal nucleus was destroyed by dual electrolytic lesions placed from a contralateral approach in order to involve a maximal number of thalamocortical afferents (Fig. 8). These lesions involved parts of the anteromedial, centrolateral, paraventricular, and lateral nuclei of the thalamus as well as much of the habenular nuclei and stria medullaris. Lesions of the anterior nuclei were placed with an angled electrode to avoid involvement of the

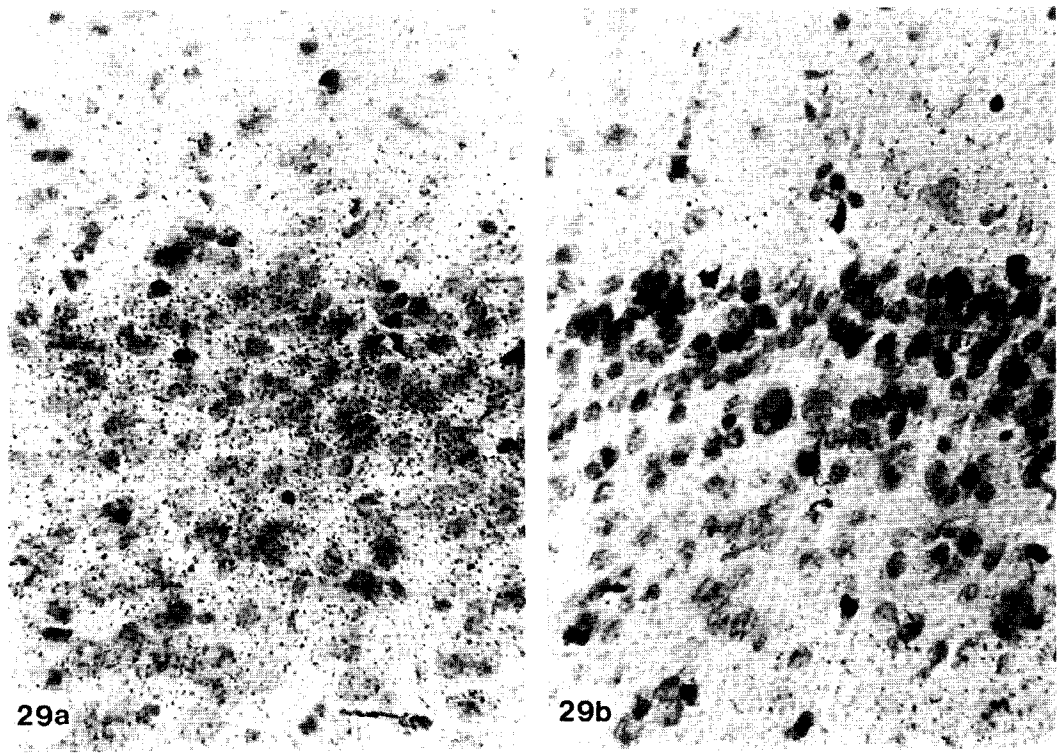

Fig. 6. Degeneration in layers I–IV of areas 29a and 29b following a callosal lesion. Fink-Heimer stain, × 377.

cingulum. These lesions involved all subdivisions of the anterior nuclei and destroyed fibers passing laterally from the medially located spared parts of the anterodorsal and anteromedial nuclei and possibly from the ventromedial and reuniens nuclei. The ventral anterior, lateral, and reticular nuclei of the thalamus were also damaged.

Despite the known problems in making estimates of the density of terminal degeneration, it is reasonable to compare the distribution and relative density of thalamic and callosal input to cingulate cortex when lesions in both systems are essentially complete and the degeneration is analyzed at an optimal postoperative survival time for each system. Furthermore, although silver procedures impregnate both degenerating small axons and terminals, the validity of these light microscopic observations can be verified, as shown in the next section, with quantitative electron microscopy of terminal degeneration.

Extensive as well as restricted lesions of the mediodorsal nucleus result in silver-impregnated degeneration in both areas 24 and 32 primarily in layer IIIb with less in layers Ia and Ib. Termination of callosal afferents in area 24 differs from that of the thalamic afferents in two prominent ways. First, most contralateral afferents terminate in those layers which receive fewest thalamic afferents. These include layers Ib and Ic, II, IIIa, Vb,and VI. Second, the overall quantity of degeneration produced by contralateral lesions appears to be five to ten times as great as that produced by mediodorsal thalamic lesions. Figure 7 compares the differential laminar distribution of degeneration originating from thalamic and callosal sources. Although these observations indicate a substantial segregation of these two systems of afferents in area 24, there are two laminae in which thalamic and callosal afferents overlap: layer Ib and layer IIIb.

Segregation of thalamic and callosal afferents is also apparent in posterior cingulate cortex (area 29c). Lesions of the anterior nuclei result in silver-impregnated degeneration in area 29c which is most conspicuous in layers I and IV, with less in layer II–III (Fig. 7). In contrast, termination of contralateral affer-

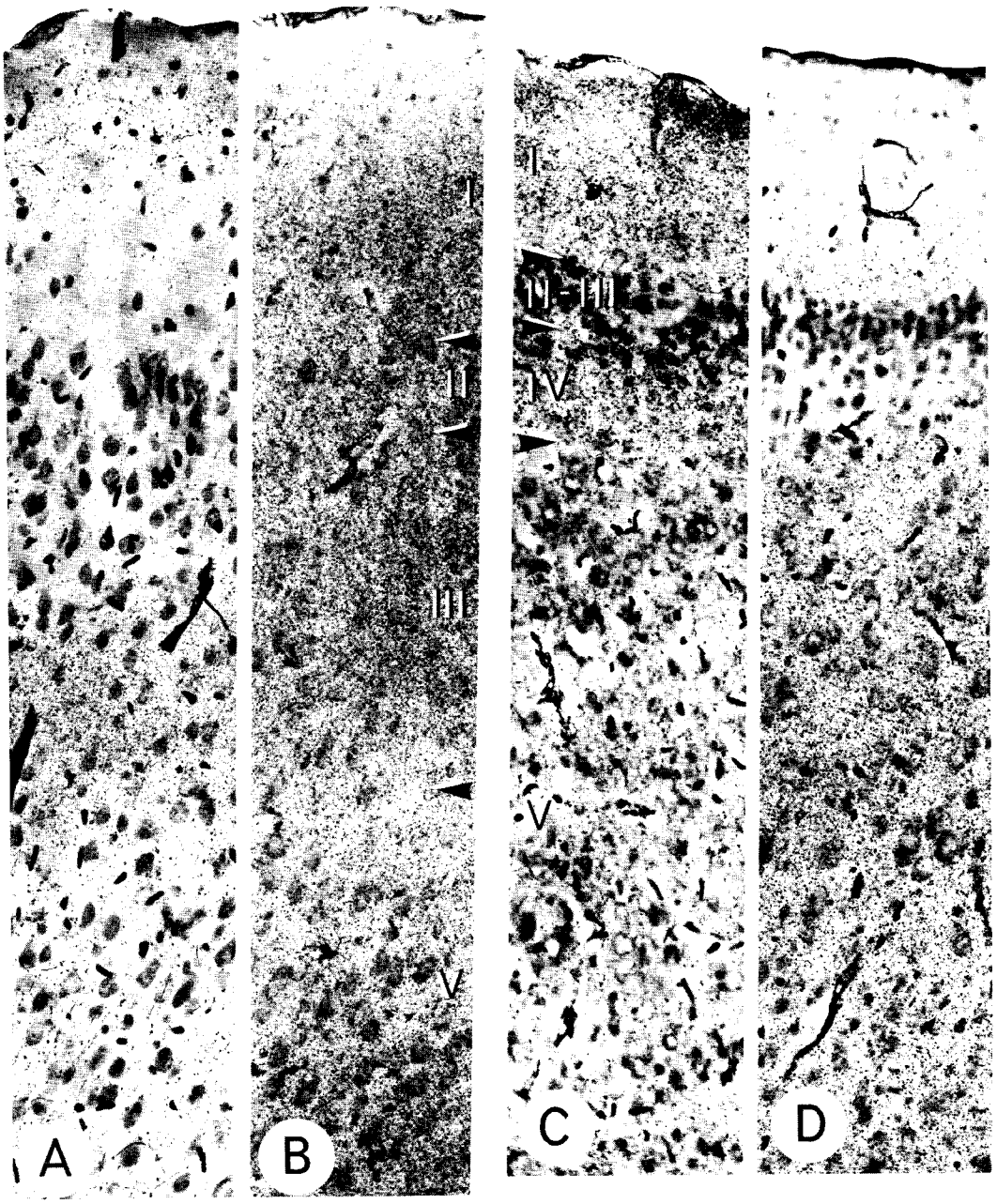

Fig. 7. Laminar distribution of degeneration in area 24b following mediodorsal thalamic (A) and callosal (B) lesions and in area 29c following anterior thalamic (C) and callosal (D) lesions. Fink-Heimer stain, × 150.

ents in area 29c is most obvious in layers V and VI with little in layer Ic and virtually none in either layers Ia and Ib or layers II–IV. Limited overlap of thalamic and callosal afferents occurs in layer Ic.

A number of questions remain regarding the interpretation of these light microscopic observations. These include whether degeneration in layers V and VI after thalamic lesions is composed of terminal degeneration as well

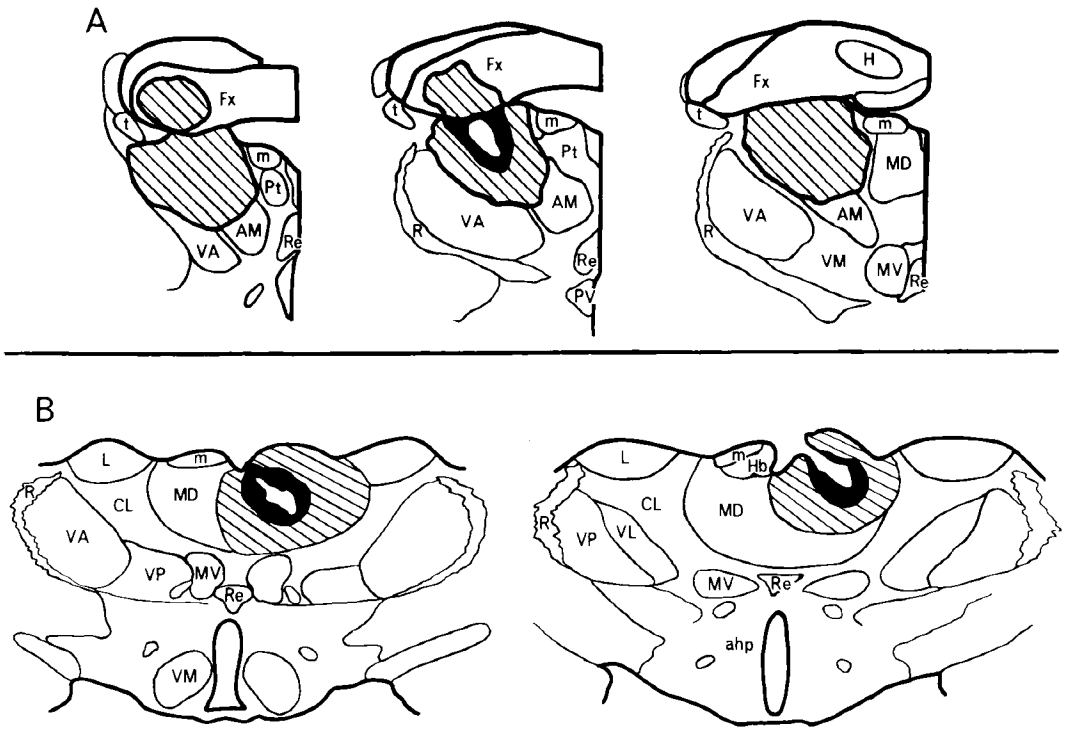

Fig. 8. Reconstruction of lesions placed in the anterior (A) and mediodorsal (B) thalamic nuclei.

as fibers of passage and whether, following contralateral lesions, terminal degeneration is present but not made apparent by silver staining. It is also uncertain whether the dense degeneration in area 24 following a contralateral lesion and the moderate amount following a mediodorsal thalamic lesion reflects real differences in the number of synaptic contacts formed by these afferents and/or differences in the form of the terminal axonal arbor; i.e., the callosal axons have more numerous branches than thalamic axons but each forms the same number of synaptic contacts. An ultrastructural analysis of the type, distribution, and quantity of degenerating axon terminals addresses these issues.

Ultrastructure of degenerating thalamic and callosal afferents

A survey of the ultrastructure of cingulate cortex indicates a number of characteristics of area 29c which are not present in area 24b. First, in area 29c layers I and IV have a particularly high density of heavily myelinated axons. Second, layer Ia, which is adjacent to the glial limiting membrane and occupies approximately one-quarter of the thickness of layer I, contains some especially large synaptic terminals. These terminals are approximately 2–4 μm in diameter and form asymmetric synaptic contacts mainly with both large and small dendritic spines (Fig. 9A). Third, neurons in layer II–III are very closely apposed (i.e., no intervening neuropil elements) in groups of three or more reflecting the dense packing of somata seen in light microscopic preparations.

Thalamic afferents. The degeneration produced in area 24b following mediodorsal thalamic lesions is similar in form and distribution to that reported for other thalamocortical afferents. Thus, degenerating terminals form exclusively asymmetric synapses, predominantly with dendritic spines (Fig. 10A), and occasionally with smooth dendritic processes (approximately 5%). The greatest numbers of degenerating terminals are present in layer

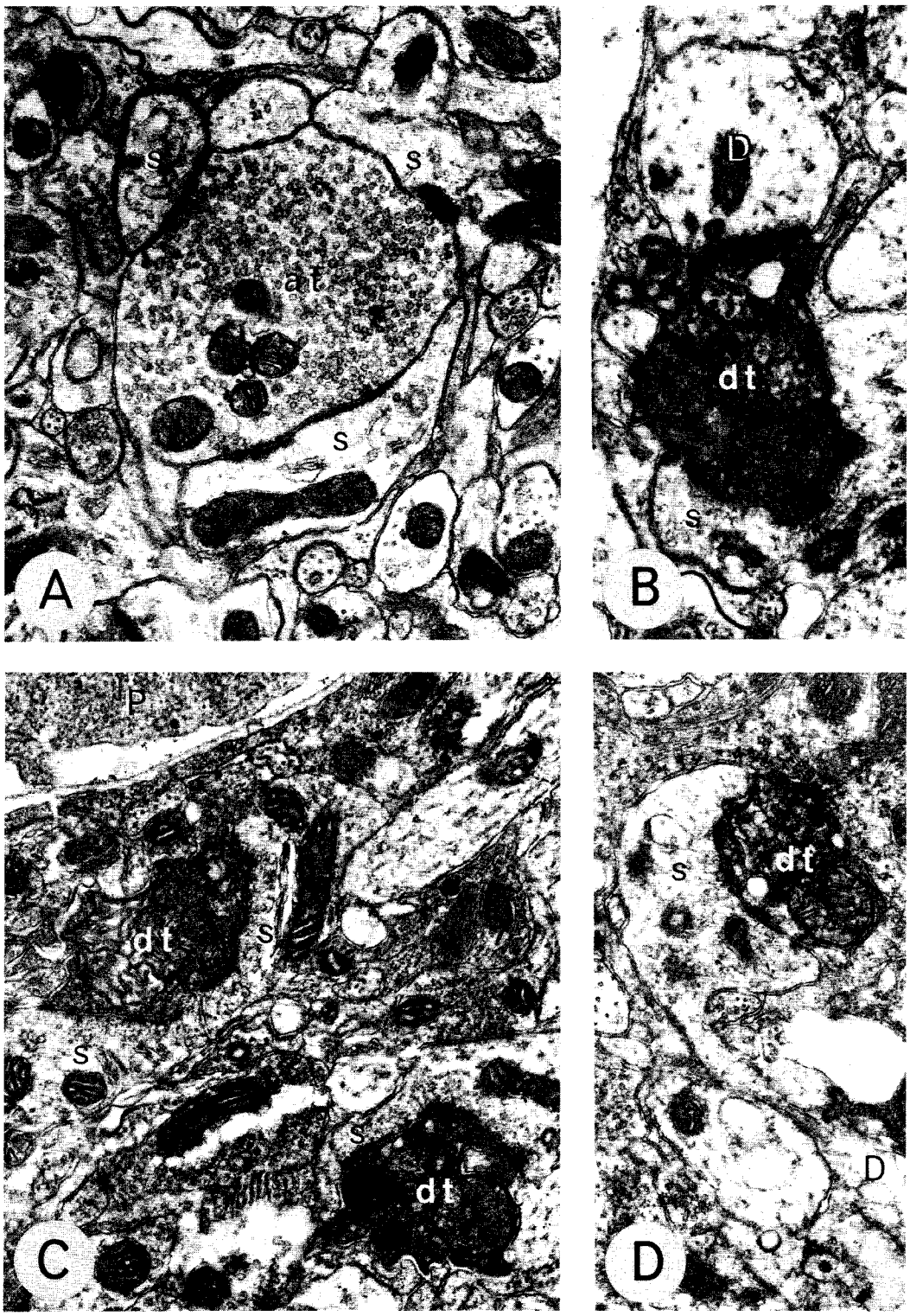

Fig. 9. Large axonal terminals in layer Ia of area 29c. A. Normal, large axonal terminal (at) forming asymmetric synapses with three spines (s). B, C, and D are photomicrographs of large, degenerating axonal terminals (dt) forming synapses primarily with spines but also with a smooth dendritic process. × 27,000.

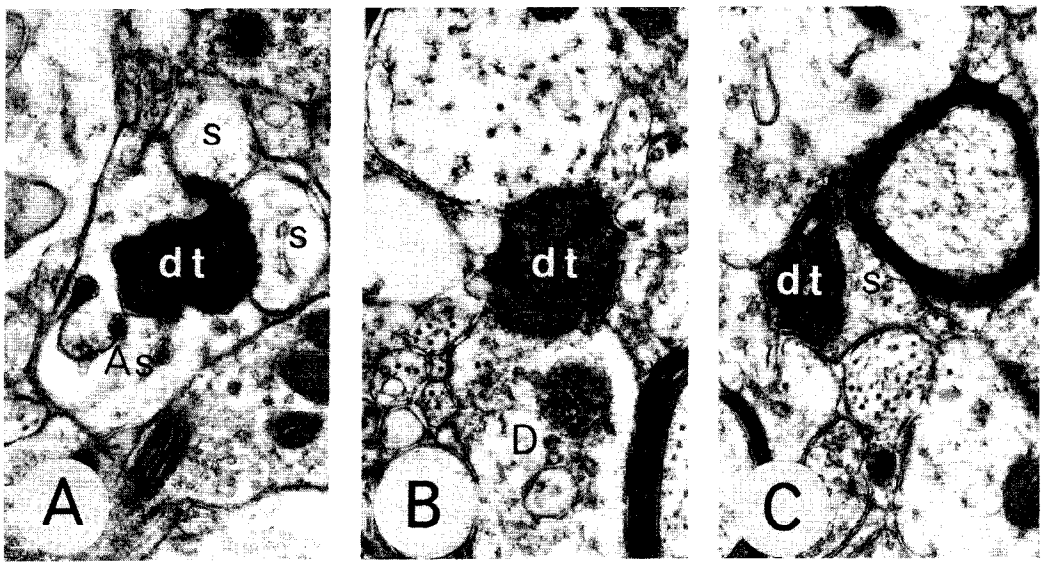

Fig. 10. Small, degenerating axonal terminals (dt) following mediodorsal (A) and anterior (B, C) thalamic lesions. A. dt forming asymmetric synapses with spines (s) in layer III of area 24b. B. dt forming an asymmetric synapse with a dendrite (D) in layer I of area 29c. C. dt forming an asymmetric synapse with a spine in layer IV of area 29c. × 30,000.

IIIb (≈11/bin), with fewer in layer Ib (≈5/bin), and least in layers V and VI (≈1–3/bin) and in layers II and IIIa (≈0–2/bin), as indicated in Figure 12.

In contrast, degenerating anterior thalamic afferents to area 29c are of two distinct types. One type is a large terminal in layer Ia directly beneath the pial surface (Fig. 9B,C,D). These terminals, though slightly shrunken in comparison to the large, nondegenerating terminals, form extensive asymmetric contacts mainly with small and large dendritic spines. The second type of terminal is smaller and also makes synapses primarily with spines and occasionally with smooth dendritic processes (Fig. 10B and C). These smaller terminals are present mainly in layers Ib and Ic and II–V.

The laminar distribution of degenerating thalamocortical terminals in area 29c is presented in Figure 12 and indicates that terminals are present mainly in layer Ia (≈23/bin), with fewer of them in layers Ib–IV (≈2–5/bin), less in layer V (≈0–2/bin), and none in layer VI. It is clear that although axons of passage and a limited number of thalamocortical axon terminals are present in layers V and VI of areas 24 and 29, the primary site of thalamic termination is in the superificial layers as indicated by light microscopic observations. It should also be noted that the number of terminals in layer II–III of area 29c is included with that of layer IV because layer II–III is narrower than one 300-mesh grid square and because there is great variability in the density of neuronal somata in this layer. In places where there are many neuronal somata and few neuropil elements, the number of degenerating terminals is greatly reduced. The actual number of degenerating terminals in layer II–III, therefore, is less than in layer IV.

Finally, in addition to degenerating terminals, the myelinated axons of layer Ib in area 29c undergo extensive degeneration following anterior thalamic lesions. Although degenerating myelinated axons are also present in deeper layers, there appears to be less degeneration of horizontally oriented axons in layer IV (i.e., the inner plexiform layer).

Callosal afferents. Degenerating callosal afferents form an essentially homogeneous population of terminals which synapse mainly with spines but occasionally with smooth dendritic processes (Fig. 11). Only one degenerating terminal forming an asymmetric, axosomatic synapse was encountered. In contrast to the results of the thalamic lesions, none of the large terminals in layer Ia of area 29c degenerate following contralateral lesions.

As shown in Figure 12, quantitation of the number of degenerating terminals following contralateral lesions demonstrates the following facts. First, the distribution of callosal af-

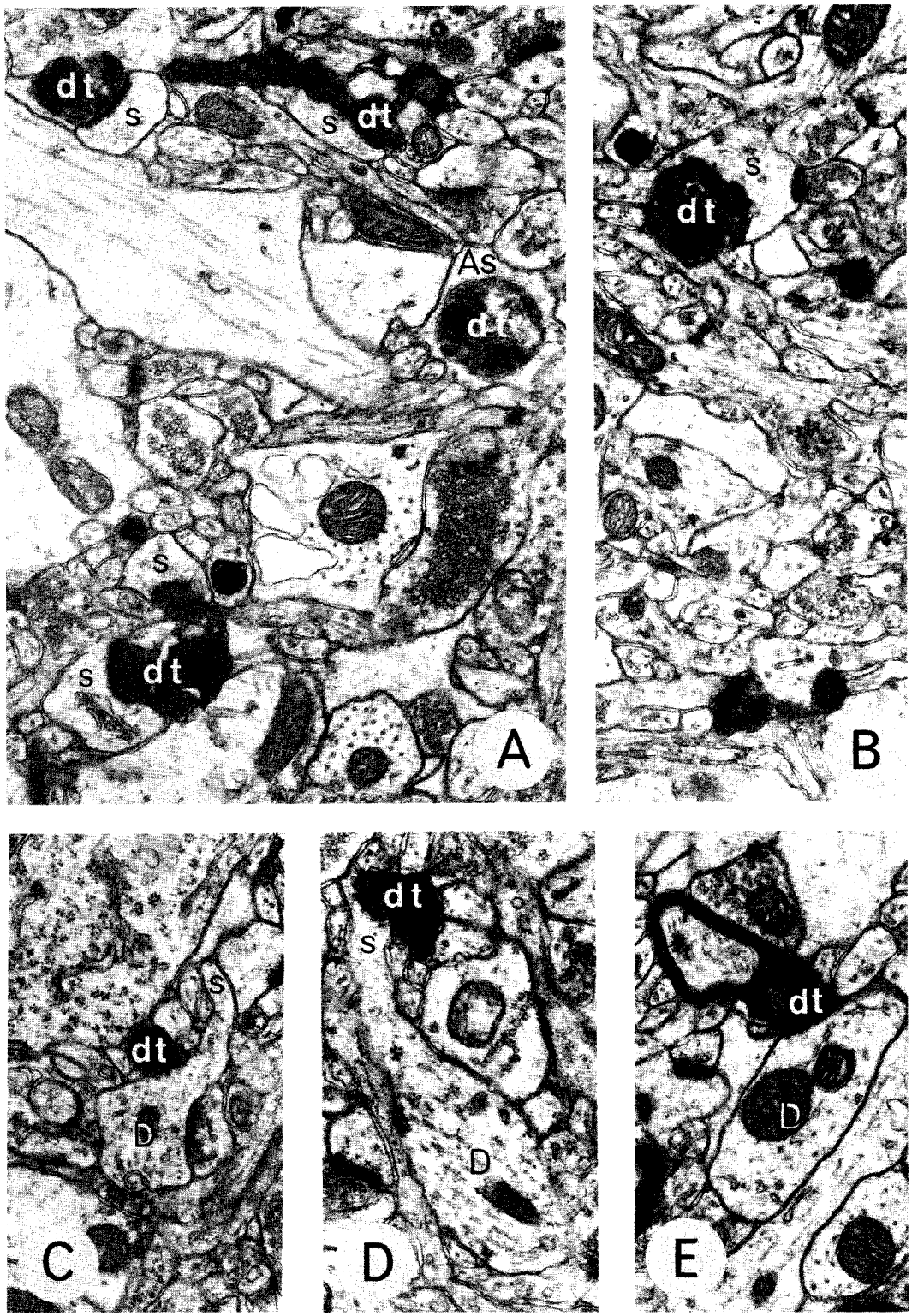

Fig. 11. Degenerating axon terminals (dt) in area 24b following a callosal lesion. dt's present in layer V (A), layer III (B), layer II (C and D), and layer I (E). × 23,000.

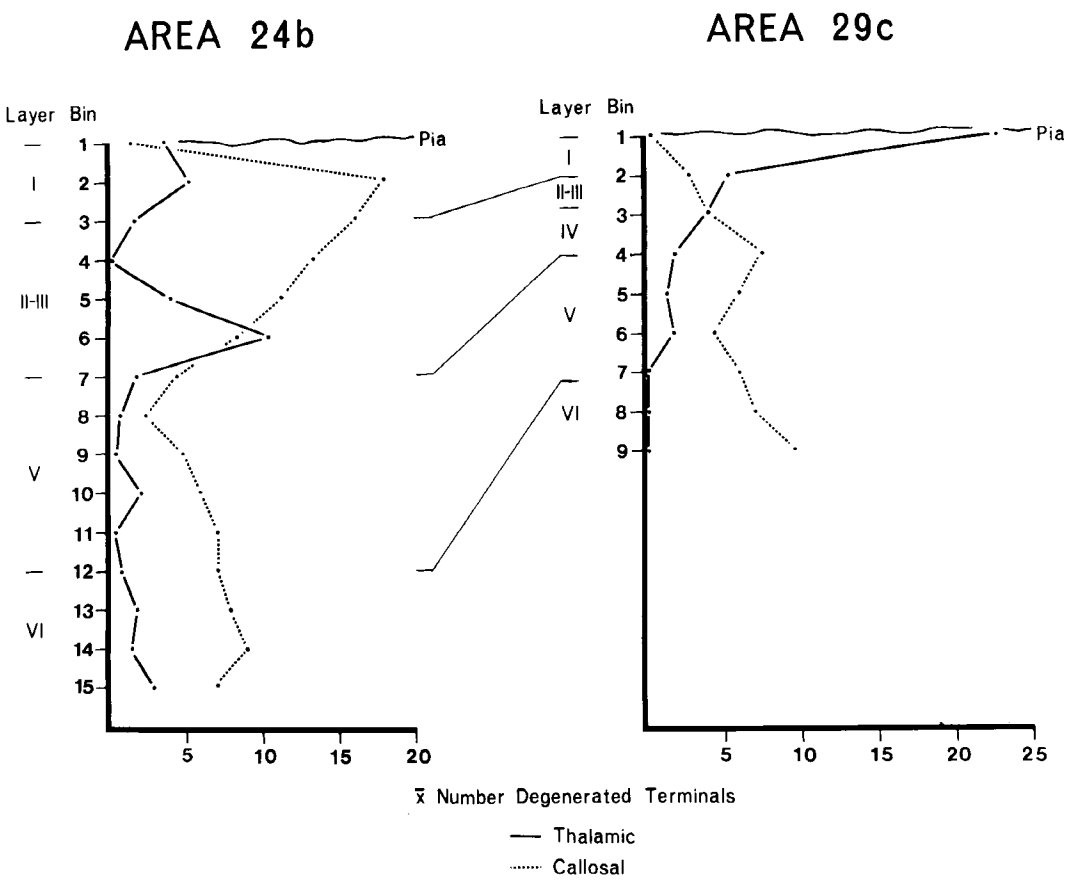

Fig. 12. Distribution of degenerating axon terminals in areas 24b and 29c following thalamic (mediodorsal and anterior nuclei respectively) or callosal lesions. Bin number represents the cortical depth for which each mean is calculated.

ferents and the segregation from thalamic afferents indicated by light microscopic observations are essentially correct. Most degenerating terminals in area 24 occur in layers Ib, Ic, II, and IIIa (≈12–18/bin). There are fewer in layers V and VI (≈5–9/bin), and least in layer Va. The peak number of mediodorsal thalamic afferents occurs in layer IIIb, while the least callosal and few thalamic terminals are present in layer Va.

Second, there are more degenerating callosal terminals in area 24b than there are in area 29c following the same contralateral lesion. The peak number of degenerating terminals in area 24 occurs in layer Ib (≈18/bin) with fewer in layers V and VI (≈5–9/bin), while in area 29c the greatest density of terminals is in layers V and VI (≈5–10/bin) and there are few in layers I–IV (≈0–5/bin). The mean number of degenerating terminals per bin is 8.3 for area 24b and 5.1 for area 29c, and, since area 24 is thicker than area 29, the total number of degenerating terminals in equivalent widths of areas 24b and 29c are 692 and 127 respectively. Thus, there are more than five times as many callosal terminals present in area 24b as there are in an equivalent width of area 29c.

Finally, in area 24b there are substantially more synaptic contacts formed by callosal than by mediodorsal thalamic afferents. Except in layer Ia and in one part of layer III (Fig. 12), the number of degenerating callosal terminals is consistently higher in each layer than that of thalamic origin. Furthermore, the total number of degenerating terminals following

thalamic or callosal lesions is 109 and 692 respectively in equivalent units of area (15 bins $\times$ 2,916 μm^2). Thus, there may be as many as seven times more callosal than thalamic terminals in this cortex. Another indication of the extent of callosal input to area 24b is the extensive proliferation of microglial cells in response to axonal degeneration, particularly in layers Ib–III where the greatest number of degenerating terminals is present. Mediodorsal thalamic lesions do not evoke this response from microglial cells.

DISCUSSION

Thalamocortical afferents

Light microscopic studies of thalamocortical afferents have reported termination in the superficial layers of cingulate cortex (Leonard, '72; Domesick, '72; Krettek and Price, '77; Herkenham, '79; Caviness and Frost, '80). Electron microscopic observations of the present study indicate that in addition to layers I and III or IV the mediodorsal and anterior thalamic afferents have limited termination in the deep cortical layers V and VI. Furthermore, terminals of these myelinated axons are both large and small and form asymmetric synapses primarily with spines and occasionally with smooth dendritic processes.

There are two reasons to expect that these afferents produce excitatory responses in cingulate cortical neurons. First, asymmetric synapses are also formed by the axons of other thalamic nuclei which terminate in visual (Peters et al., '79) and somatosensory (White, '78) cortices. Activation of these thalamocortical pathways produces excitatory responses in both visual (Creutzfeldt et al., '74; Toyoma et al., '74) and somatosensory (Hellweg et al., '77) cortices. Furthermore, the large axon terminals which degenerate in layer Ia of cingulate cortex following anterior thalamic lesions are similar to the large axon terminals formed by hippocampal mossy fibers (Hamlyn, '62). The mossy fiber terminals form asymmetric synapses and, when activated in *in vitro* hippocampal preparations, produce excitatory responses in hippocampal neurons (Yamamoto, '72).

A second reason to expect excitatory responses in cingulate cortex following activation of thalamic neurons is that this afferent may contain the neurotransmitter acetylcholine (ACh), which produces excitation in other cortical neurons (Krnjević and Phillis, '63; Krnjević et al., '71; Dodd et al., '81). Three lines of evidence indicate that ACh may be present in the anterior thalamic afferent to cingulate cortex. First, the irreversible ACh receptor ligand α-bungarotoxin binds in layers I and V–VI of posterior cingulate cortex (Hunt and Schmidt, '78) where we have shown that thalamic terminals synapse. Second, lesions of the anterior thalamic nuclei almost completely abolish cholinesterase activity in layers I and IV of posterior cingulate cortex (Lewis and Shute, '67). Finally, the anterior thalamic nuclei have the highest content of acetylcholinesterase activity in the thalamus (Parent and Butcher, '76). Thalamocortical afferents from the anterior nuclei, therefore, are the only thalamic afferents for which there is evidence for synaptic mediation via ACh.

Callosal afferents

The present analysis of silver-impregnated degeneration and quantitative electron microscopy following contralateral lesions indicates that layers Ib through IIIa of areas 32 and 24 receive the greatest amount of callosal input of all cingulate cortex areas, while areas 29b–c receive a "light to moderate" number of synaptic terminals in layers V and VI. In contrast, Jacobson ('70) reports that callosal input to area 24 terminates primarily in the deeper layers. Jacobson's ('70) inability to identify the dense termination in the superficial layers of area 24 may have been due to very lateral placement of the contralateral lesions, resulting in incomplete section of callosal afferents, or variations in the Fink-Heimer silver impregnation procedure which may have suppressed staining of terminals in the external layers. Although Yorke and Caviness ('75) describe a differential laminar distribution of callosal inputs to anterior and posterior cingulate cortex in the mouse similar to that reported in the present analysis of the rat, they describe termination of the anterior cingulate afferent as "light" in quantity and were unable to demonstrate a callosal input to area 25. The contradiction between their findings and our own in the rat may also be due to the technical considerations described above.

In the present study it is noted that following HRP injections into contralateral cingulate cortex few neurons label in layers II–III of area 24 when the injection primarily involves deeper layers. Indeed, most HRP-positive neurons are in layer V, suggesting that reciprocal interhemispheric connections exist between

superficial and deep layers respectively. Ribak ('77), using the anterograde autoradiographic procedure in rat visual cortex (area 18), made a similar observation, since injections of radioactively labeled amino acids in layer V and the white mater resulted in terminal labeling in layer V contralaterally, while full depth injections also labeled terminals in the external layers.

Additional connectional considerations

The termination of thalamic and callosal afferents in different layers occurs in both anterior and posterior cingulate cortices. The observation that these afferents largely terminate in different layers is not limited to cingulate cortex, since afferents from the medial geniculate nucleus of the thalamus and from contralateral cortex are also relatively separate in auditory cortex of the rat (D. Vaughan, personal communication). Therefore, the separation of these two afferent systems may be a general feature of most neocortical areas in the rat.

Although the role of callosal afferents to cingulate cortex in behavioral responses is not yet known, area 24 is unique in that it may receive as many as seven times more callosal than thalamic axon terminals. However, the number of thalamic afferents may be underestimated if terminals degenerate at different rates or if the lesions are incomplete. Electron microscopic evaluation of the time course of degeneration following mediodorsal thalamic lesions indicates that not all terminals degenerate at the same rate. After 2 days few terminals exhibit signs of degeneration, after 3 days the numbers of terminals reported in this study are clearly degenerating, and after 4 days many degenerating terminals are enclosed in glial sheaths and many more myelinated axons show degenerative changes. The numbers reported, therefore, reflect the greatest quantity of obviously degenerating thalamic terminals present at any one time. Finally, the lesions placed in the mediodorsal nucleus are extensive and involve parts of the anteromedial nucleus which is also known to project to area 24 in the rat (Krettek and Price, '77). Thus, the primary thalamic afferents to area 24 have been destroyed but thalamic terminals originating in undamaged parts of the anteromedial, parataenial, and ventromedial (Herkenham, '79) nuclei are not included in this count, nor are brainstem afferents from the substantia nigra and ventral tegmental area (Beckstead, '79). The ratio of callosal to brainstem afferent terminals would be reduced if these additional afferents had been destroyed.

The presence of two distinct types of degenerating terminals in area 29c following anterior thalamic lesions raises questions about whether there are two separate populations of neurons in the thalamus which give rise to terminals of different sizes. If not, does the axon of an individual thalamocortical neuron emit collaterals as it ascends in the cortex, some of which form small terminals and others the large terminals in layer Ia?

Two distinct axonal plexuses are located in layers Ib and IV of area 29c, which have been referred to as the outer and inner plexiform layers (Vogt and Peters, '81). Following thalamic lesions, numerous myelinated axons degenerate in layer Ib but apparently fewer degenerate in layer IV following thalamic and almost none following contralateral lesions. This suggests that other afferents may course through the inner plexiform layer. One such possibility are afferents from the postsubiculum (Swanson and Cowan, '77) and subiculum (Sørensen, '80) which are known to also terminate in the external layers of retrosplenial cortex.

Throughout the present analysis the complementary laminar termination of thalamic and callosal afferents has been described as well as those layers in which the termination of these two afferents significantly overlap. Since the ultrastructure of synapses formed by these afferents has also been investigated, it is now appropriate to ask whether these two afferents form synapses with the same classes of neurons or whether they each synapse preferentially with different neuronal types (i.e., pyramidal vs. nonpyramidal). Such anatomical studies focusing on specific neuronal connections will form the basis for understanding the sequence of responses produced by cingulate cortex neurons during motor and learning processes.

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