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**THE PRIMATE CINGULATE CORTEX: CHEMOARCHITECTURE,
PROJECTIONS TO THE PRIMARY MOTOR CORTEX, AND NEURONAL
SPECIALIZATION**

by

ESTHER A. NIMCHINSKY

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial
fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

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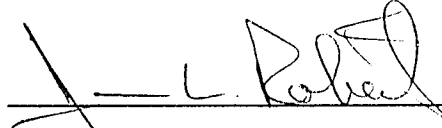
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ABSTRACT**THE PRIMATE CINGULATE CORTEX: CHEMOARCHITECTURE,
PROJECTIONS TO THE PRIMARY MOTOR CORTEX, AND NEURONAL
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Esther A. Nimchinsky

Advisors: John H. Morrison, PhD and Patrick R. Hof, MD

The primate cingulate cortex is characterized by great anatomic and functional diversity. In an effort to further understand its anatomic organization and to relate its architecture to its described functional domains, the following set of experiments was designed. First, a chemoarchitectonic analysis of the cingulate cortex was undertaken in the macaque monkey, using neurofilament protein and calcium-binding proteins parvalbumin, calbindin and calretinin as cellular markers. Second, the analogous chemoarchitectonic analysis was performed of the cingulate cortex in the human, with special emphasis on features not found in the nonhuman primate. Third, a neurochemical study of two projections from the cingulate motor areas to the primary motor cortex was performed, using retrogradely transported dyes and neurofilament protein, a marker that emerged from the first two studies, to analyze at the cellular level the relationship between the neurochemical architecture of these areas and neurochemical characteristics of its corticocortical projection neurons. Lastly, the distribution of the dopamine D3 receptor protein was analyzed in the macaque monkey and rat cortex, with the aim of determining its usefulness as a specific marker for particular areas or projections within the cingulate cortex. The results of these studies demonstrated the chemoarchitectonic heterogeneity of the cingulate cortex, which is generally comparable in the macaque monkey and the

human. However, the human cingulate cortex contains certain cell types not encountered in the macaque, such as spindle neurons and calretinin-containing pyramidal neurons in the anterior cingulate cortex. Neurofilament protein proved to be a useful marker for the cingulate motor areas in the macaque monkey, and the two cingulate motor projections had comparable proportions of neurofilament protein-containing neurons. Finally, the D3 receptor protein, at the light and confocal microscopic levels, had a nearly ubiquitous distribution in both the rat and macaque monkey, and was thus deemed not appropriate as a selective marker for certain areas or projections. The cingulate cortex in the human and nonhuman primate contains numerous discernable cortical areas whose functions are only beginning to be understood. Selective neurochemical markers and morphologic specializations may prove very useful in the further understanding of this cortical region.

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DEDICATION

This work is dedicated to my parents, for their constant encouragement,
to Dr David Raab, for putting a bee in my bonnet,
and to Patrick, for seeing me through.

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Chapter 1

INTRODUCTION

One of the most striking features of the primate cerebral cortex is its regional heterogeneity. This variability takes origin in two processes: differentiation and specialization. Differentiation is the process by which "primitive" areas with only few cellular layers, such as three-layered allocortex, acquire a granular layer and external pyramidal layers, eventually arriving at the six-layered structure characteristic of "isocortex." Specialization is the process by which a given area's laminar structure is adapted to suit its unique functional requirements. For instance, primary sensory cortex manifests an elaboration of its thalamorecipient layer IV, whereas in primary motor cortex, this layer's characteristic cells have disappeared altogether, but layer V and its corticofugal connections are dominant.

It is notable that the mammalian brain, with its high degree of cortical differentiation, contains areas whose general cytoarchitecture seems not to have changed since the mammals' putative common ancestor. Thus, certain areas, such as the hippocampus, are highly conserved, and are evident in insectivores, rodents, carnivores, and, in fact, in most mammalian species (Kaas, 1987). These "primitive" areas coexist along with highly differentiated neocortex. The primate cingulate gyrus represents an outstanding illustration of this exercise in contrasts in that it contains the whole range from the most basic allocortex through fully differentiated isocortex, to areas of marked specialization for certain aspects of motor function.

ANATOMY OF THE PRIMATE CINGULATE CORTEX

Macroscopic anatomy

The term "cingulate cortex" applies to all of the cortical areas on the medial surface of the hemisphere surrounding the corpus callosum. It forms a limbus, or border, around the edge of the hemisphere, so it is, in the most literal sense, a limbic structure.

The callosal sulcus lies between the corpus callosum and the cingulate gyrus, and is thus the only sulcus with cortex lining only one of its banks. It is deeper posteriorly than anteriorly; in the monkey its anterior portion barely extends beneath the surface. The cingulate sulcus marks the anterior border of the cingulate cortex rostral to the genu of the corpus callosum (the anterogenu region) and its superior border as it curves around the genu and follows the contour of the corpus callosum (Fig. 1.1). The cingulate sulcus is one of the primary fissures in the brain. It is notable for being highly conserved both in its position and as a cytoarchitectonic border (Ariëns Kappers et al., 1936). Caudally, the cingulate sulcus turns dorsally toward the edge of the hemisphere, but the superior border of the cingulate cortex follows the trajectory established anteriorly by the cingulate sulcus and curves posteriorly and ventrally around the splenium (Fig. 1.1). It is important to appreciate the topographic location of the retrosplenial area. In its posterior extent in the anterior limb of the calcarine sulcus, it abuts the secondary visual cortex, and elsewhere, its location is sufficiently caudal to contact numerous higher-order visual areas. This proximity to a visual area has implications for some of the connections and functions of the more caudal cingulate areas, which will be discussed below.

In both human and nonhuman primates, the posterior half of the cingulate gyrus usually has more intracingulate sulci and dimples than the anterior half. In the nonhuman primate, this may mean one or two relatively shallow indentations; in the human it means numerous, highly variable anteroposterior and dorsoventral folds, which may be of such complexity that critical cytoarchitectonic borders may lie buried far from view (Vogt et al., 1995).

The cortical area ventral to the genu of the corpus callosum (Brodmann's area 25) which lies very close to the caudal orbitofrontal cortex is not, topographically, part of the cingulate gyrus. However, on the basis of its cytoarchitecture and its thalamic and cortical connections, it may be considered part of the cingulate cortex, and will be considered as such in the present study.

Due to its huge expanse and comprehensive sweep across the hemisphere, the cingulate gyrus has many neighboring cortical fields. In the monkey, anteriorly to posteriorly, they are the medial orbitofrontal cortex (Brodmann's area 14), medial prefrontal area (area 32), premotor (area 6), motor (area 4), primary somatosensory (areas 3, 1, and 2), anterior parietal (area 5), medial posterior parietal (area 7m), visual areas V3 and V2, parahippocampal gyrus and subiculum. It is thus well suited to act as a zone of convergence of many types of information at various stages of processing from all over the brain.

Microscopic anatomy

Microscopically, the cingulate gyrus is far from a homogeneous structure. In rodents, the cingulate cortex is divisible into an agranular anterior region (area 24) and a granular posterior region (area 29). Area 24 is divided into a ventral portion and a dorsal portion, referred to as areas 24a and 24b, respectively. Area 24b is considerably more differentiated than area 24a, whose layers are very difficult to resolve (Vogt and Peters, 1981). Area 29 is subdivided on the basis of granularity and connections (Vogt, 1993) into areas 29a-d, from ventral to dorsal, and 29e, which is caudal to all four. The cingulate cortices of lagomorphs represent variations and elaborations on this theme (Brodmann, 1909; translation by Garey, 1994; Vogt et al., 1986).

The primate cingulate cortex is much more extensive and contains cytoarchitectonically defined areas not found in other species (Vogt et al., 1987; Vogt et al., 1995). Specifically, there is a well-developed caudal granular area dorsal to and distinct from area 29 which is designated as area 23 (Fig. 1.2). Like area 24, area 23 is divisible along the ventrodorsal axis into less and more differentiated areas (23a and 23b), although all subdivisions of area 23 are, by definition, granular. Other areas specific to primates include area 30 in the callosal sulcus between areas 29 and 23a, and an area 31 dorsal to 23c. In addition, with the expansion of the cerebral cortex in general, the cingulate cortex invaded the ventral bank of the cingulate sulcus, creating area 24c

rostrally and 23c caudally. Similarly, the retrosplenial cortex and the ventral-most areas of posterior cingulate cortex were displaced into the callosal sulcus. In human, this shift is also evident anteriorly. The human cingulate cortex is further distinguished by its area 33 anteriorly in the depths of the callosal sulcus (Fig. 1.3; Brodmann, 1909; translation by Garey, 1994; Vogt et al., 1995). The cytoarchitecture of the cingulate cortex will be described first for the nonhuman primate.

Cytoarchitecturally, area 25 has the lowest degree of laminar differentiation (Vogt et al., 1987). It consists of three layers: a broad molecular layer and two pyramidal cell layers, designated II-III and V-VI. Layer V-VI consists of pyramidal cells packed much more densely and stained more intensely in Nissl-stained material than in layer II-III. In area 24a, layers II and III are difficult to separate, but they are discernible from layers V-VI, and layer V is very prominent with large, densely packed pyramidal cells. However, the distinction between layers II-III and V-VI is less prominent than in area 25. In area 24b, layer II is somewhat distinguishable from layer III, a layer Va is evident and discernable from layer Vb, and there is clear separation between layers Vb and VI. In area 24c, these features are more clearly visible, and layer III is more conspicuous (Vogt et al., 1987; Matelli et al., 1991). Area 23a has the thinnest supragranular layers II and III, and its layer IV is also narrow. Areas 23b and 23c are fully differentiated, with a fully developed layer IV and equally represented layers II-III and V-VI. These areas are therefore regarded as isocortex (Vogt et al., 1987).

The change between area 24 and area 23 does not occur abruptly. There is a zone of transition, which has been called area 24' or 24d (Fig. 1.2; Matelli et al., 1991). In this area, and extending beyond it dorsally in the depths of the cingulate sulcus, there is a field characterized by very large pyramidal cells in layer V, comparable in size to the giant pyramidal cells in the primary motor cortex. Braak (1976) described this field in the human brain as a presumptive primitive secondary motor area, distinct from primary motor cortex both topographically and by its limbic characteristics, namely its dense layer

II and conspicuous layer V with poorly organized giant pyramidal cells. The fact that this area has been observed in the nonhuman primate (Matelli et al., 1991), where it is known to contain a somatotopically arranged motor field (Luppino et al., 1991) most likely supports this notion.

Area 29 consists of subareas designated 29l, or 29a-c (by analogy to the rodent brain) laterally and area 29m (or 29d) medially. Area 29l is considered a periallocortical structure (Vogt et al., 1987), implying a region of transition between the allocortical hippocampal rudiment found ventral to the cingulate gyrus and the proiso- and isocortex represented by areas 30 and 23. It is characterized by a broad layer I and a densely packed layer II-IV which consists largely of small pyramidal cells (Vogt, 1976). Deep to this is layer Va, which is more densely packed than Vb, and a sparse layer VI. The transition to area 29d is marked by the emergence of small-to-medium-sized pyramidal neurons just deep to layer I and the appearance of an incipient layer IV. With area 30, the layer III that began in area 29c becomes more developed. Layer IV is also slightly more visible. Therefore, this area is still considered dysgranular, or possessing a real but ill-defined layer IV, and it, as well as area 23a, is categorized as proisocortex (Vogt et al., 1987). This designation distinguishes it from periallocortical area 29, where only a few of the cortical layers found in isocortex are observed, and from isocortical area 23b, where all six layers are not only present, but also well defined.

The ventrodorsal and anteroposterior trends observed in the nonhuman primate also pertain to the human cingulate cortex, and the cytoarchitectonic areas are comparable, but with a few additions in the human (Fig. 1.3). Firstly, a poorly laminated field is apparent in the depth of the callosal sulcus anteriorly and is called area 33 (Brodmann, 1909; translation by Garey, 1994; Vogt et al., 1995). Areas 24a and 24b are characterized by the presence of clusters of elongated, spindle-shaped pyramidal cells in layer Vb that are not present in the macaque and are not seen elsewhere in the human brain with the exception of the anterior insular cortex (Von Economo, 1926; Ngowyang,

1932). Lastly, there is an area interposed between areas 23c and 7, designated by Brodmann as area 31, which has a cytoarchitecture intermediate between that found in the cingulate sulcus and the overlying parietal cortex (Brodmann, 1909; translation by Garey, 1994; Vogt et al., 1995).

The dichotomy between anterior and posterior cingulate cortex is maintained in their respective subcortical and cortical connections, as discussed below.

CONNECTIVITY

Subcortical connections

Thalamus. The most well known input to the cingulate cortex is that from the anterior nuclei of the thalamus, which receive inputs from the hippocampal formation (Aggleton et al., 1986). However, the anterior and posterior divisions of the cingulate cortex have very different sets of thalamic afferents. Specifically, area 24 receives input from the intralaminar nuclei and from the midline nuclei, including the central densocellular, parafascicular and mediodorsal densocellular nuclei (Vogt et al., 1987). Area 29 receives afferents from the anterodorsal, anteroventral and laterodorsal nuclei (Vogt et al., 1987), the nuclei that have historically been linked to the limbic cortex (Papez, 1937). Area 23 receives no input from the anterior nuclear complex, aside from afferents from the anteromedial nucleus, which represent its most sizable thalamic contribution. It also receives projections from the mediodorsal, the lateroposterior, and the medial pulvinar nuclei (Vogt et al., 1979; Baleyrier and Manguière, 1985; Vogt et al., 1987). As elsewhere, the thalamocortical projections to the cingulate cortex are reciprocal (Jones, 1985).

It is interesting to note that, with respect to the thalamocortical connections at least, anterior cingulate and area 29 do not differ greatly from their counterparts in nonprimate species. In the rat, for instance, the major thalamic afferents to area 24 come from midline and intralaminar nuclei (as well as the anteromedian nucleus), while the

input to area 29 is predominantly from the anterior and the laterodorsal nuclei (Domesick, 1969; Thompson and Robertson, 1987). This observation is in keeping with the premise that there is a degree of homology of these areas among species. Thus area 23, the only one of these areas not found in rodents, is unusual among cingulate areas not only in its cytoarchitecture, which achieves a degree of differentiation characteristic of isocortex, but also in its thalamic innervation. It is the only cingulate area to receive thalamic afferents from the visually dominated lateroposterior and medial pulvinar nuclei whose expansion in the primate reflect those species' increased reliance on vision.

Brainstem. Anterior cingulate cortex receives afferents from the periaqueductal gray (Jürgens and Ploog, 1970). Both anterior and posterior divisions of the cingulate cortex project to the pons. Moreover, there is a topographic arrangement, with areas 24 and 25 projecting medially and area 23 projecting more laterally (Vilensky and Van Hoesen, 1981). Furthermore, both anterior and posterior cingulate cortex receive noradrenergic, serotonergic, and dopaminergic projections from the locus coeruleus, raphe nuclei, and substantia nigra/ventral tegmental area, respectively.

Area 23 in the macaque monkey has a denser noradrenergic innervation than area 24 (Crino et al., 1993), which, however, is more densely innervated than nearby areas 11, 12, 13, 25, 10 and 46 (Lewis and Morrison, 1989). In both divisions of the cingulate cortex, the highest density occurs in layers I, V, and VI (Lewis and Morrison, 1989; Crino et al., 1993). The noradrenergic innervation of area 29 is most pronounced in its superficial layers (Crino et al., 1993).

In area 24, serotonergic fibers ramify in all layers, albeit with a decreased density in layers IIIa and IIIc (Crino et al., 1993). In area 23, there is a high density of fine varicose fibers throughout layer I, and in layers V and VI. Area 29 is characterized by a denser pattern in layers I and III (Crino et al., 1993).

In the primate, as in the rodent, the anterior cingulate receives one of the densest dopaminergic innervations in the brain (Lindvall and Björklund, 1984; Lewis et al.,

1987). It is the sole recipient of the supragenual dopaminergic system which takes origin largely from the substantia nigra (Foote and Morrison, 1987), and also receives projections from the ventral tegmental area (Porrino and Goldman-Rakic, 1982). In the primate, there is a higher density of dopamine fibers in area 24 than in area 23. In both divisions, the highest density is observed in layers Ic, II, V, and VI; however, in the dorsal portion of area 24, dopamine fibers are restricted to layers I-III (Crino et al., 1993). Interestingly, area 29 is practically devoid of dopaminergic innervation (Crino et al., 1993).

Telencephalic subcortical structures. The laterobasal, accessory basal and central nuclei of the amygdala project to the anterior cingulate cortex, terminating in layers I and II (Porrino et al., 1981; Vogt and Pandya, 1987). These projections extend throughout the ventrodorsal extent of the anterior cingulate cortex and throughout the anteroposterior extent of area 24, except for its caudalmost segment (Vogt and Pandya, 1987). Area 24 in return projects to the lateral basal nucleus of the amygdala (Pandya et al., 1973; Baleyrier and Mauguère, 1980; Pandya et al., 1981). Posterior cingulate cortex, like most other neocortical areas, is not connected with the amygdala (Baleyrier and Mauguère, 1980; Vogt and Pandya, 1987).

Area 24 projects to the nucleus accumbens (Baleyrier and Mauguère, 1980), and to the head of the caudate nucleus and the rostral putamen bilaterally, although more heavily on the ipsilateral side (Baleyrier and Mauguère, 1980), whereas area 23 projects only to the caudate nucleus (Baleyrier and Mauguère, 1980). Finally, both area 23 and 24 project to the ventral portion of the claustrum (Baleyrier and Mauguère, 1980).

Cortical connections

In general, the posterior regions of the cingulate cortex have more widespread corticocortical connections than the anterior cingulate (Vogt et al., 1979). Area 24 receives afferents from prefrontal, lateral orbitofrontal, insular and parahippocampal cortical areas TF and TH (according to von Bonin and Bailey (1947); Vogt et al., 1979;

Baleydier and Mauguière, 1980; Vogt and Pandya, 1987). It projects to premotor, orbitofrontal, inferior parietal, anterior insular and perirhinal cortices (Pandya et al., 1981). It is noteworthy that the transition area 24c' and part of 23c are reported to be extensively connected with primary motor, premotor, and supplementary motor areas in a somatotopic fashion (Morecraft and Van Hoesen, 1992), and have been shown to project directly to the red nucleus (Humphrey et al., 1984) and spinal cord (Dum and Strick, 1993). This anatomic evidence, along with equally strong physiological evidence (see below) implies that the cingulate gyrus, in addition to its other functions, contains numerous motor areas with unique properties (Dum and Strick, 1993).

Area 23 receives afferents from prefrontal (areas 9 and 46), medial orbitofrontal (11, 14), parahippocampal (areas TF, TL and TH), posterior parietal, and higher order visual (area 19) cortices (Vogt et al., 1979; Vogt and Pandya, 1987; Andersen et al., 1990; Neal et al., 1990), as well as from area 8b at the inferior edge of the dorsal limb of the arcuate sulcus, entorhinal cortex, and superior temporal cortex (Baleydier and Mauguière, 1980). Posterior cingulate cortex projects throughout the medial cortex dorsal to the cingulate sulcus as well as to prefrontal areas 9, 10 and 46, orbitofrontal area 11, the dorsal bank of the superior temporal sulcus, the parahippocampal gyrus, entorhinal cortex, and the presubiculum (Baleydier and Mauguière, 1980; Pandya et al., 1981). A ventral portion of area 23b forms the bulk of the caudomedial lobule (Vogt et al., 1987), which is heavily interconnected with the prefrontal cortex and is thought to serve as a relay between the prefrontal cortex and the hippocampus (Goldman-Rakic et al., 1984).

Another group of connections that are very prominent in the cingulate gyrus are the intracingulate connections. The anterior and posterior divisions are very closely interconnected in an elaborate network of corticocortical connections that Vogt and Pandya (1987) organize into three tiers. The ventral tier is the level where area 25 projects to area 24, and areas 29 and 30 project to each other and to area 23. The

intermediate tier is that in which area 24a is linked with 23a and 23b. The dorsal tier is where areas 24b and 24c are connected with 23b and 23c. The inclusion of area 25 in this tight-knit group of cortical areas is further evidence that it is, in fact, a cingulate area.

A certain degree of caution must be used in interpreting the currently available data on the connectivity of the primate cingulate cortex. First, relatively few studies have analyzed the cingulate gyrus of the primate, and some of these were based on fairly large injections or ablations that often involve the underlying white matter. Consequently, the present synopsis relies on the consensus of the most cautiously executed studies. Second, the most ventral and posterior areas 29 and 30 are virtually inaccessible for direct, localized manipulations, so their connections must be derived as a result of injections or ablations elsewhere. Therefore, until an exhaustive area-by-area study is undertaken, the projections to and from these areas must be considered sketchy, at best.

In summary, then, anterior cingulate cortex is reciprocally interconnected with orbitofrontal and insular cortices (although it receives prefrontal, parahippocampal, and subicular inputs as well) and its major outputs are to motor and limbic structures. The posterior cingulate cortex entertains more robust reciprocal projections with prefrontal, parietal, temporal, orbitofrontal, and parahippocampal cortices. It also receives input from areas linked to the visual or oculomotor systems (frontal eye field, area 8) and projects to the motor system and the ventromedial temporal lobe which presumably connects it with the hippocampus. Finally, different cingulate areas are strongly connected to each other. This organization, reminiscent of the strong interareal connections of the prefrontal cortex (Barbas and Pandya, 1989), supports the assertion that, although in the primate, anterior and posterior cingulate cortex differ widely in cytoarchitecture, certain extrinsic connections and functions, they are nonetheless parts of a single functional system.

FUNCTIONAL ANATOMY

The cingulate cortex subserves a variety of functions consonant with its cytoarchitectonic diversity. These functions can usually be localized to a greater or lesser degree to some site along the gyrus. Most of the studies of cingulate cortical function have been performed in rodents and rabbits. While certain of the properties have been found to be common to more than one of these species, and may occasionally be presumed to have been conserved in the primate, these studies must be considered with care. Another consequence of the predominance of nonprimate studies in the cingulate gyrus is that the most intensively investigated areas are anterior cingulate and retrosplenial cortex, and not area 23, which cannot truly be studied in rodents or lagomorphs.

The functions of the cingulate gyrus fall into the following categories: In the anterior cingulate gyrus: autonomic and visceral processes; nociception; vocalization; learning and conditioning; attention. In the posterior cingulate gyrus: somatomotor; oculomotor; visual. In addition, the dorsal cingulate gyrus, both anteriorly and posteriorly is home to numerous "cingulate motor areas (CMA)."

Anterior cingulate gyrus

Autonomic and visceral processes. Numerous studies dating as far back as the mid-1940's have implicated the primate anterior cingulate cortex in autonomic functions such as changes in respiration, excess salivation, cardiac irregularity, pupillary dilation, shivering, piloerection, penile erection, urination, defecation, and alterations in gastric motility (Smith, 1945; Kaada, 1951; Showers and Crosby, 1958; Dua and MacLean, 1964; Robinson and Mishkin, 1968; Talairach et al., 1973). Most of these functions are localized to the infralimbic, anterogenua, and most rostral portions of the cingulate gyrus. These responses tend to be distributed throughout the area and are likely attributable to the projections, described in the rat and rabbit, from anterior cingulate cortex to the nucleus of the solitary tract, nucleus ambiguus, and the dorsal motor nucleus

of the vagus (Terreberry and Neafsey, 1983; Hurley et al., 1991; Buchanan and Powell, 1993). Another, possibly related response to anterior cingulate stimulation is facial movements, including grimaces and expressions associated with alarm and aggression (Showers and Crosby, 1958).

These findings in the nonhuman primate are bolstered by studies in the human, where changes in pulse and respiration, as well as facial movements were elicited upon stimulation of the anterior cingulate gyrus (Pool and Ransohoff, 1949; Pool, 1954). Furthermore, cingulate seizures are characterized by autonomic signs (Goldensohn, 1992).

Nociception. The anterior cingulate gyrus has long been implicated in the subjective experience of pain. This has been the basis of one of the neurosurgical approaches to extreme pain, the cingulotomy (Foltz and White, 1962). The fact that it has been shown to be effective at least some of the time provides indirect evidence of the involvement of this area with the response to pain, if not its actual sensation. In addition, a recent study in the human utilizing positron emission tomography and magnetic resonance imaging identified the anterior cingulate cortex as one of only three cortical areas that respond strongly to thermal nociceptive stimuli (Talbot et al., 1991). (The other areas were primary and secondary somatosensory cortex.) Finally, another study showed activation of the anterior cingulate cortex during the experience of angina pectoris (Rosen et al., 1994).

Experimental evidence of this involvement has been largely derived from nonprimate species. In the rabbit, Sikes and Vogt (1992) identified units responsive to one or more type of pain (thermal, mechanical or transcutaneous electrical) in area 24. The distinguishing features of these cells were large receptive fields, such as the entire dorsal surface of the animal, and high thresholds (Sikes and Vogt, 1992).

Indirect evidence for the existence of this pain system in nonprimates comes from studies of active avoidance. It has been shown that rabbits that have ablations of their

anterior cingulate cortex exhibit deficits in a task that requires them to actively avoid a painful unconditioned stimulus by jumping in response to a neutral conditioned stimulus (Gabriel et al., 1991). It has been hypothesized that the basis for this deficit is not impaired learning (although, as discussed below, some forms of learning are linked to this region), but the absence of the unpleasantness associated with pain. This assumption is based on studies in humans who underwent cingulotomy. These subjects feel and even localize the stimulus; it merely has no aversive content (Foltz and White, 1962).

The involvement of anterior cingulate cortex in the subjective response to pain is probably related to the facts that 1) it receives afferents from the intralaminar and midline thalamic nuclei, which receive fibers from the spinothalamic tract (Mantyh, 1983), 2) it is reciprocally connected with the periaqueductal gray (Mantyh, 1982; Jürgens and Ploog, 1970), a region known to be implicated in the processing of noxious stimuli (Liebeskind et al., 1973), and that 3) it contains a very high concentration of mu opioid binding sites (Wamsley et al., 1982; Sharif and Hughes, 1989), the type which is believed to mediate analgesia and whose presence is a frequent characteristic of structures involved in the processing of painful stimuli (Jaffe and Martin, 1990).

Vocalization. Numerous brain regions in the nonhuman primate can elicit vocalizations, but virtually the only cortical area that, upon stimulation, can evoke meaningful vocalizations (i.e., vocalizations that are known to have some content reflecting the internal state of the animal) is the region surrounding the genu of the corpus callosum (Dua and MacLean, 1964; Jürgens and Ploog, 1970). Animals with ablations of this area do not produce spontaneous calls (Aitken, 1981). Vogt observes that the autonomic and nociceptive connections of this area make it well placed to evoke sounds associated with emotional states. MacLean goes even further, characterizing the particular vocalization that is absent upon ablation of the anterior cingulate cortex in young primates as the "separation cry." This cry is essential to maintain contact between a mother and her offspring and MacLean considers it one of the hallmarks of mammalian

behavior and evidence of the fundamental nature of the cingulate cortex (MacLean, 1990). Interestingly, lesions in this region have been implicated in the production of gelastic seizures, events characterized by uncontrollable laughter, but without feelings of amusement (Arroyo et al., 1993), and bilateral infarction of the anterior cingulate gyri in humans gives rise to a syndrome of akinetic mutism (Nielsen and Jacobs, 1951; Barris and Schuman, 1953; Nakasu et al., 1991). The mutism may be due to the disruption of vocalization centers, although these have not yet been unambiguously demonstrated in the human.

Learning, conditioning and attention. Numerous types of learning are thought to be subserved by the cingulate gyrus. As noted earlier, active avoidance conditioning is selectively affected in anterior cingulate cortex lesions (Gabriel et al., 1980). Single unit recordings confirm the activity of this area in this paradigm, and demonstrate that different layers of the cingulate cortex are active during different stages of discriminative learning in this paradigm (Foster et al., 1980). It is intuitive that the nociceptive center in the anterior cingulate cortex plays a role in this learning paradigm. Similarly, the cingulate autonomic center is presumably responsible for the role of the cingulate cortex in classical (Pavlovian) conditioning (Buchanan and Powell, 1982). However, the cingulate cortex is implicated in some learning paradigms which do not involve aversive stimuli or autonomic unconditioned responses. Specifically, different components of a spatial discriminative learning task in mice have been attributed to anterior and posterior cingulate cortex. Anterior cingulate cortical lesions interfere with improvement in performance during the course of the study, while posterior cingulate cortical lesions affect acquisition and long-term retention of the behavior (Meunier et al., 1991).

One explanation for the akinetic mutism observed after bilateral lesions of the anterior cingulate gyri is that the cingulate cortex plays a role in attention that, when disrupted, leaves a syndrome of extreme apathy to surroundings, and even to communication, that may manifest as akinetic mutism. Consistent with this view is the

neglect syndrome occasionally observed after cingulectomy (Watson et al., 1973). Functional investigations in human subjects using positron emission tomography support this explanation (Posner et al., 1988; Pardo et al., 1990).

Posterior cingulate gyrus

In keeping with its connections with higher-order somatosensory, oculomotor and visual structures, posterior cingulate cortex participates to some extent in all of these functions. In the behaving cat, Olson and Musil, (1992a) demonstrated units in posterior cingulate cortex that began to fire at the time of onset or immediately after saccades, indicating that their role is to monitor, rather than trigger, eye movements. Other neurons fire tonically during fixation on a target, with a frequency that depends on the angle of the eye in the orbit. Others fire in relation to quick phases of vestibulo-ocular nystagmus. Such movement-associated firing does not require visual input, since they also occur in the dark. In addition, they identify units that fire in response to somesthetic, visual, and/or auditory stimuli (Olson and Musil, 1992a). In the macaque monkey, they made similar observations regarding saccades and visual stimuli (Olson et al., 1993). The role played by the cingulate cortex in this process seems most similar to that played by the posterior parietal cortex, with which it is heavily connected (Baleydier and Mauguière, 1980; Vogt and Pandya, 1987; Cavada and Goldman-Rakic, 1989a; Cavada and Goldman-Rakic, 1989b). It is also significant in this context that the portion of the posterior cingulate cortex that comprises the bulk of the caudomedial lobule lies in very close proximity to the superior colliculus.

The cingulate cortex has also been implicated in aspects of spatial memory (Murray et al., 1989). Although no distinction has yet been made between anterior and posterior cingulate in this function, the very strong interconnections between posterior cingulate cortex and posterior parietal cortex make the posterior division a more likely candidate for this role as well.

The role of the retrosplenial cortex is difficult to study, since it is very inaccessible and difficult to isolate in the primate. One possible function of this area is suggested both by studies of patients with unilateral retrosplenial lesions and experimentally lesioned rats. In these cases, the subject fails to habituate to stimuli presented in the contralateral visual field (Kwon et al., 1990). Orienting responses are unaffected. This may point to a role of this area in the discrimination of relevant from irrelevant visual information, in a manner distinct from the attentional functions of the anterior cingulate described above. Another interesting possible function is suggested by retrosplenial amnesia, an unusual syndrome that occurs following retrosplenial infarctions (Ebata et al., 1991). Aside from anterograde and retrograde amnesia, which could be partly attributed to involvement of the nearby subiculum and posterior hippocampus, unique features include the perception that the contralateral side of the patient's face (or the face of anyone the patient looks at) is somehow shrunken or distorted. When asked to draw the face, the patients draw the distortion they perceive. Inanimate objects do not appear distorted, nor do other parts of the body. A visual effect is not surprising given the visual inputs to this area, in particular from visual cortical areas and from the lateroposterior and medial pulvinar nuclei. However, the striking specificity of this deficit implicates high-order connections that have yet to be elucidated in the primate.

The cingulate motor areas

An important functional component of the primate cingulate gyrus is a group of cortical areas that are connected with primary and supplementary motor (Muakkassa and Strick, 1979) and premotor (Pandya et al., 1981) cortical areas, as well as with the red nucleus (Humphrey et al., 1984) and spinal cord (Hutchins et al., 1988). They have been designated according to their positions along the cingulate sulcus as the rostral cingulate motor area (CMAr), the ventral cingulate motor area (CMAv), and the dorsal cingulate motor area (CMAd) continuous with and immediately dorsal to CMAv in the dorsal bank of the cingulate sulcus (Dum and Strick, 1992). Although this last area is not, strictly

speaking, part of the cingulate gyrus, its connections and physiological properties indicate that it belongs with CMAr and CMAv. Stimulation of these areas elicits movements which differ from those elicited from primary motor cortex in having a higher threshold for excitability and in often being complex, involving more than one limb or joint (Luppino et al., 1991; Dum and Strick, 1992). These motor areas do not merely represent the "rest of the body" counterpart to the oculomotor areas in posterior cingulate cortex, since the latter are characterized by neuronal firing after a saccade, and thus receive an efference copy of the information, whereas the neurons of the cingulate motor areas fire in advance of the movement (Shima et al., 1991). Although the functions of these areas are not clear, they are well placed to play a unique role among the motor areas, for they are the only motor areas that receive amygdalar and anterior thalamic input. They may thus represent a means by which the limbic system could influence motor functions (Brooks, 1986). In addition, the suggestion that the CMAv and CMAd reside in area 23c is noteworthy, since that would make these the only known cortical motor areas in the primate that contain a granular layer IV.

A brief survey of the above segregation of functions points out a major contrast between anterior and posterior cingulate cortex. Specifically, the anterior cingulate gyrus seems to be involved in basic functions necessary to preserve the organism and keeps it oriented. The posterior cingulate cortex seems to function as a polymodal sensory area which, in turn, projects anteriorly to influence the anterior areas.

RELEVANCE OF NEUROCHEMICAL STUDIES

There are certain elements that unite the anterior and posterior cingulate cortices into a single, unique structural unit, including projections from the anteromedial thalamic nucleus and an exclusive and elaborate intracingulate corticocortical network, as well as roles in learning and motor control. Nonetheless, whether examined from an anatomic or functional point of view, there is a clear dichotomy that impels one to ask what

relationship, if any, exists between the cytoarchitecture of an area and the connections which subserve its functions. In other words, could the differences between (and within) anterior and posterior cingulate cortex be "read" in their anatomic attributes?

If it were possible to define the cellular features common and unique to all neurons participating in a given circuit, and then do the same for all other projections, one could then describe the efferents of an area on both a regional and laminar basis on the strength of its cytoarchitecture. A similar procedure might elucidate the afferents to the area. However, this requires a redefinition of "cytoarchitecture." Clearly the Nissl stain does not provide adequate information about the neurons it labels for this type of analysis, and other tools must come into play. The laminar position of a neuron, something that can be observed with the Nissl stain, can give an indication of its possible role. For instance, subcortical projections arise in deep (infragranular) cortical layers, and corticocortical projections arise in deep and superficial layers (Jones, 1984). On a more subtle note, Barbas (1986) observed that the corticocortical projections from less well differentiated cortical areas tend to originate preferentially in the deep cortical layers. A shift to the more superficial layers is correlated with an increased level of laminar differentiation. However, it is still not possible to point to a neuron in layer V of area 24b and say definitively that it is a corticocortically projecting cell, much less that it is projecting to area 9.

Some cellular markers can be localized predominantly to nonpyramidal cells. For instance, the calcium-binding proteins parvalbumin, calbindin and calretinin are small low-molecular weight, soluble proteins that bind 2 to 4 calcium ions, depending on their structure. Interestingly, they are found almost exclusively in nonpyramidal neurons, specifically GABAergic interneurons. Furthermore, in the neocortex, these three proteins are present in three distinct and non-overlapping, morphologically identified populations of interneurons, with parvalbumin present in basket and chandelier cells (DeFelipe et al., 1989b; Hendry et al., 1989), calbindin in double bouquet cells (Hendry et al., 1989), and

calretinin in a different population of bitufted cells (for review see Andressen et al., 1993).

Although no similarly clear-cut cellular markers exist to identify classes of pyramidal neurons, certain neurochemical markers can greatly contribute to the description of a pyramidal neuron and its projection. For instance, in the primate, among corticocortically projecting cells, only a subpopulation contains nonphosphorylated neurofilament protein (Campbell et al., 1991; Hof et al., in press). On a regional level, neurofilament protein proves an interesting cellular marker because the distribution of neurofilament protein-containing neurons exhibits regional and laminar heterogeneity. In fact, the distribution of neurofilament protein-containing neurons is so specific to individual cortical areas that it has been used to delineate many visual areas in the macaque monkey that can be virtually indistinguishable using the Nissl stain (Hof and Morrison, 1995). Significantly, the proportion of neurofilament protein-containing cells of origin varies with the circuit (Campbell et al., 1991; Hof et al., in press), suggesting that its regional distribution may be related at some level to the projections of individual areas.

Other markers of projection neurons could be molecules with a definable functional significance for the neuron. For example, neurotransmitter receptors might also be useful markers for discrete sets of cells. The monoaminergic systems innervate the cortex in an area- and lamina-specific manner. This is very evident in the cingulate cortex, as noted above. This method of organizing afferents ensures that parts of neurons, that are also organized in a laminar fashion, are contacted in the most efficient manner. However, that does not necessarily mean that, for instance, every dendrite in layer II of anterior cingulate cortex requires a dopaminergic input. It is clear that in this respect, the distribution of the relevant receptors is critical for allowing cells to access the monoaminergic input where and when it is relevant, and otherwise to ignore it. Furthermore, in these systems as well, there are numerous receptor subtypes which

further enable neurons to respond in an optimal way to the available neurotransmitters. It is probable that not all corticocortically projecting pyramidal cells will possess precisely the same complement of neurotransmitter receptors and receptor subtypes. Such heterogeneity permits countless interactions of great subtlety between the glutamatergic component of corticocortical connections and the sweeping influences of the more divergent and diffuse systems. Furthermore, if this cellular feature varies with the nature of the target of the projecting cell, then even within a given lamina pyramidal cell populations should be differentiable, and perhaps even identifiable. This information could be extrapolated to the regional level and contribute to the interpretation of regional heterogeneities.

GOALS OF THE PRESENT STUDY

The goals of the present study were to describe further the anatomic organization of the cingulate cortex using the neurochemical markers described above, and to relate the observed architectonic patterns to the neurochemical properties of individual neurons. To this end, the following studies were performed:

1. A chemoarchitectonic analysis of the cingulate cortex in the macaque monkey.
2. A chemoarchitectonic analysis of the cingulate cortex in the human, with special emphasis on features not found in the nonhuman primate.
3. A detailed analysis of a particular set of projections from the cingulate cortex, namely the corticocortical projections to the primary motor cortex, with the specific aim of relating the neurochemical features of the cingulate motor areas to the neurochemical characteristics of some of their constituent projections.
4. A description of the distribution of the dopamine D3 receptor in the rat and macaque monkey cortex, with the intention of determining the appropriateness of this marker for detailed distributional studies in the primate cingulate cortex similar to that described in (3).

The ultimate goal of this research is to be able to use this information obtained in the nonhuman primate indirectly to infer the connections of the human cingulate cortex and to understand its regional diversity. Such information should be helpful for the organization and comprehension of the profusion of functional data now beginning to emerge.

FIGURE LEGENDS

Fig. 1.1 The medial surfaces of the human (A) and macaque monkey (B) cerebral hemispheres. The cingulate cortex is delineated by arrows, and the asterisk indicates the approximate location of the border between areas 24 anteriorly and 23 posteriorly. Anterior cingulate cortex occupies a proportionally larger portion of the cingulate gyrus in the human than in the monkey. The human brain was from a 92 year old woman with no history of neurologic or psychiatric disorders. A block had been removed from the medial temporal lobe and another from the occipital pole for pathologic analysis.

Fig. 1.2 Distribution of cytoarchitectural areas in monkey cingulate cortex. CC, corpus callosum; CS, cingulate sulcus; SpS, splenial sulcus; RS, rostral sulcus; RhS, rhinal sulcus; POS, parieto-occipital sulcus; CaS, calcarine sulcus. Reproduced and adapted from Vogt (1987).

Fig. 1.3 Flattened representation of the human cingulate cortex, showing cytoarchitectural boundaries. The star indicates the approximate location of the anterior commissure. Shaded lines indicate the brain surface and apices of gyri; depths of sulci are not shown in order to simplify the map. Bold dashed lines outline the cingulate areas, and thinner dashed lines show borders between areas. Dotted lines indicate borders between subdivisions of each area. PaC, paracentral sulcus; CaS, callosal sulcus; CS, cingulate sulcus; SpS, splenial sulcus. Reproduced from Vogt et al., 1995.

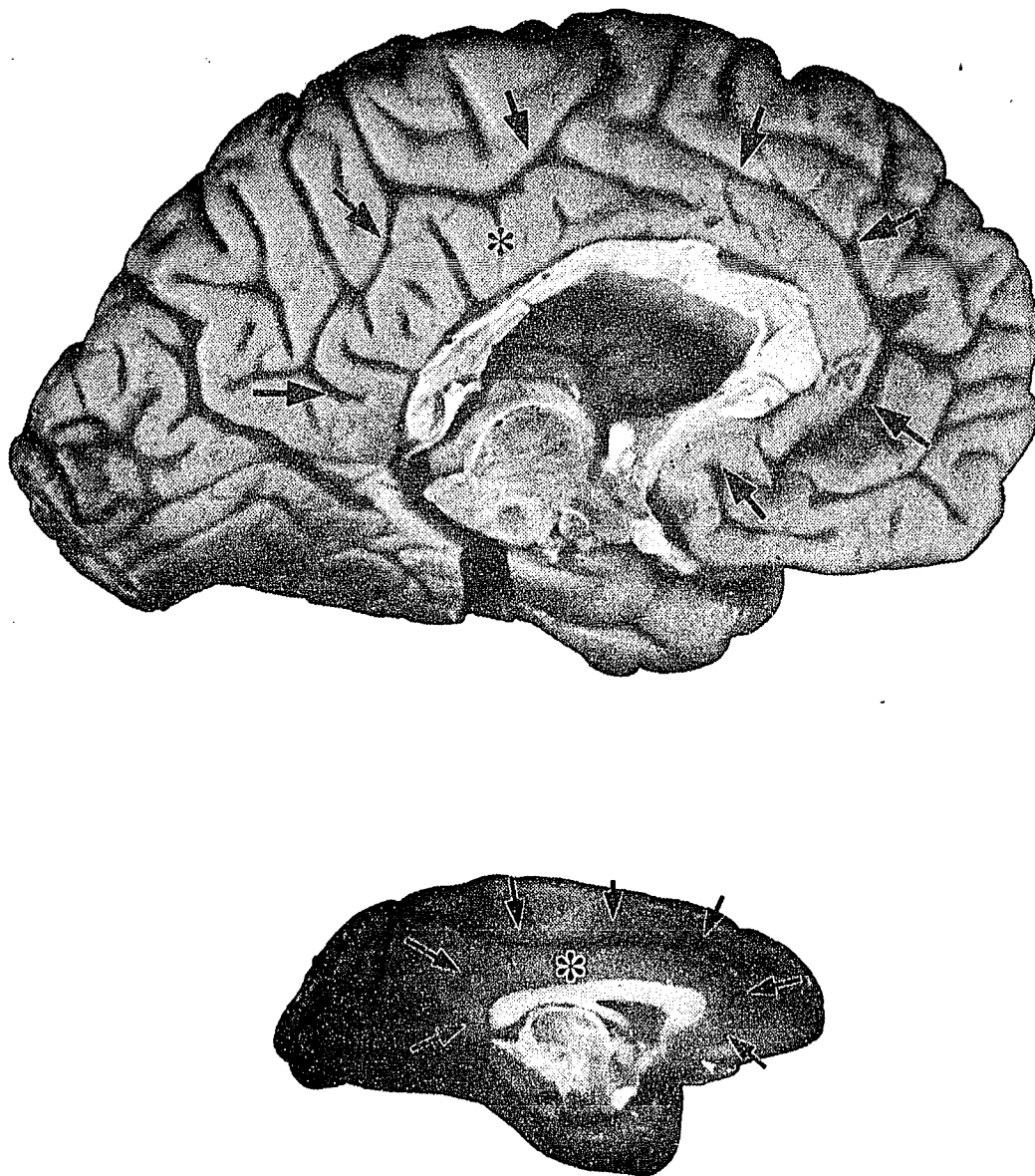


Fig. 1.1

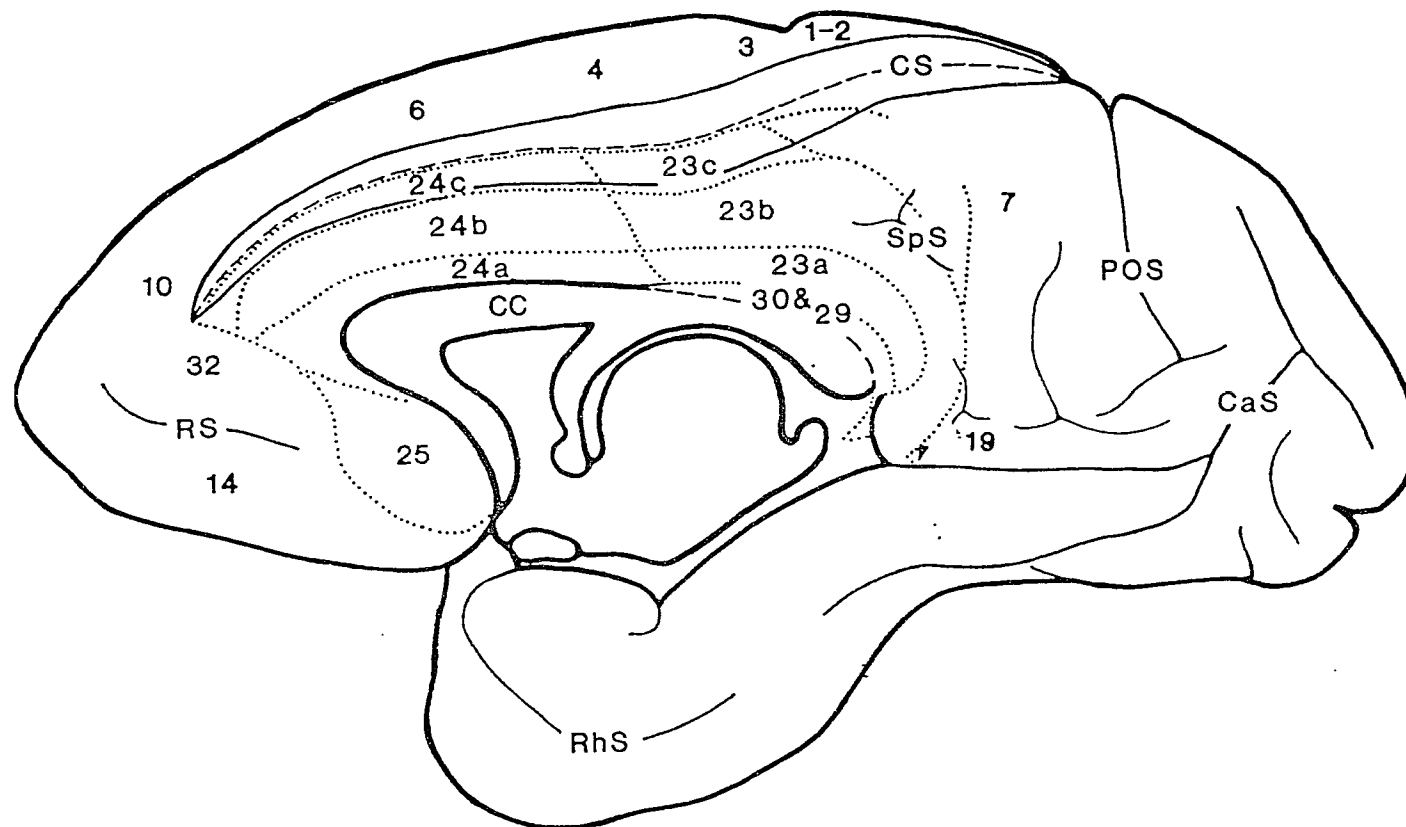


Fig. 1.2

Fig. 1.2

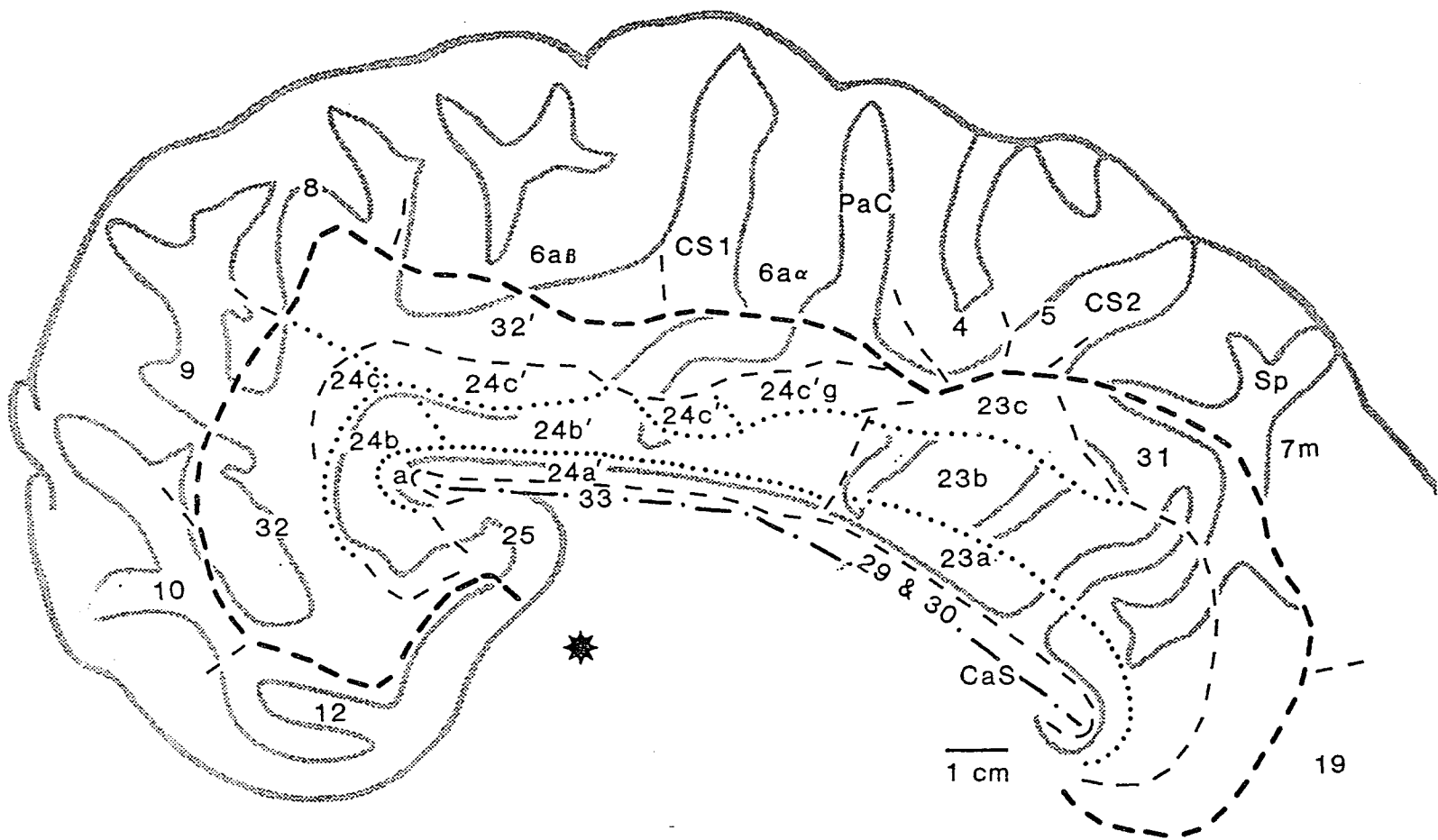


Fig. 1.3

Chapter 2

Regional Distribution of Neurofilament and Calcium-Binding Proteins in the Cingulate Cortex of the Macaque Monkey

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(Cereb. Cortex 2:456-467, 1992)

SUMMARY

The cingulate cortex is composed of morphologically and functionally distinct areas. It is considered to be a major component of the limbic system and has been shown to subserve a wide range of autonomic and somatic motor functions. The anterior and posterior regions of the cingulate cortex can be differentiated according to their thalamic afferents as well as their patterns of corticocortical connectivity. The primate cingulate cortex is traditionally divided into a series of cytoarchitectonic zones that can be distinguished along a ventral to dorsal axis of differentiation in both the anterior (areas 25, 24a, 24b and 24c), and posterior (areas 29, 30, 23a, 23b and 23c) regions. However, little is known about the precise cellular organization of these subareas. In the present study, we attempt to define the neuronal morphological and biochemical composition of the different cingulate cortex subareas, using antibodies to the neurofilament triplet protein and calcium-binding proteins. Results indicate that there is a strong correlation between the structure and functions of the cingulate cortex and the immunostaining patterns. For instance, distribution of neurofilament protein-rich pyramidal neurons parallels that of specific corticocortical and cortico-subcortical systems and is a useful marker to delineate the cingulate motor area. Calcium-binding protein-containing neurons display a high degree of regional and laminar specialization. In particular, parvalbumin-positive interneurons are codistributed with neurofilament protein-immunoreactive pyramidal cells along the ventro-dorsal and rostro-caudal axes of the cingulate cortex. Calbindin- and calretinin-positive immunostaining show more monotonous laminar and

regional patterns, although they exhibit a particular labeling in area 29 that may correspond to the termination of select thalamocortical afferents. These chemoarchitectural patterns of regional and laminar neuronal specialization may be envisioned as the reflection of the richness of cortical diversity in the cingulate gyrus, and makes it an ideal place to explore the interplay of the distributions of various neuron types in cortical areas of known function.

INTRODUCTION

The cingulate cortex is an important component of the limbic system and contains a number of anatomically and functionally distinct subfields (Vogt, 1985; Vogt et al., 1987). Experimental evidence shows that the cingulate cortex in rodent and primates is involved in a variety of tasks encompassing a broad range of sensorimotor functions. For instance, the anterior cingulate cortex appears to be concerned with attention (Kennard, 1955; Watson et al., 1973; Pardo et al., 1990; Pardo et al., 1991), memory and learning processes (Gaffan and Harrison, 1987; Murray et al., 1989), complex somatic and visceral motor activities (Ward, 1947; Kaada et al., 1949; Kaada, 1951; Showers and Crosby, 1958; Showers, 1959), and vocalization (Jürgens and Ploog, 1970; Jürgens, 1983), as well as response to pain (Foltz and White, 1962; Jones et al., 1991; Talbot et al., 1991). The posterior cingulate cortex appears to be mainly involved in the integration of specific visuomotor and somatic motor stimuli (Olson and Musil, 1992a; Olson and Musil, 1992b; Musil and Olson, 1993; Olson et al., 1993). Thus, there is an apparent segregation of functions between the anterior and posterior cingulate areas.

In addition, many studies have indicated that cingulate cortex is heavily interconnected with motor structures at both the cortical and subcortical levels (Muakkassa and Strick, 1979; Vilensky and Van Hoesen, 1981; Glickstein et al., 1985; Hutchins et al., 1988; Luppino et al., 1990; Dum and Strick, 1991; Luppino et al., 1991; Matelli et al., 1991; Dum and Strick, 1992; Dum and Strick, 1993; Kurata, 1994; He et

al., 1995). The anterior cingulate cortex possesses a motor area distinct from the supplementary motor field (Muakkassa and Strick, 1979; Dum and Strick, 1991; Shima et al., 1991; Dum and Strick, 1993), that is somatotopically connected to the primary motor cortex, and projects in a topographic manner to the spinal cord (Hutchins et al., 1988; Dum and Strick, 1991; He et al., 1995). In the human brain, a specific field containing giant pyramidal neurons located in the dorsal aspect of the cingulate cortex (Braak, 1976; Braak and Braak, 1976) is thought to be the equivalent of the monkey cingulate motor area 24c' that is characterized by a higher density of large pyramidal cells in layers III and V than in the adjacent fields (Matelli et al., 1991).

Furthermore, the anterior and posterior cingulate areas can be differentiated by their thalamic inputs. For example, the thalamic outflow to anterior cingulate cortex is dominated by projections from the midline and intralaminar nuclei, whereas the posterior cingulate cortex receives mostly from the anterior group of thalamic nuclei and from the pulvinar (Vogt et al., 1979). These differences in thalamic innervation as well as cortical connectivity of specific fields within the cingulate cortex parallel the functional diversity and distribution of information processing in this anatomically complex region of the brain (Vogt et al., 1979; Baleyrier and Mauguière, 1980).

Despite the numerous studies on the distribution of physiologically-defined areas in cingulate cortex and their cytoarchitectural description using Nissl-stained materials, there are only limited data on the cellular organization of these areas in the primate. In particular, correlations between the cytoarchitecture and the distribution of select neuronal populations is still lacking. Previous studies in the monkey and human neocortex have demonstrated the existence of a strong correlation of the regional variations of the Nissl-staining pattern with the distribution of subsets of pyramidal neurons and interneurons characterized by specific morphological criteria as well as biochemical phenotype (Campbell and Morrison, 1989; Kondo et al., 1994; Hof and Morrison, 1995; Hof et al., 1995). For instance, calcium-binding proteins and certain

components of the cytoskeleton such as neurofilament and microtubule-associated proteins have been found to be reliable markers for select neuronal classes exhibiting region-specific distribution (Campbell and Morrison, 1989; Blümke et al., 1990; Peters and Sethares, 1991) and allowing in the case of the visual system for a refined definition of certain areas that was not possible on the basis of Nissl stain alone (Hof and Morrison, 1995).

In order to establish chemoarchitectural criteria to define the specific subregional organization patterns of efferent and local circuit systems in parallel with the distribution of functional processes, we have used an antibody to non-phosphorylated epitopes on the medium and heavy molecular weight subunits (168 and 200 kDa, respectively) of the neurofilament protein triplet that labels a subset of pyramidal neurons displaying very high degree of regional and laminar specialization (Campbell and Morrison, 1989; Hof et al., 1990; Hof and Morrison, 1990; Hof and Morrison, 1995; Hof et al., 1995). In addition, this group of neurofilament protein-containing pyramidal neurons corresponds to a particular population of corticocortically projecting cells that display a highly specific regional and laminar distribution in the monkey neocortex (Hof et al., in press; Hof et al., submitted).

To complement the study of pyramidal neurons we analyzed the regional and laminar staining patterns obtained with antibodies to the calcium-binding proteins parvalbumin, calbindin D-28k, and calretinin. These calcium-binding proteins have been demonstrated in morphologically distinct and non-overlapping GABAergic interneuron classes in the vertebrate central nervous system (Demeulemeester et al., 1988; Hendry et al., 1989; Celio, 1990; Seress et al., 1991; Résibois and Rogers, 1992; Hof et al., 1993). Thus, in the primate cerebral cortex, parvalbumin is found in large basket cells and in a subset of chandelier cells (DeFelipe et al., 1989b; Lewis and Lund, 1990; Ribak et al., 1990; Hof et al., 1991; Hof et al., 1993), calbindin is present in some double bouquet neurons as well as in a subset of layer III pyramidal neurons (DeFelipe et al., 1989a; Hof

and Morrison, 1991; DeFelipe and Jones, 1992; Hof et al., 1993), and calretinin is observed in multipolar, bipolar and double bouquet cells (Jacobowitz and Winsky, 1991; Résibois and Rogers, 1992; Rogers, 1992; Hof et al., 1993; Hof et al., 1993; Lüth et al., 1993; Condé et al., 1994). Calbindin and calretinin are structurally closely related; however, they are present in separate neuronal populations. Furthermore, calcium-binding proteins are reliable markers of different classes of thalamic relay cells and their cellular and neuropil staining patterns within the neocortex correlate with the presence of select thalamocortical inputs (Jones and Hendry, 1989; Blümke et al., 1990; Blümke et al., 1991; DeFelipe and Jones, 1991; Blümcke et al., 1994). In the present study, we have investigated the possible relationships between the regional and laminar distribution of pyramidal cells and interneurons and the localization of areas related to specific functions in the monkey cingulate cortex.

MATERIALS AND METHODS

Tissue preparation

The brains of ten adult cynomolgus monkey (*Macaca fascicularis*) were used in this study. Some of these animals were used in other studies (Campbell et al., 1991; Hof et al., in press; Hof et al., submitted), and all protocols were conducted within NIH guidelines for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC) at Mount Sinai School of Medicine. The materials were prepared according to Campbell et al. (1987). Briefly, the monkeys were deeply anesthetized with ketamine hydrochloride (25 mg/kg i.p.) and pentobarbital sodium (20 mg/kg i.v.), intubated and ventilated with 100% oxygen. The chest was then opened, the heart exposed, and 1-2 ml of 0.1% sodium nitrite was injected into the left ventricle. The descending aorta was clamped and the monkeys were perfused transcardially with cold 1% paraformaldehyde in phosphate buffer for 30 to 60 seconds followed by cold 4%

paraformaldehyde for 8 to 10 minutes. The brains were then removed from skull and cut into 3-5 mm-thick blocks and postfixed for 6 to 10 hours. On three animals, the entire cingulate gyrus was dissected out from both hemispheres to allow for parasagittal sectioning of the dorsal and ventral aspects, and horizontal sectioning of the medial wall of the cingulate cortex. These preparations were useful for a better definition of the transition zones between the different cytoarchitectonic fields of the cingulate cortex in the rostrocaudal axis. Coronal blocks from the anterior and posterior cingulate gyrus were obtained from the other animals. We report below the chemoarchitectural organization of the ventral aspect of the monkey cingulate cortex (i.e., anterogenua area 25, anterior area 24a, and posterior areas 29, 30 and 23a) in comparison with the medial and dorsal areas 24b, 24c and 24c' anteriorly, and 23b and 23c posteriorly. The nomenclature for the cingulate cortex areas was adapted from Vogt et al. (1987).

Staining procedure

Following postfixation, the blocks were washed in a series of sucrose solutions (12%, 16%, and 18%) in phosphate-buffered saline (PBS), frozen and cut on a cryostat. Adjacent 40 μ m-thick sections were collected for histological and immunohistochemical purposes. The sections were incubated overnight at 4°C with a monoclonal antibody to non-phosphorylated epitopes on the medium and heavy subunits of the neurofilament protein (SMI-32, Sternberger Monoclonals, Jarrettsville, MD) or with fully characterized monoclonal antibodies to the calcium-binding proteins parvalbumin and calbindin (Celio et al., 1988; Celio, 1990), and with a fully characterized polyclonal antibody to calretinin (Schwaller et al., 1993). Antibodies were used at a dilution of 1:10,000 (SMI-32), 1:3,000 (parvalbumin), 1:2,500 (calbindin) and 1:3,000 (calretinin) in PBS containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin. The sections were then processed by the avidin-biotin method with Vectastain ABC kits (Vector Laboratories, Burlingame, CA) and diaminobenzidine. The immunoreactivity was then intensified in 0.005% osmium tetroxide. Adjacent sections were stained with thionin in order to clarify

the cytoarchitecture. In addition, a double-labeling experiment was carried out in order to visualize the relationships between pyramidal cells and parvalbumin-immunoreactive terminals from basket cells in select cingulate cortical regions. Briefly, sections were incubated overnight at 4° C with antibody SMI-32 (1:2500) and a polyclonal antibody to parvalbumin (1:1000). They were then incubated in biotinylated anti-mouse IgG (1:200) and fluorescein-conjugated anti-rabbit IgG (1:100) for 3 hours at room temperature, and then in streptavidin-Texas red (1:200) for 1 hour. They were mounted with glycerin and water (3:1), and viewed with the appropriate set of filters.

RESULTS

Areas 25 and 24a

Area 25 is an agranular field with intermediate-sized pyramidal neurons in layers III and V. The pyramidal cells are more abundant in layer III than in layer V, and are smaller and sparser in layers II and VI. SMI-32 stains a select population of pyramidal neurons restricted to layers V and VI (Fig. 2.1A). These neurons are dense in layer V, whereas they are much sparser in layer VI. The deep layers are characterized by a band of parvalbumin-ir neuropil in layer V and isolated large parvalbumin-ir basket neurons in layer VI (Fig. 2.1B). Calbindin is present in two distinct populations. First, it is found in a population of double bouquet interneurons located in all cortical layers except layer I. The second population consists of small pyramidal cells which exhibit a punctate staining pattern lighter than that of the interneurons. This population resides exclusively in layer II and the upper two-thirds of layer III. The neuropil as stained with calbindin is homogeneous throughout all the cortical layers. Calretinin marks a population of double bouquet cells concentrated in the deep portion of layer II but also scattered throughout layers III through VI. The dendrites of these cells extend in radially oriented cascades through the cortex. The neuropil labeling pattern is homogeneous except for a deeply stained rim in the most superficial portion of layer I.

Area 24a is characterized in Nissl-stained sections by the absence of a distinct layer IV, very large pyramidal cells in layer III and smaller pyramidal cells in layers II, V and VI (Fig. 2.2A). As in area 25, immunostaining with SMI-32 is restricted to the small- to medium-sized pyramidal cells of layers V and VI (Fig. 2.2B). Parvalbumin immunostaining reveals neuropil homogeneously labeled throughout the cortex and the presence of immunoreactive cells evenly distributed in layers II through VI. The axons of these cells are frequently varicose and can be observed to take an oblique or horizontal course through the cortex (Fig. 2.2C). Calbindin-containing interneurons are distributed as in area 25, but with a slightly higher density in layer II. Calbindin-containing pyramidal cells are located in layers II and III as in area 25 (Fig. 2.2D). Fewer cells are labeled with calretinin than with calbindin in this area. The portion of layer II immediately underlying layer I is relatively free of calretinin-immunoreactive neuronal somata and is traversed by many fine and long tangential fibers (Fig. 2.2E).

Areas 29, 30 and 23a

Area 29 is a granular periallocortex with an undifferentiated granular layer in Nissl stain. On parasagittal preparations, the rostral-most aspect of area 29 shows a dense and thick band of small neurons in layer II, and layer III appears as relatively thin band of large pyramidal cells that are distinct from the deeper layers V and VI where sparser and smaller pyramidal cells predominate (Fig. 2.2F). Neurofilament protein-containing neurons are denser in layers V and VI, with the largest cells in layer V. A few neurofilament protein-containing neurons are observed in layer III. Layer VI exhibits a peculiar staining pattern in that the small pyramidal neurons are arranged in a series of longitudinal rows (Fig 2.2G). This labeling pattern is observed exclusively in area 29. Interestingly, layer VI contains longitudinally-running bundles of fibers labeled by all three calcium-binding proteins. This pattern is particularly evident on calcium-binding protein-stained materials in the parasagittal plane (Fig. 2.2H-J). Parvalbumin-immunoreactive neurons are present in all layers of area 29 except layer I, and are less

dense in layer VI than in layers II to V (Fig. 2.2H). Calbindin-immunoreactive pyramidal neurons are scarce in this area. Calbindin-immunoreactive interneurons predominate in layer II, and layers V and VI exhibit a sparser population (Fig. 2.2I). Layer III, which is virtually devoid of labeled neurons, exhibits a band of intense neuropil labeling. Calretinin-positive neurons are mostly located in layer II and V, while layer III is characterized by a strong labeling of the neuropil comparable to that observed with calbindin, and a lower density of calretinin-immunoreactive neurons (Fig. 2.2J)

The dysgranular area 30, lying more medially to area 29, displays staining patterns comparable to those observed in area 29. There are however more neurofilament protein-containing pyramidal neurons and large parvalbumin-immunoreactive basket cells in layer III in area 30. In addition, the deep layers of area 30 do not display the longitudinal arrangement of neurofilament protein-containing pyramidal neurons that was observed in area 29. Layer II exhibits an increase in calretinin-immunoreactive neuron density as compared to area 29. The neuropil staining by calbindin and calretinin of layer III in area 29 disappears in area 30.

Area 23a, in the continuation of area 30, is distinguished by the presence of layer IV. Neurofilament protein-containing neurons are distributed in both layers III and V-VI. This bilaminar distribution is strikingly different from that observed in the anterior cingulate areas 25, 24a, 24b and 24c where no neurofilament protein-containing neurons are found in the superficial layers. At this level, the neuropil is divided in two bands of parvalbumin labeling corresponding to deep layer III and layer V. Area 23a contains large basket cells in layers III, V and VI, and a greater number of calretinin-positive neurons in layer III than areas 29 and 30. Calbindin-immunostaining shows a pattern similar to that found in area 30.

Areas 24b and 24c

Nissl stain of area 24b reveals a dense population of relatively large pyramidal neurons in layer II and the upper third of layer III, whereas the lower two-thirds of layer

III exhibit a sparser population. Layer V can be subdivided into two sublayers Va and Vb, with layer Va containing large pyramidal cells, and layer Vb and layer VI being characterized by fewer and smaller neurons (Fig. 2.3A). In the continuation of area 24a, neurofilament protein staining shows a progressive increase in the density of labeled pyramidal neurons in layers V and VI, although the global staining pattern is qualitatively comparable to that observed in area 24a. In addition, the deep portion of layer III contains a few lightly stained pyramidal neurons and some apical dendrites from layer V neurofilament protein-containing neurons (Fig. 2.3B). Neurofilament protein staining parallel the Nissl stain in layer V, in that there are larger neurofilament protein-containing pyramidal cells in layer Va, whereas layer Vb appears as a neuron-sparse zone. Parvalbumin staining shows a more differentiated pattern than in area 24a in that two distinct bands of neuropil labeling are present in layers III and V. Layer VI appears lightly stained and contains fewer parvalbumin-immunoreactive neurons than layers II to V (Fig. 2.3C). The labeling pattern for calbindin is comparable to that in area 24a, with a slightly increased number of interneurons in layer II and the upper part of III. Layer II and the superficial part of layer III contain a moderate number of lightly stained small pyramidal neurons which are not observed in layers V and VI (Fig. 2.3D). Calbindin-immunoreactive interneurons are denser in layer VI than in layer V. Finally, area 24b has fewer calretinin-immunoreactive neurons in layer II and more in layer III than area 24a (Fig. 2.3E).

Area 24c is recognizable in Nissl-stained sections by the presence of large pyramidal neurons in both layers III and V, whereas layers II and VI contain smaller pyramidal cells, and the absence of a distinct layer IV (Fig. 2.3F). The neurofilament protein-containing neuron distribution is comparable to that observed in areas 25, 24a and 24b, in that most of the stained pyramidal cells reside in layers V and VI (Fig. 2.3G). There is an increased bundling of dendrites in layer V as compared to areas 24a and 24b, with apical dendrites reaching the lower portion of layer III. Layer III displays sparse

immunoreactive neurons, located mostly in its lower one-third. More dorsally, the number of neurofilament protein-immunoreactive neurons in deep layer III increases and delineates a subarea located in front of the area of transition to the posterior cingulate area 23c. This area may correspond to the cingulate motor area located in area 24c'. Parvalbumin immunoreactivity in area 24c is comparable to that in area 24b, with the deeper band of parvalbumin-immunoreactive neuropil staining in a distinctively patchy manner (Fig. 2.3H). In area 24c' however, there is an increased density of parvalbumin-immunoreactive neurons in layers II to V and the presence of numerous pericellular baskets around unstained pyramidal neurons in layers III and V. Staining patterns for calbindin (Fig. 2.3I) and for calretinin (Fig. 2.3J) are comparable to those observed in area 24b.

Areas 23b and 23c

On Nissl stained sections, the more dorsal parts of posterior cingulate cortex are truly isocortical. Layer IV is well developed and the flanking pyramidal cell layers contain densely packed medium- to large-sized pyramidal neurons, with further increases in density in both the deep portion of layer III and the superficial portion of layer V where they abut layer IV (Fig. 2.4A). In area 23b, neurofilament protein immunostaining reveals intensely labeled medium- to large-sized pyramidal cells in layer III and more lightly stained cells in layers V and VI (Fig. 2.4B). Parvalbumin immunostaining homogeneously labels the neuropil in layers II through VI while parvalbumin-immunoreactive cells are found predominantly in layer II and the superficial part of layer III. There is relatively sparser parvalbumin-immunoreactive cells in the deeper portion of layer III and then a denser band of cells in layer IV and the superficial part of layer V. Layer VI is characterized by the presence of more sparsely distributed large, multipolar cells with varicose dendrites (Fig. 2.4C). Large numbers of calbindin-immunoreactive interneurons are here, as elsewhere in the cortex, located primarily in layer II and the superficial portion of layer III, with their density increasing slightly in the more dorsal

part of area 23b and into 23c. As in the anterior cingulate, there is a sparser population of cells in layers V and VI. Unlike the distribution in anterior cingulate, however, there is also a band of calbindin-immunoreactive interneurons in layer IV, some of which display widely ramifying dendrites. There are slightly fewer calbindin-immunoreactive pyramidal cells in these posterior areas than anteriorly, but there is still a considerable population, again strictly confined to layers II and III (Fig. 2.4D). Calretinin immunostaining reveals a comparatively large population of interneurons, concentrated as elsewhere in layer II but present throughout the thickness of the cortex. An increased density of calretinin-immunoreactive cells also exists in layer IV and there is a discontinuous band of interneurons in layer V (Fig. 2.4E).

Nissl staining of area 23c shows a narrow layer of densely packed pyramidal cells in layer II just below the molecular layer, and a granular layer IV more compactly arranged than in more ventral areas (Fig. 2.4F). Staining for neurofilament protein reveals groups of large, very intensely stained pyramidal cells in layer III. These groups include the largest such cells in the cingulate cortex. Interestingly the neurofilament protein staining pattern does not parallel the Nissl stain, since there are several large neurofilament protein-immunoreactive pyramidal neurons in layer III where relatively small-sized neurons are visible in Nissl stain. It should be noted that these very large neurofilament protein-immunoreactive neurons may represent a small population of pyramidal cells that may not be clearly apparent on a Nissl preparation. Additionally, apical dendrites of pyramidal cells in layers V and VI can be seen rising through layer IV in dense bundles (Fig. 2.4G). As observed elsewhere in the cortex, the neurofilament protein-immunoreactive pyramidal cells in layer VI are smaller and more lightly stained than those in layer V. The staining patterns of the calcium-binding proteins resemble those of area 23b (Figs 2.4H-J). One notable feature of parvalbumin immunostaining in area 23c and to a lesser extent in areas 23a and 23b, is the presence, in layer V, of numerous parvalbumin-immunoreactive pericellular baskets.

DISCUSSION

The diversity of functions subserved by the cingulate cortex, as described elsewhere in this issue is clearly reflected in a striking diversity of cyto- and chemoarchitecture. The Nissl stain, upon which most cytoarchitectonic parcellations have been based, permits the discrimination of different types of cortex, but such observations have a limited value beyond pure subdivision, since very few cell types can be reliably distinguished. On Nissl-stained preparations, two anatomic trends are evident from an analysis of the cingulate cortex architecture. One is a gradient in the ventrodorsal direction, progressing in anterior cingulate from the ventral, less differentiated area 24a through the intermediate area 24b, to the more differentiated area 24c, and similarly, in posterior cingulate from the less differentiated areas 29 and 30, to the differentiated areas 23a, 23b and area 23c. The second gradient is in the rostrocaudal direction, from the agranular anterior cingulate areas 24a, 24b, 24c, and 24c', through the transition to the isocortical areas 23a, 23b, and 23c of the posterior cingulate cortex. Differences in the patterns of immunostaining with various antibodies delineate different regions and subregions and both confirm and underscore the more subtle differences observed with the Nissl stain. For instance, immunostaining with antibody SMI-32 or with antibodies to the calcium-binding proteins not only reveals distinctive patterns of labeling; it also labels populations of cells which have certain properties which may then be related in some way to their possible function within the area in question, and ultimately to the function of the area itself.

Nonphosphorylated neurofilament protein and parvalbumin immunostaining most clearly exemplify these patterns and do so in a complementary manner. Areas and layers marked by significant numbers of neurofilament protein-containing pyramidal cells are enriched in parvalbumin-immunoreactive fibers in the neuropil. In the absence of layer IV, in anterogenuial area 25 and area 24a, neurofilament protein immunostaining is restricted to layers V and VI. More dorsally and posteriorly, neurofilament protein-

immunoreactive pyramidal cells begin to appear in layer III. Parvalbumin-immunoreactive neuropil is thus restricted to these layers. Since there is still no layer IV, there is a bilaminar pattern of neuropil labeling, centered at the levels of the pyramidal cell perikarya in layers III and V-VI. This immunoreactivity would appear to be due to the numerous parvalbumin-immunoreactive basket cells that populate all of the cortical layers below layer I. This assumption is substantiated by the presence of patchy neuropil staining in pyramidal cell layers that represents clusters of pericellular baskets, and further by the demonstration of parvalbumin-immunoreactive baskets surrounding neurofilament protein-immunoreactive perikarya. The emergence of a granular layer in posterior cingulate cortex is accompanied by the increased number of neurofilament protein-immunoreactive pyramidal cells and by some degree of regional specialization such as the presence of large neurofilament protein-immunoreactive neurons in layer III of area 23c that are not conspicuous on Nissl-stained sections. Layer IV itself is marked by a low density of parvalbumin-immunoreactive basket cells and a band of parvalbumin-immunoreactive neuropil. This band fills the space between the two parvalbumin-immunoreactive stripes found in anterior cingulate layers III and V and this may be what gives the neuropil a homogeneous appearance in these areas. Calbindin and calretinin immunostaining results in a more monotonic pattern of labeling. Both proteins are present in populations of cells that predominate in superficial layers but are present throughout the cortex. Exceptions include layer IV, which, when it appears, is characterized by an increased density of these cells, and area 29 which is remarkable for its virtual absence of calbindin-immunoreactive pyramidal cells. It is notable that the neuropil as stained by both these labels is fairly homogeneous, except in area 29.

The observations summarized above lend support to certain notions regarding efferent (corticocortical and corticofugal) projections from and afferent (thalamocortical) projections to limbic and non-limbic cortex. Retrograde tracing studies have shown that neurofilament protein is found in a subset of corticocortically projecting cells in the

monkey neocortex (Campbell et al., 1991; Hof et al., in press; Hof et al., submitted). In fact, depending on the functional nature of the projection, the proportion of neurofilament protein-immunoreactive retrogradely labeled neurons participating to the connections between different neocortical association areas ranges between 25% and 90% (Campbell et al., 1991; Hof et al., in press). It has been noted that corticocortical projections from limbic cortex are furnished preferentially by cells in the infragranular layers, while those from more differentiated isocortex originate in supragranular layers (Barbas, 1986). Clearly, the distribution of neurofilament protein-immunoreactive cells throughout the cingulate gyrus substantiates this view. The least differentiated parts of the cingulate cortex, i.e. anterogenual cortex and areas 24a, 29 and 30 contain these corticocortically projecting cells only in layer V, while the more isocortical areas dorsally and posteriorly are characterized by a population of cells in layer III that increases along the limbic-to-isocortical gradient. Interestingly, neurofilament protein-immunoreactive pyramidal cells have been shown to participate in projections to the region of the principal sulcus from ipsi- and contralateral anterior cingulate cortex which originate exclusively from layer V. Up to 40% of the neurons forming these particular projections were found to be neurofilament protein-immunoreactive (Campbell et al., 1991; Hof et al., in press). In contrast to the less differentiated anterior cingulate areas, the corticocortical connections from the posterior cingulate cortex originate from both layers III and V, and 18% to 30% of the projection neurons in both layers III and V are neurofilament protein-immunoreactive (Hof et al., in press). Furthermore, the cells contributing to the two major intracingulate streams of information, i.e., the rostral outflow and caudal feedback systems (Van Hoesen et al., 1993) originate in layers with high densities of neurofilament protein-immunoreactive pyramidal cells. In view of the laminar distribution of corticocortically-projecting neurofilament protein-immunoreactive neurons, it is possible that the forward rostral outflow system from posterior to anterior cingulate cortex originates in layer III and V of the posterior cingulate areas, while the caudal feedback

system would be expected to originate in deeper layers, because it takes origin from a more rudimentary type of cortex (Barbas, 1986).

Neurofilament protein is also found in some corticofugal cells. For example, Betz cells in primary motor cortex are intensely neurofilament protein-immunoreactive (Campbell and Barbas, 1991). Dum and Strick (1991) physiologically defined regions in areas 24c and 23c with distinct motor functions, which they designated cingulate motor areas (CMA). The rostral-most portion, CMAr, may correspond to an area located in the depths of the cingulate sulcus which has been described physiologically (Luppino et al., 1991; Shima et al., 1991) and has been designated area 24d (Matelli et al., 1991) or 24c' (Vogt, 1993). Similarly, the posterior area 23 contains a region termed CMAv (Dum and Strick, 1993). Several authors also showed that the CMA are reciprocally connected both with primary motor cortex and with each other, and project to the pons and spinal cord (Muakkassa and Strick, 1979; Vilensky and Van Hoesen, 1981; Glickstein et al., 1985; Hutchins et al., 1988; Dum and Strick, 1993). The observation of discrete fields in areas 24c and 23c which are characterized on neurofilament protein immunostaining by large, possibly corticocortically and cortico-subcortically projecting cells in layers III and V, supports the notion that the area so defined by chemoarchitecture corresponds to this highly specific functional area. For both corticocortical and corticofugal projections from the cingulate gyrus, then, neurofilament protein immunostaining can to some extent be correlated with function.

There is evidence that calbindin and calretinin belong to phylogenetically older systems than parvalbumin (Glezer et al., 1992). Calbindin and calretinin are present in older and less specific ascending spinothalamic pathways and their nuclei, whereas parvalbumin is found in the more specific and younger lemniscal system (Jones and Hendry, 1989; Rausell and Jones, 1991b; Rausell and Jones, 1991a; Rausell et al., 1992a; Rausell et al., 1992b). Thus, projections belonging to the older calbindin and calretinin systems might be expected to have more diffuse terminations in the cortex, while

afferents from the newer, more specific parvalbumin system might be restricted to certain cortical areas and layers. For instance, parvalbumin-immunoreactive thalamic relay neurons have been shown to project to layer IV of restricted cortical areas, whereas calbindin- (and possibly calretinin-) immunoreactive thalamocortical connections project principally to layer I in a less area-specific manner (Jones and Hendry, 1989; Blümke et al., 1991; DeFelipe and Jones, 1991; Hashikawa et al., 1991; Rausell et al., 1992a). The pattern of calcium-binding protein immunoreactive afferent terminations is also in agreement with the hypothesis proposed by Rausell and coworkers (1992) that the calbindin-immunoreactive cortically projecting cells are distributed throughout the thalamus, irrespective of traditional nuclear boundaries, forming a "matrix" upon which the more specific parvalbumin-immunoreactive cell-containing nuclei are superimposed. This scheme might be continued into the cortex in the form of different specific- and non-specific afferent terminations as described above. In addition, the neuropil of layer III of area 29, the phylogenetically oldest area of posterior cingulate cortex (Vogt, 1993) displays a very specific pattern of calbindin and calretinin immunostaining. This could represent a region-specific pattern of calbindin- and calretinin-immunoreactive thalamocortical afferent termination that antedates the patterns of parvalbumin-immunoreactive thalamocortical termination found elsewhere in the cingulate cortex.

The anteromedian nucleus of the thalamus projects to all cingulate areas. This projection is so striking that it has been proposed that it be used as a criterion to define the extent of cingulate cortex (Vogt et al., 1987). The cells of the anteromedian nucleus, are calbindin-immunoreactive (Jones and Hendry, 1989). This could account for the consistent and even labeling of the neuropil in all of the cingulate areas with calbindin. In the primate, area 23 is distinguished by afferents from the lateral posterior nucleus and the medial pulvinar (Vogt et al., 1987), nuclei that label not only with calbindin, but also with parvalbumin (Jones and Hendry, 1989). Such a parvalbumin-immunoreactive projection might be expected to produce an additional band of neuropil staining in this

area, restricted to the thalamic recipient layers IV and deep III. Therefore, limbic or paralimbic cortical areas receiving afferents from limbic thalamic nuclei which presumably belong to the calbindin- and calretinin-containing relay systems might be expected to exhibit a monotonic pattern of neuropil labeling with calbindin and calretinin, while more differentiated cortical areas receiving input from parvalbumin-immunoreactive thalamic nuclei would have parvalbumin-immunoreactive labeling in the thalamic input layer(s). Thus, the staining properties and patterns of termination of thalamic afferents could be correlated with the types of nuclei they originate from. This may explain the striking pattern of calretinin and calbindin neuropil staining in layer III of area 29. The discrete band of intense neuropil staining observed here may correspond to the termination of afferents from the laterodorsal, anterodorsal and anteroventral nuclei of thalamus. These nuclei have been shown to project preferentially to area 29 (Vogt, 1985; Vogt et al., 1987). Interestingly, the presence of calbindin has been demonstrated in relay neurons of these three nuclei in the monkey (Jones and Hendry, 1989), and calretinin is present in fibers in the anterodorsal nucleus of the rat (R  sibois and Rogers, 1992).

The cingulate cortex has been described as a bridge between the thalamus on one side and limbic and isocortex on the other (Powell, 1978). As such, it plays a central, integrative role and is involved in the processing of convergent information from many regions of the brain. It is only to be expected that its cortical anatomy should be so diversified and highly specialized for all of its many tasks. It is anticipated that as more cellular markers become available, greater insight may be gained into the functional and anatomic complexity of this central brain area.

FIGURE LEGENDS

Fig. 2.1. Sections through the macaque monkey anterogenual portion of the cingulate cortex (area 25), cut coronally. Serial sections were immunostained with SMI-32 (A) or antibody to the calcium-binding protein parvalbumin (B). Note that SMI-32 labels a population of pyramidal neurons in layers V and VI (A), and that this pattern is paralleled by the dense staining of layer V by parvalbumin (B). Scale bar = 100 μ m.

Fig. 2.2. Sections through the macaque monkey cingulate cortex, cut parasagittally. A-E, Area 24a. F-J, Anterior level of areas 29. Serial sections were stained with Nissl (A and F), or immunostained with antibody SMI-32 (B and G) or antibodies to the calcium-binding proteins parvalbumin (C and H), calbindin (D and I), or calretinin (E and J). Note the absence of neurofilament protein-immunoreactive pyramidal cells in layer III (B) and the presence of a bilaminar pattern of neuropil labeling with parvalbumin (C) in area 24a, and the distinctive staining patterns in area 29 of the deep layers with antibody SMI-32 (G) and of layer III with calbindin and calretinin (I and J). The cingulum bundle can be seen here to contain many intensely calbindin-immunoreactive fibers (I). Layers are indicated by Roman numerals on the left panel and are separated by circles on the other panels. Scale bar = 100 μ m.

Fig. 2.3. Sections through the macaque monkey cingulate cortex, cut horizontally to visualize areas 24b (A-E) or parasagittally to visualize area 24c (F-J). Serial sections were stained with Nissl (A and F), or immunostained with antibody SMI-32 (B and G), or antibodies to the calcium-binding proteins parvalbumin (C and H), calbindin (D and I), or calretinin (E and J). Note the continued bilaminar pattern of neuropil staining with parvalbumin (C and H), and the paucity of neurofilament protein-containing pyramidal cells in superficial layers (B and G). Layers are indicated by Roman numerals on the left panel and are separated by circles on the other panels. Scale bar = 100 μ m.

Fig. 2.4. Sections through the macaque monkey cingulate cortex, cut horizontally to visualize areas 23b (A-E) or parasagittally to visualize area 24c (F-J). Serial sections were stained with Nissl (A and F), or immunostained with antibody SMI-32 (B and G), or antibodies to the calcium-binding proteins parvalbumin (C and H), calbindin (D and I), or calretinin (E and J). Note the presence of layer IV (A and F), the appearance of neurofilament protein-immunoreactive pyramidal cells in layer III (B and G), the presence of calcium-binding protein-positive neurons in layer IV of these areas (compare to Figs 2.1, 2.2), and the homogeneity of the neuropil as stained with parvalbumin. The presence of very large neurofilament protein-immunoreactive pyramidal cells in layer III (G) may indicate that this area corresponds to the cingulate motor area. Layers are indicated by Roman numerals on the left panel and are separated by circles on the other panels. Scale bar = 100 μ m.

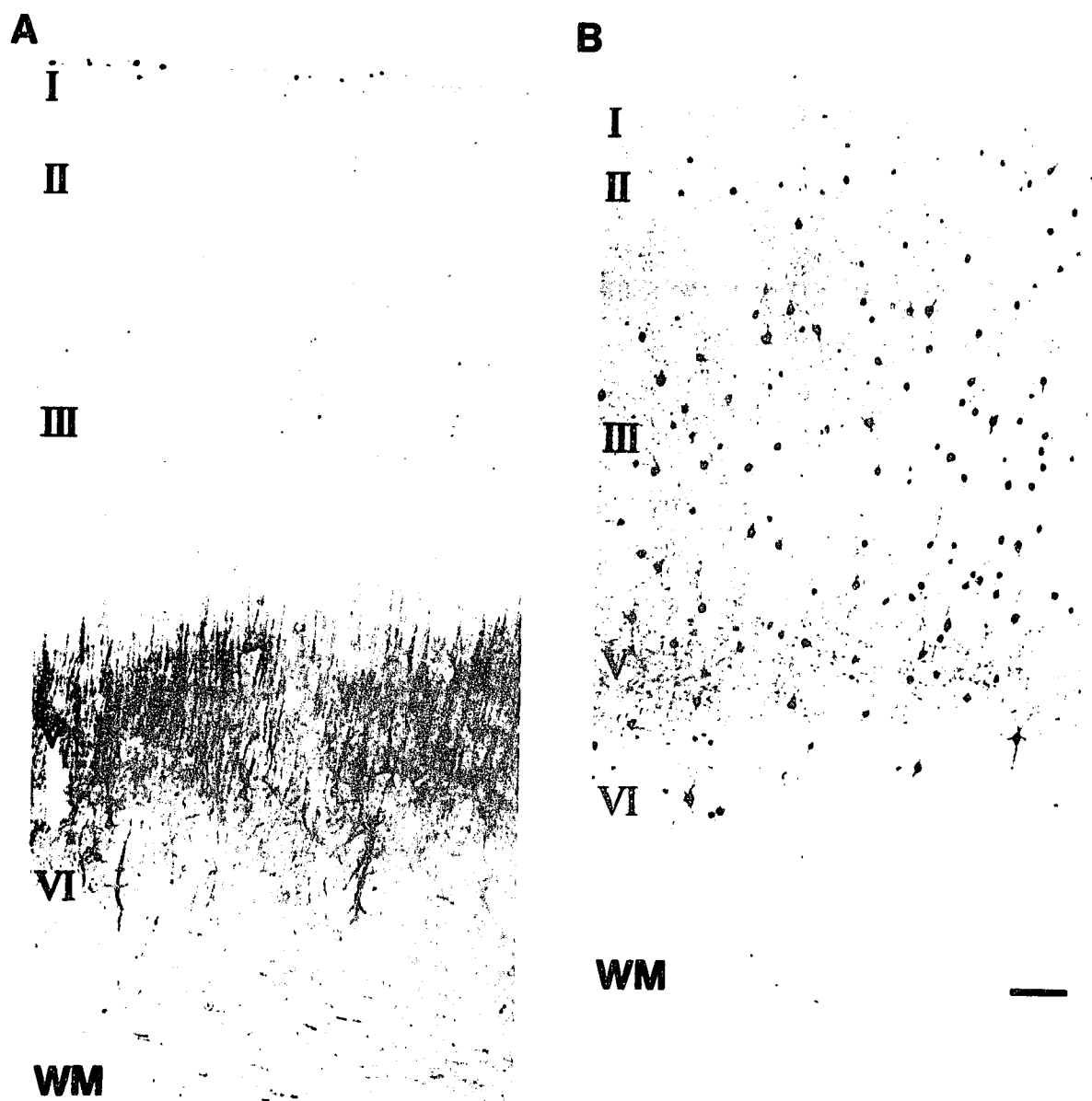


Fig. 2.1

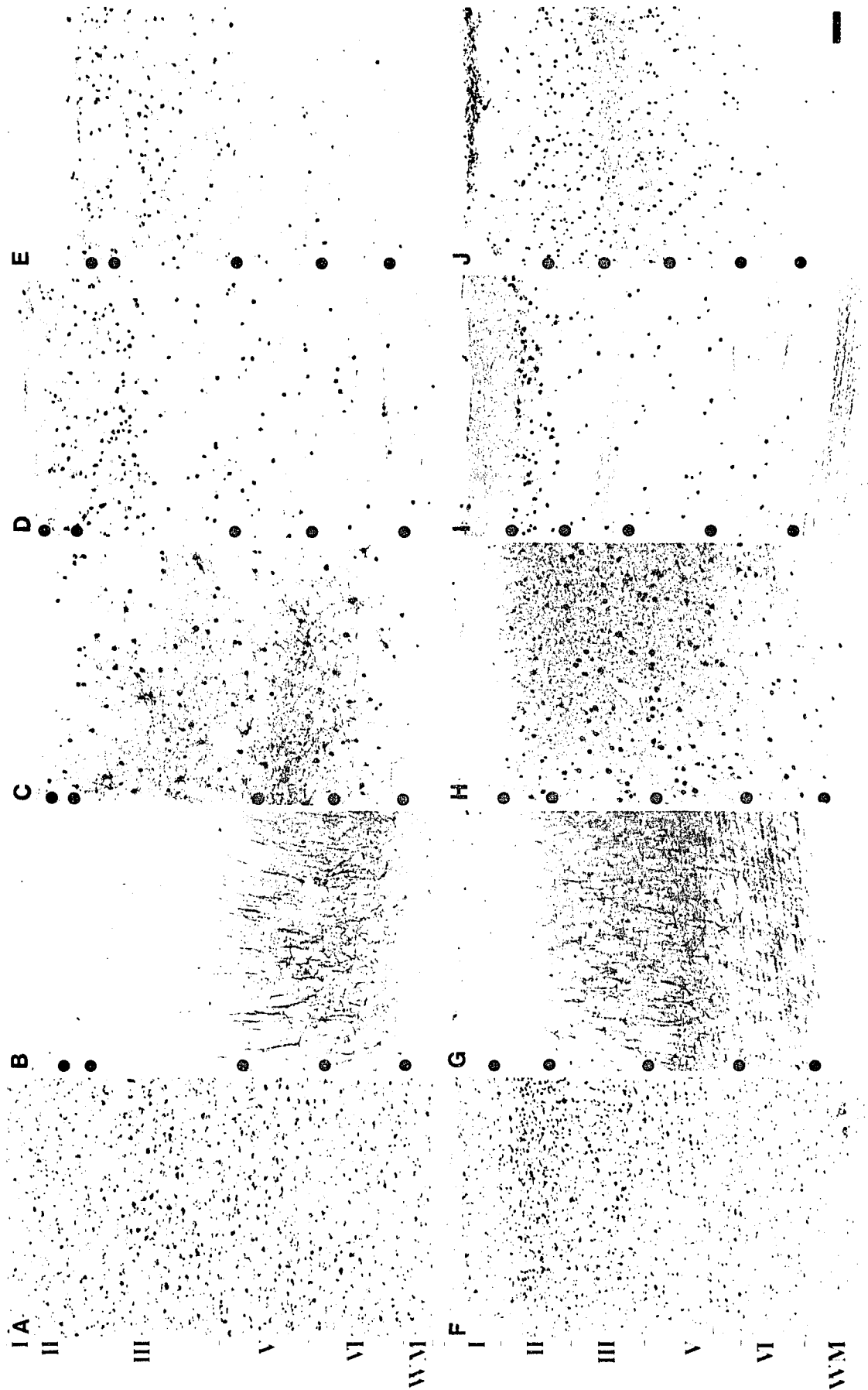


Fig. 2.2

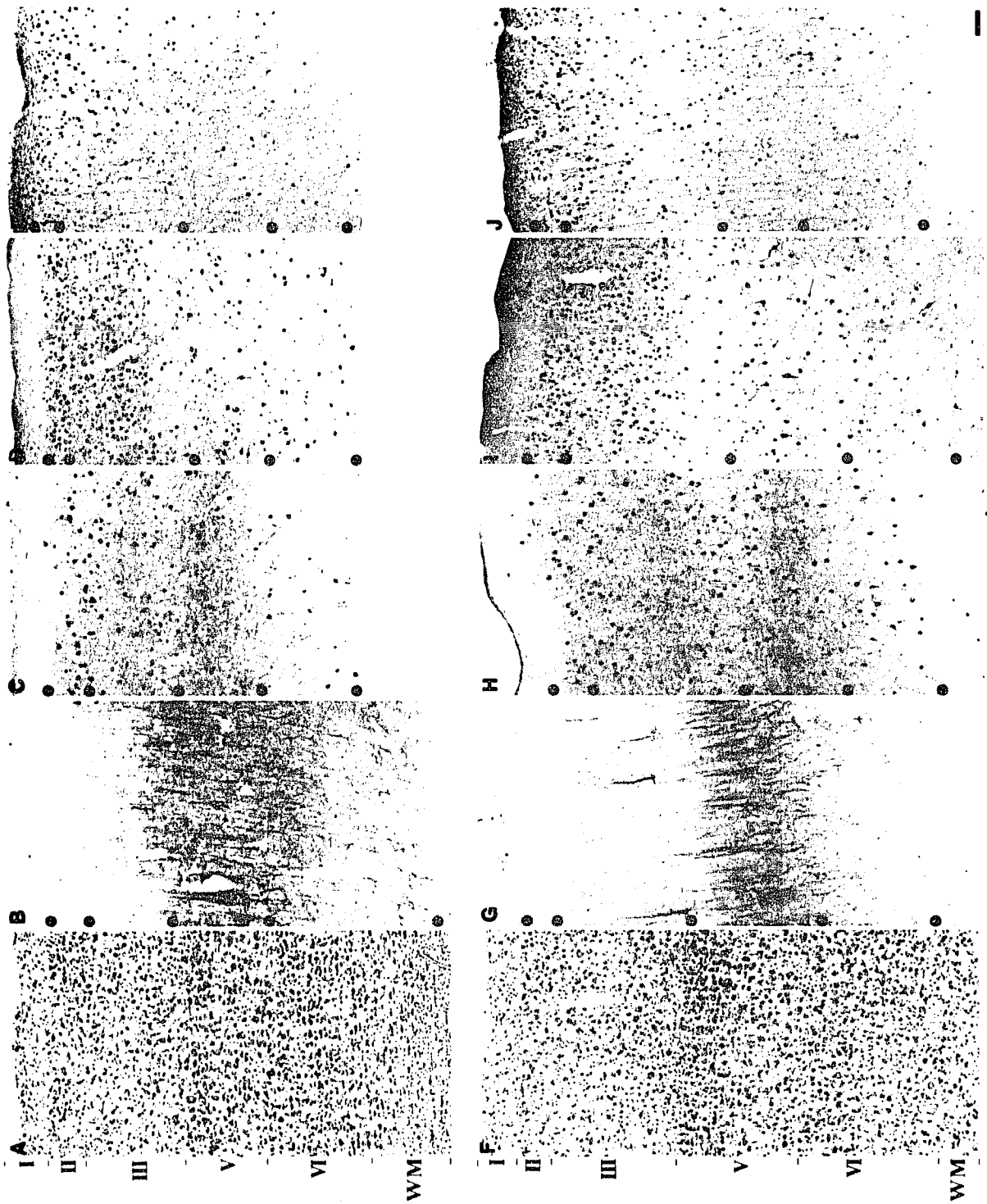


Fig. 2.3

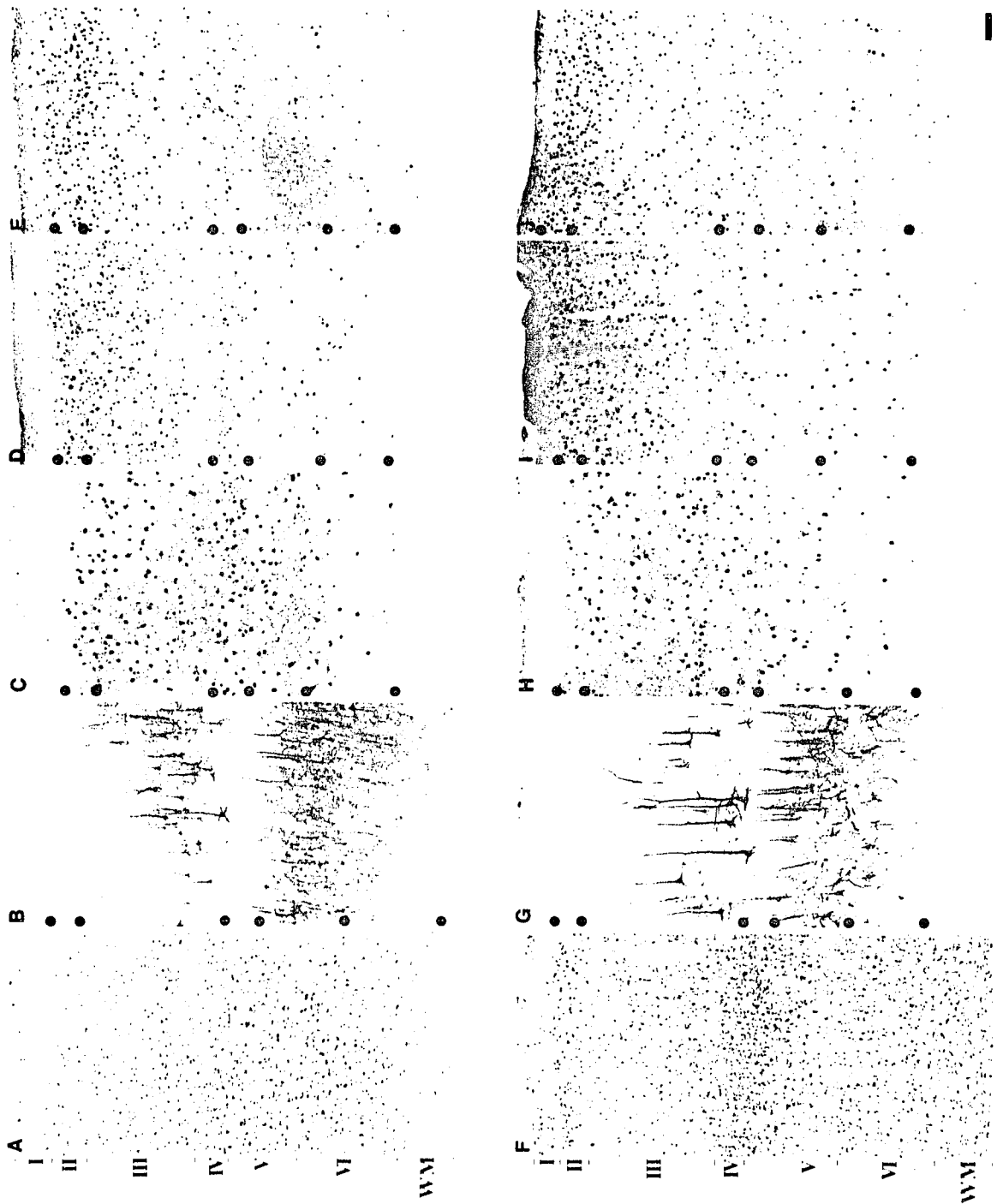


Fig. 2.4

Chapter 3

Chemoarchitecture of the Human Cingulate Cortex

SUMMARY

In recent years, numerous functional imaging studies in the human have suggested the involvement of the cingulate gyrus in a wide variety of cognitive, motor and sensory functions. These studies have highlighted the need for detailed anatomic analyses to better delineate its many cortical fields. In the present study, neurofilament protein, and the calcium-binding proteins parvalbumin, calbindin and calretinin were used as neurochemical markers to help study the differences among areas and subareas in the distributions of particular cell types or neuropil staining patterns. As observed in the macaque monkey, the most rostral parts of the anterior cingulate cortex were marked by a lower density of neurofilament protein-containing neurons, and the virtual restriction of these cells to layers V and VI. Neurofilament protein-immunoreactive layer III neurons were more numerous in the caudal portions of the anterior cingulate cortex, and reached maximal densities in the posterior cingulate cortex. Similarly, neurofilament protein-containing layer III neurons were more prevalent in dorsal than in ventral portions of the gyrus. Parvalbumin-immunoreactive neurons generally reflected this distribution, while calbindin- and calretinin-immunoreactive nonpyramidal neurons had a more consistent distribution along the gyrus. Calbindin-immunoreactive pyramidal neurons were more abundant anteriorly than posteriorly, and an unusual population of calretinin-immunoreactive neurons in layer V was found largely in the most anterior and ventral portions of the gyrus. Parvalbumin and calbindin immunoreactivity also presented complex neuropil labeling patterns that varied along the cingulate gyrus, and could represent, in part, thalamic termination zones. Calretinin immunoreactivity showed a uniform neuropil labeling pattern, with the exception of areas 29 and 20. Certain chemoarchitectonic features appear to correlate with functional domains of the cingulate

cortex, and may represent important regional markers as the functional relevance of regional specialization in the human cingulate cortex emerges.

INTRODUCTION

Studies of the cytoarchitecture of the cingulate gyrus in a range of mammalian species have demonstrated the existence of numerous areas distinguishable on the basis of the Nissl stain (Rose, 1927; Vogt, 1976; Vogt and Peters, 1981; Vogt et al., 1986; Vogt et al., 1987; Vogt et al., 1995). In the human and the nonhuman primate, the most obvious distinction is that the anterior portion of the cingulate gyrus dorsal to the corpus callosum lacks a clear layer IV, and is designated area 24, distinguishing it from the posterior portion of the gyrus, area 23, which does have a layer IV (Vogt et al., 1995). More subtle differences in cortical architecture, such as the relative prominence of layer II, the thickness of layer III, and the width, sublayering, and cellular composition of layer V, help to divide further these areas into consecutive strips of cortex beginning close to the depth of the callosal sulcus with 24a and 23a (in the anterior and posterior parts of the cingulate gyrus, respectively), continuing onto the medial wall of the gyrus (24b and 23b), and extending to or even dorsal to the depth of the cingulate sulcus (24c and 23c) (Vogt et al., 1987; Vogt, 1993; Vogt et al., 1995). The transition from anterior to posterior cingulate cortex is not an abrupt one, and occupies a distinct cortical area designated 24' which is also subdivided in the ventrodorsal direction into areas 24'a, 24'b, and 24'c. The cortical areas that lie immediately rostral and ventral to the genu of the corpus callosum are also considered part of the cingulate cortex, and are designated areas 32 and 25, respectively (Vogt et al., 1987; Vogt, 1993; Vogt et al., 1995). Finally, a cytoarchitectonically distinctive complex of cortical areas that lie caudally in the depth of the callosal sulcus, laterally to area 23a, is designated area 29, which is subdivided into areas 29a-d, and area 30, which is located between area 29 and 23a. This multiplicity of areas in the ventrodorsal direction was recognized by Von Economo (1927) and Rose

(1927), and more recently by Braak (1979b; 1979a) and Vogt (1976; Vogt et al., 1987; Vogt, 1993; Vogt et al., 1995).

The Nissl stain, despite its usefulness in anatomic parcellation, conveys limited functional information. Other cellular markers have been developed recently, however, that correlate with functional or connectional anatomic attributes, and thus provide crucial information beyond regional parcellation. One such marker is neurofilament protein, which has been visualized immunocytochemically using an antibody that recognizes a nonphosphorylated epitope on the neurofilament protein triplet (Sternberger and Sternberger, 1983; Lee et al., 1988). Neurofilament protein is found in a subpopulation of cortical neurons that vary in their distribution in different regions of the cortex (Campbell and Morrison, 1989; Hof and Morrison, 1995; Hof et al., 1995). A recent study in the macaque monkey showed that the distribution of neurofilament protein could be used to identify all cytoarchitecturally defined visual areas, including many cortical regions whose boundaries are not readily discernable on the basis of the Nissl stain (Hof and Morrison, 1995). Another study used this marker to delineate chemoarchitectural subdivisions of the human orbitofrontal cortex (Hof et al., 1995). Additionally, this marker has been shown to be a reliable indicator of the presence of the cingulate motor areas in the macaque monkey (Nimchinsky, submitted). Another set of useful markers are the calcium-binding proteins parvalbumin, calbindin and calretinin. The distribution of cortical neurons containing these calcium-binding proteins has been shown to differ in certain instances from area to area (Ferrer et al., 1992; Hof and Nimchinsky, 1992; Kondo et al., 1994; Hof et al., 1995). Moreover, with the exception of a population of pyramidal neurons that contain calbindin, the neurons that contain these proteins have been shown to be GABAergic interneurons (Celio, 1990; DeFelipe and Jones, 1992, PV? CR). Thus variations in their distribution might reflect differences in the functional organization of various cortical areas. These soluble cytoplasmic proteins are also present in projection neurons in thalamic nuclei (Hashikawa et al., 1991; Rausell and

Jones, 1991a; Rausell et al., 1992a; Rausell et al., 1992b; Diamond et al., 1993; Blümcke et al., 1994). As a result, the terminals of these projection neurons in the cortex also contain them, producing patterns of neuropil staining that vary with cortical areas and define thalamocortical terminal fields (Blümcke et al., 1990; Blümcke et al., 1991; DeFelipe and Jones, 1991; Diamond et al., 1993; Blümcke et al., 1994). Such biochemical approaches to cytoarchitecture, or chemoarchitectonics, are thus potentially powerful tools for the definition of cortical areas, particularly in human cortex, where experimental manipulation is not feasible. The present study was designed to determine whether changes in cortical cytoarchitecture in the cingulate gyrus are accompanied by variations in chemoarchitecture, and whether these changes respect the borders that can be defined on Nissl stained materials. If this were the case, these markers could facilitate the identification of cortical areas that might otherwise be very difficult to resolve. Furthermore, to the extent to which such markers can be linked to aspects of thalamocortical connectivity, staining patterns with these markers, combined with a knowledge of the connections in the nonhuman primate, could provide clues to the subcortical connectivity of these cortical areas in the human brain.

MATERIALS AND METHODS

Human brains were obtained at autopsy from seven patients with no history of neurologic or psychiatric disorders (five women, two men; age 82.3 ± 9.5 years old, range: 70-96). Clinical data on the cases were available from the medical records of the Division of Neuropathology, Mount Sinai Medical Center, New York, the Department of Psychiatry, University of Geneva School of Medicine, Switzerland, and the Department of Anatomy, University of Navarra, Pamplona, Spain. The materials were collected according to appropriate ethical guidelines and all protocols were reviewed and approved by the relevant institutional committees. In all of the cases neuropathological evaluation revealed scarce neurofibrillary tangles restricted to layer II of the entorhinal cortex.

Neurofibrillary tangles were not observed within the cingulate cortex and rare senile plaques or amyloid deposits were found in the hippocampal formation and neocortex. The postmortem delay ranged from 1 to 10 hours. In one case, the brain was perfused shortly after death through the carotid arteries with 4% paraformaldehyde. A series was available from this brain of the medial portion of the hemisphere from the frontal pole through the entire cingulate gyrus. In all other cases, the brains were hemisected at autopsy, and one hemisphere was fixed whole in 4 liters of cold 4% paraformaldehyde overnight. The entire cingulate gyrus was dissected out of the hemispheres and removed intact. The brain samples were placed into fresh 4% paraformaldehyde and postfixed for up to 72 hours longer. The samples of the cingulate gyrus were then dissected either into 1 cm thick coronal blocks (in three cases) or parasagittally (in three cases), separating the medial face containing largely areas 24b and 23b from the rest of the gyrus. The blocks were then cryoprotected by immersion in solutions of increasing concentrations of sucrose, from 12 to 30% in phosphate-buffered saline (PBS). Blocks were frozen on dry ice and cut at 40 μ m on a cryostat or sliding microtome. Two series were taken every 25 sections, with the result that the entire gyrus was sampled once every millimeter. After sectioning, the sections were rinsed thoroughly in PBS, treated in a solution of 0.75% hydrogen peroxide in 75% methanol for twenty minutes to eliminate endogenous peroxidase activity, rinsed again, and then placed into a solution of primary antibody in a diluent containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin. Four consecutive series of sections were incubated with antibodies that recognize, respectively, a nonphosphorylated epitope on the neurofilament protein triplet (SMI-32, Sternberger Monoclonals, Baltimore, MD; 1:5,000; (Sternberger and Sternberger, 1983; Lee et al., 1988)), or the calcium-binding proteins parvalbumin, calretinin (Swant, Bellinzona, Switzerland; 1:3,000; Celio, 1990; Andressen et al., 1993) or calbindin (Swant; 1:1,500; Schwaller et al., 1993). Tissue was incubated for a minimum of 40 hours on a rotating table at 4°C. Following incubation, sections were processed by the avidin-biotin method

using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and intensified in 0.0067% osmium tetroxide. Another series was stained with cresyl violet.

Analyses were performed using a computer-assisted morphometry system consisting of a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage (Zeiss, Oberkochen, Germany), a Zeiss ZVS-47E video camera system (Zeiss, Thornwood, NY), a Macintosh 840 AV microcomputer, and custom designed morphometry software developed in collaboration with the Scripps Research Institute (La Jolla, CA; Young et al., 1995). For the generation of unfolded cortical maps, every fourth section was mapped at low magnification. At the crests and fundi of all gyri and sulci, the border between layers III and V or the middle of layer IV was indicated as a landmark, and the distances between adjacent landmarks were measured. These measurements were plotted to make a flat rendering of the cingulate gyrus so that the cortex buried in the callosal and cingulate sulci was exposed. No attempt was made to flatten vertical sulci. Neurons were plotted using NeuroZoom, and the length of the cortex divided into 1000 μm bins. The mapped neurons in each bin were counted, and the frequency distribution plotted on the flattened map. The parcellation and terminology of the cingulate gyrus used was that of Vogt et al. (1995).

RESULTS

The cytoarchitecture of the human cingulate cortex has been fully described in a study related to the present work (Vogt, 1995; see enclosed reprint). For orientation and for a diagram of the distribution of the cingulate cortical subfields, see Figures 1.1 and 1.2 in the Introduction to this thesis.

Distribution of neurofilament protein-immunoreactive neurons

Immunocytochemical staining with an antibody to a nonphosphorylated epitope on the neurofilament protein yielded a staining pattern with changes in the anterior-to-posterior direction, as well as in the dorsoventral axis. The most obvious change

observed was between the anterior cingulate cortex, where neurofilament protein-immunoreactive neurons were present only in layers V and VI, and posterior cingulate cortex, where neurofilament protein-immunoreactive neurons were found also in layer III (Fig. 3.1). This change happened gradually in the rostrocaudal direction, with neurofilament protein-immunoreactive layer III neurons appearing, sporadically at first, in the dorsalmost portions of the gyrus, and then increasing in density and appearing progressively more ventrally in the gyrus, until layer III neurofilament protein-immunoreactive neurons were visible deep in the callosal sulcus.

In anterior areas 25 and 24, neurofilament protein-immunoreactive neurons were scarcer overall than in cortical areas dorsal to the cingulate sulcus (Fig. 3.2). As shown in Figure 3A, most were confined to layers V and VI, although a few lightly labeled neurons could be seen in layer III, and intensely labeled stellate neurons appeared periodically in layer II. In layer VI, there was a large number of medium-to-light neurofilament protein-immunoreactive neurons whose sizes ranged from small to medium and whose shapes ranged from truly pyramidal to multipolar, horizontal or fusiform. The most prominent population was found in layer Va, where clusters of immunoreactive typical pyramids were found. Layer Vb was sparser, and contained numerous immunoreactive spindle neurons (Nimchinsky et al., 1995b). In the most anterior portions of area 24, this pattern was the same for all the subdivisions of area 24. More caudally, immunoreactive neurons began to appear in layer III, first in the most dorsolateral portions of the gyrus, then progressively more ventrally and medially. The transition zone 24' between areas 24 and 23 was characterized by an increasing frequency of layer III neurofilament protein-immunoreactive pyramidal neurons (Fig. 3.3B). However, the area marked anteriorly by the appearance of the first of these neurons and posteriorly by a densely populated layer III was much larger than that occupied by the transition zone between anterior and posterior cingulate cortex as defined by the Nissl stain (Fig. 3.1). Thus in area 24', subarea 24a' contained very few immunoreactive layer III neurons, subarea 24b'

contained a few, and subarea 24c' contained a considerable number. In area 23, all three subdivisions contained immunoreactive neurons in layer III. These neurons were more prominent in areas 23a and 23c than in 23b. In area 23a, the immunoreactive pyramidal neurons formed clusters and were distributed throughout the thickness of layer III (Fig. 3.4B). In contrast, in areas 23b and 23c, the layer III pyramidal neurons were much more organized, and tended to be located in the deepest portion of layer III (Fig. 3.4C). In the caudal portion of the callosal sulcus, area 29 contained two layers with immunoreactive neurons: layers III and V (Fig. 3.4A). The transition to area 23a was gradual, occurring through area 30, with the progressive appearance of more immunoreactive neurons in layer III and an increase in the number of immunoreactive neurons in layer VI.

Distribution of calcium-binding protein immunoreactivity

Parvalbumin. Immunoreactive neurons. In the anterior-most portions of area 24, parvalbumin immunoreactivity for both neurons and neuropil was much more intense dorsally than ventrally (Fig. 3.5). In most of area 24, parvalbumin-immunoreactive neurons were found mostly in layer V. In anterior area 24a and in area 25, however, virtually none were found, even in layer V (Fig. 3.6A). More caudally in subarea 24a and in subarea 24b, more layer V neurons appeared, and still more were found in area 24c (Fig. 3.6B, 3.6C). These neurons were frequently clustered, giving the neuropil labeling of layer V a patchy, discontinuous quality (Fig. 3.6B, 3.6C). Parvalbumin-immunoreactive layer III neurons began to appear in area 24a, but these were very sparse. More were apparent in area 24c (Fig. 3.6C). In addition, parvalbumin-immunoreactive layer II neurons appeared in area 24c (Fig. 3.6C). In area 24b, there was a zone between the layer III and layer V parvalbumin-immunoreactive neurons that was virtually devoid of immunoreactivity. This zone did not appear in the lateral portion of area 24c, where immunoreactive neuronal somata were found throughout layers II-VI. Vertically oriented parvalbumin-immunoreactive cartridges, possibly representing the terminals of chandelier

neurons on the initial segments of layer V pyramidal cell axons (DeFelipe et al., 1989b; Lewis and Lund, 1990; Williams et al., 1992) were abundant in layers V and VI in the anterior cingulate cortex, in all of its subdivisions (Figs 3.6C and 3.8D), while far fewer were found in layers II and III. In areas 24a' and 33, layer II was characterized by a considerable population of occasionally large immunoreactive somata (Fig. 3.7A). In addition, in areas 33 and 24a', labeled neurons were very scarce in the deep portion of layer VI, whereas in areas 24b' and 24c', clustered immunoreactive neurons were frequently encountered in this layer (Figs 3.7A, 3.7B). These patterns continued into area 23, where, as described above, layer IV contained parvalbumin-immunoreactive neurons (Figs 3.8A and 3.8B).

Neuropil labeling patterns. In area 25, very light neuropil labeling was detectable (Fig. 3.6A). Most of the labeling appeared to be due to the presence of immunoreactive neuronal somata in layer V. Increased neuropil labeling was observed in layers III and V of area 24a (Fig. 3.6B). In areas 24b and 24c, a band of parvalbumin-immunoreactive neuropil appeared in the deep portion of layer III, and it was more intense dorsally, such that in area 24c, it was very intense (Fig. 3.6C). This band coincided with the presence of immunoreactive layer III neurons, but it was also evident ventrally, where immunoreactive neurons were very rare. More caudally within area 24, in area 24', this immunoreactive band appeared progressively more ventrally, until it reached area 24a', and even area 33 in the depth of the callosal sulcus (Fig. 3.7A). In areas 33, the caudal portion of subarea 24a' and 23a, the neuropil was labeled from layer I through layer V (Fig. 3.8A), in contrast to the pattern more rostrally, where the neuropil immunoreactivity was restricted to layers III and Vb (Figs 3.7A and 3.7B). In particular, layer II was characterized by very dense neuropil labeling, from which fibers extended into layer I (Fig. 3.8A). In one case, the pattern of immunoreactivity observed in area 24a' extended onto the gyral surface, stopping at the dimple parallel to the cingulate sulcus, which thus delineated the border between area 24a' and 24b', as described by Vogt (1995). In area

24', the emphasis of the neuropil labeling shifted gradually from dorsal to more ventral regions. These patterns continued into area 23, where the deep portion of layer III and layer IV was occupied by dense parvalbumin-immunoreactive neuropil labeling (Fig. 3.8B). In areas 29 and 30, layer IV contained immunoreactive neurons, but there was no obvious neuropil labeling to demarcate these areas from area 23 (Fig. 3.8C). The regional variations in parvalbumin-immunoreactive neuropil labeling are summarized schematically in Fig. 3.9A. On a flattened map of the cingulate cortex, the patterns of neuropil labeling with parvalbumin can be seen to vary in a manner largely irrespective of sulcal and traditional cytoarchitectonic boundaries (Fig. 3.9A).

Calbindin. Immunoreactive neurons. Calbindin immunoreactivity was obtained in only two cases. In area 25, calbindin-immunoreactive neurons were found mostly in superficial cortical layers (Fig. 3.10A). Intensely immunoreactive nonpyramidal neurons were concentrated in layer II and the superficial portion of layer III. Their somata were generally round-to-ovoid, and were comparable in size to those immunoreactive for calretinin. In layer VI, a separate population of neurons was present, and these tended to be multipolar and somewhat less intensely immunoreactive. Lightly immunoreactive pyramidal neurons were visible in layers II and III (Fig. 3.10A). In area 24, immunoreactive neurons appeared in layer III and, to a lesser extent, layers V and VI (Figs 3.10B and 3.10C). The cellular patterns were comparable among areas 24a, 24b and 24c. However, in ventral area 24a, there were very few immunoreactive neurons in the labeled neuropil of the deep portion of layer III (Fig. 3.10B). A few appeared in area 24b, but area 24c was characterized by a considerable number of nonpyramidal neurons in this sublayer (Fig. 3.10C). Area 33 contained very few immunoreactive nonpyramidal neurons, and virtually no immunoreactive pyramidal neurons. In area 24', immunoreactive pyramidal neurons were somewhat reduced in comparison with area 24 (Figs 3.11A and 3.11B). With the appearance of layer IV, this layer became populated

with immunoreactive neurons (Figs 3.12A, 3.12B and 3.12C). Area 23 contained the fewest calbindin-immunoreactive pyramidal neurons in the cingulate gyrus (Fig. 3.12B).

Neuropil labeling patterns. Area 25 was characterized by the lightest calbindin-immunoreactive neuropil staining in the cingulate gyrus (Fig. 3.10A). In area 24, the neuropil was stained from layer II through the deep portion of layer III. This labeling was incremental, with layer II lightest and the labeling increasing in intensity with the cortical depth (Figs 3.10B and 3.10C). Layers I, V and VI were largely unstained, except for immunoreactive patches in layer V. The neuropil staining was not homogeneous. Intensely labeled beaded fibers were visible in the deep layers, and could often be attributed to immunoreactive nonpyramidal neurons. The intensity of the staining in the neuropil increased from area 24a through area 24c, and what appeared in area 24a as an increasing superficial-to-deep gradient of neuropil immunoreactivity became resolved in area 24c as an immunoreactive band in layer III (Fig. 3.10C). The neuropil in area 33 was also very lightly labeled. In area 24', the patches of immunoreactive fibers in layer V became more pronounced, especially in area 24a' (Fig. 3.11A). In area 24', the neuropil in layers V and VI became labeled, leaving only layer I unstained (Fig. 3.11B). Within area 24', the band of immunoreactive neuropil gradually extended ventrally until it reached the depth of the callosal sulcus, thus labeling areas 33 and 24a'. In area 23a, the band of neuropil occupied layers II through V (Fig. 3.12A), and was thus reminiscent of the pattern observed with parvalbumin (Fig. 3.8A). Area 23c still contained an immunoreactive band only in layers II and III, not throughout its thickness (Fig. 3.12B). In areas 29 and 30, the staining pattern resembled that observed in area 23a. No distinctive neuropil staining was observed in these areas (Fig. 3.12C).

Calretinin In the anterior cingulate cortex, as described elsewhere in the primate neocortex, intensely immunoreactive calretinin-containing neurons were found mostly in layer II and the superficial portion of layer III (Hof et al., 1993; Condé et al., 1994). These were nonpyramidal neurons, whose morphology resembled that of bitufted

neurons. Occasionally, immunoreactive neurons were found that resembled bipolar neurons. Their somata were ovoid-to-fusiform, and their dendrites, frequently beaded, could often be seen coursing radially through the cortex. Calretinin-immunoreactive neurons were occasionally encountered in layer I, and were presumed to represent Cajal-Retzius cells (Vogt Weisenhorn et al., 1994). Scattered immunoreactive neurons could be seen in the deep portions of layer III and layers V-VI. In layer VI, calretinin-immunoreactive neurons assumed numerous morphologies, including multipolar and horizontal forms. In addition, both areas 25 and 24 contained a unique population of immunoreactive pyramidal-like neurons in layer V (Figs. 3.13A and 3.13B). This population was most numerous in area 25, and in anterior and ventral portions of area 24. These neurons were more lightly labeled than the nonpyramidal neurons, and only their somata and most proximal dendritic arborizations were visible (Fig. 13C).

In area 24', the patterns of immunoreactivity were generally the same as for area 24. One difference, however, was the virtual absence of calretinin-immunoreactive layer V pyramidal neurons (Fig. 3.14B). This was the most salient difference between subareas 24c and 24c' (Figs 3.14A and 3.14B). However, in subarea 24c'g, occasional very large calretinin-immunoreactive pyramidal neurons appeared (Fig. 3.13D). This area corresponded with Vogt's (1995) area 24c'g, or the gigantocellular area (Braak, 1976; Braak and Braak, 1976). With the definitive appearance, in area 23, of layer IV, calretinin-immunoreactive neurons were found in layer IV (Fig. 3.14C), but they were not as numerous as those labeled in this layer with parvalbumin. In the posterior portion of the gyrus, a few immunoreactive pyramidal neurons were found in layers V and VI in areas 29 and 30. Area 23 contained essentially no calretinin-immunoreactive pyramidal neurons (Fig. 3.14C). The distribution of calretinin-immunoreactive pyramidal neurons is represented in Fig. 3.9B. The greatest density, expressed as the number of neurons observed per 1000 μm cortical distance, was observed in the most anterior and ventral portions of the cingulate cortex, corresponding approximately to areas 24a and 24b.

Scattered calretinin-immunoreactive neurons were also found through much of the anterior cingulate cortex up to the border with area 23, but their distributions were too sparse to appear on the figure, since bins containing fewer than 10 neurons were discounted. Area 25 also contained large numbers of these neurons, but was omitted from this figure.

Layer V of area 24 contained a meshwork of immunoreactive neuropil which lent a multilaminar appearance to the cortex. This meshwork was very dense, and appeared to be associated with the presence of calretinin-immunoreactive pyramidal neurons. With the disappearance of these neurons, the immunoreactive band of neuropil in layer V became less intensely labeled (Figs 3.14A and 3.14B). In areas 29 and 30, there was an intensification of the neuropil labeling in layer IV (Fig. 3.14D). In cross-section, the immunoreactive band in layer V was patchy, and could be seen to shift from a more lateral position rostrally to a more medial position caudally.

DISCUSSION

Comparison with the Nissl stain

The present study demonstrates that the human cingulate cortex is, indeed, a region of considerable neurochemical heterogeneity. In contrast with the areal borders described in Nissl-stained materials, the boundaries defined by different distributions of neurofilament or calcium-binding proteins do not, as a rule, obey the currently accepted parcellation of the cingulate cortex into areas 24a through 24c and 23a through 23c. In fact, each staining pattern indicates a trend or trends generally independent of the Nissl-based boundaries and, often, of other staining patterns. The only consistent cytoarchitectonic difference that also holds true for all the neurochemical markers described here is the division between the anterior and posterior cingulate cortex. In no case was the labeling in the most anterior portions of the gyrus comparable to that in the most posterior portions. However, the borders between these areas are seldom as sharp as

those seen with the Nissl stain. This is especially true of the neuropil labeling patterns, which vary in the dorsoventral axis completely irrespective of cytoarchitectonic boundaries as defined by the Nissl stain, and in the anteroposterior axis show gradients rather than block-like areal patterns. Even cellular markers, however, have distributions that taper along the gyrus, rather than showing abrupt changes. In this respect, as in others, the human cingulate cortex is characterized by neurochemical differences that reflect those found in the macaque monkey (Hof and Nimchinsky, 1992). There are certain cellular features, however, that distinguish the human from the nonhuman primate.

Neurofilament protein-immunoreactive neuron distribution varies along the human and macaque monkey cingulate cortex

The changes described along the anteroposterior axis in the expression of neurofilament protein are globally comparable in the two species, in that anteriorly, neurofilament protein-containing neurons are found largely in deep layers and posteriorly, they are found also in layer III. As in the monkey, the transition from the pattern found most anteriorly to that which characterizes the posterior cingulate cortex occupies a significant portion of the gyrus in the rostrocaudal axis (Hof and Nimchinsky, 1992). However, in the human, layer III neurofilament protein-immunoreactive neurons appear relatively more rostrally in the dorsal portions of the gyrus, so that area 24b contains these neurons, whereas in the monkey, the comparable area does not. Other differences include the presence, in the human, of immunoreactive spindle neurons in layer Vb of the anterior cingulate areas 24 and 24' (Nimchinsky et al., 1995b), and a distinct population of multipolar neurons in layer II. In addition, in the human, the population of neurofilament protein-containing neurons is much denser than in the monkey. However, the overall staining pattern in the cingulate cortex is remarkably consistent in the two species.

Calcium-binding proteins label characteristic cellular features of the macaque monkey and human cingulate cortex

Parvalbumin-immunoreactive neurons manifest a pattern of distribution comparable to that observed in the nonhuman primate (Hof and Nimchinsky, 1992). Specifically, parvalbumin-immunoreactive neurons in the human cingulate cortex, as in the monkey, correlate with the presence of neurofilament protein-containing neurons on a regional basis. On the laminar level, however, this is not strictly true, since layer II frequently contains substantial numbers of parvalbumin-immunoreactive neurons, whereas this layer is generally devoid of neurofilament protein-containing neurons. One unusual finding is the presence in layers V and VI of large numbers of parvalbumin-immunoreactive structures that may represent the cartridges of chandelier neurons. These structures represent the terminations of chandelier neurons along the axon initial segment of pyramidal neurons, and as such, signify a powerful inhibitory influence on pyramidal neuron firing. They have been described elsewhere in the monkey and human cerebral cortex, but they are usually located preferentially in superficial layers (DeFelipe et al., 1989b; Lewis and Lund, 1990; Williams et al., 1992). An exception was a study in the human temporal neocortex that found an higher density of parvalbumin-immunoreactive cartridges in layers IV through VI than in layers II and III. In that study, which demonstrated a close association between parvalbumin-immunoreactive chandelier neurons and neurofilament protein-immunoreactive pyramidal neurons, it was suggested that the predominance of cartridges in superficial layers represented an association of this form of inhibitory control with corticocortically projecting neurons (Del Río and DeFelipe, 1994). The findings reported in the present study are in accord with this concept, since in the anterior portions of the primate cingulate gyrus, corticocortically projecting neurons take origin preferentially in deep cortical layers (Barbas, 1986; Nimchinsky et al., 1993). Thus the presence of neurofilament protein-containing pyramidal neurons and of parvalbumin-immunoreactive chandelier neurons and their

cartridges in deep layers in the anterior portions of the cingulate gyrus support this location as the preferred site of origin of corticocortical projections in the human as well.

Calbindin-immunoreactive nonpyramidal neurons in both species have a relatively consistent pattern of distribution throughout the cingulate gyrus. Calbindin-immunoreactive pyramidal neurons, in contrast, are much more frequent in anterior than in posterior portions of the cingulate gyrus. This observation is similar to that described in the monkey cingulate cortex (Hof and Nimchinsky, 1992). Comparable inhomogeneities in the distribution of these neurons have been reported in the visual areas of the temporal lobe (Kondo et al., 1994). Both the temporal lobe and the cingulate gyrus are characterized by progressive cytoarchitectonic changes, with the anterior portions of each described as "dysgranular" for, among other characteristics, lacking a well-defined layer IV, and their posterior portions "isocortical," containing six well-defined cortical layers (Galaburda and Pandya, 1983; Pandya and Yeterian, 1985). The cortical areas in between can be described as transitional stages between the two. In both the cingulate gyrus and the temporal lobe, the more dysgranular the area, the more likely it was to contain calbindin-immunoreactive pyramidal neurons in layers II and III. In addition, the most extremely dysgranular regions (or even agranular, as in the case of the cingulate gyrus), are characterized by a considerable reduction in the number of parvalbumin-immunoreactive neurons (Kondo et al., 1994). These two populations of neurons, of course, represent functionally distinct classes, and the significance of the expression or lack of expression of a calcium-binding protein is still unclear. One possibility is suggested by the observation that the rostral portions of the temporal lobe and of the cingulate cortex represent two of the few cortical regions known to project directly to the amygdala. In both cases, the neurons furnishing this projection are found mostly, if not exclusively, in layer III (Aggleton et al., 1980). The calbindin-containing pyramidal neurons are thus positioned to participate in this projection. However, the more common role of layer III neocortical neurons, the furnishing of corticocortical

projections, remains a distinct possibility (Jones, 1984). Nonetheless, such recurring gradients in different regions of the brain suggest that cytoarchitectonic variations may represent more than mere alternate shuffling of the same cell types found throughout the cortex.

Calretinin-immunoreactive nonpyramidal neurons populate superficial layers preferentially, as described both for the cingulate cortex in the monkey and other cortical areas in the monkey and human (Hof et al., 1993; Hof et al., 1993; Condé et al., 1994). This pattern varies little through the numerous different cingulate cortical areas examined in the present study. However, the presence and distribution of calretinin-containing pyramidal-like neurons set the cingulate cortex apart from other cortical areas. While it was not possible in the present study to unambiguously identify these neurons as pyramidal neurons, as a result of their relatively light labeling that was usually restricted to the soma, a number of features suggest that they may, indeed, be pyramidal neurons. First, their overall morphology is unarguably pyramidal, in layers that are dominated by this cell type. Second, their labeling is qualitatively different from that seen in the nonpyramidal neurons, which are labeled very intensely, throughout their cytoplasm, suggesting that they may belong to a completely different cell class. This is in contrast with the pyramidal-like neurons described by Condé et al. (1994) in the macaque monkey prefrontal cortex, which were well enough labeled to follow their dendrites for considerable distances. Finally, calcium-binding protein-containing pyramidal neurons are not without precedent, as calretinin is found in a subgroup of Betz cells in the human (Nimchinsky et al., 1992) and calbindin-containing pyramidal neurons populate layers II and III in many cortical areas in both the monkey and human (Hof and Morrison, 1991; Ferrer et al., 1992; Hof and Nimchinsky, 1992; Kondo et al., 1994; present study). However, until retrograde labeling or Golgi staining in the human is combined with immunocytochemistry, this question may remain unanswered, since comparable neurons are not observed in the nonhuman primate. Regardless of the cell type to which these

neurons belong, their restriction to the cingulate gyrus in general, and to only areas 32, 25, 24, 24' and 29 in particular suggests a unique role. Their unusual nature is underscored by the fact that they are not seen in the human orbitofrontal cortex, a region that bears numerous cyto- and chemoarchitectonic similarities to the cingulate cortex (Hof et al., 1995). Calretinin-immunoreactive pyramidal neurons have been described in the newborn rat, but they disappear early in postnatal life (Vogt Weisenhorn et al., 1994). It is possible that in the human cingulate cortex, these particular neurons continue to express this protein throughout life. Whether this cellular anomaly serves a particular function or is merely a phylogenetic curiosity, these neurons appear to be a characteristic feature of the cingulate cortex, and are useful in the parcellation of these areas. The localization of these neurons almost exclusively in layer V does not help to define their connectivity, since this layer in the nonhuman primate gives rise not only to striatal and spinal cord projections, but also to corticocortical projections, as mentioned above (Barbas, 1986; Hutchins et al., 1988; Dum and Strick, 1991; Dum and Strick, 1992; Kunishio and Haber, 1994; He et al., 1995). It is notable, however, that the distribution of this unique cell type overlaps extensively, at the species level, and on a regional and laminar basis, with that of the spindle neurons that characterize these areas in the human. Unusual cell types, then, appear with much greater than average frequency in these parts of the human neocortex, and may suggest a particular local propensity for cortical evolution at the cellular level.

Calcium-binding protein immunoreactivity defines distinctive neuropil staining patterns in the monkey and human cingulate cortex

With parvalbumin immunoreactivity, three separate patterns were observed. One consisted of the sheet-like labeling of the neuropil of layer III which began rostrally in the dorsolateral portions of the cingulate gyrus, and which gradually extended caudally, medially and ventrally, eventually to label layer III throughout the gyrus. Another consisted of the discrete labeling of the neuropil of areas 24a' and 23a, which marked

these areas in layers II through V. The third was the labeling of layer IV, wherever present. The combination of these three patterns simultaneously labeling the cingulate cortex gives rise to a complex staining pattern, which in many respects resembled that of the monkey (Hof and Nimchinsky, 1992). Unlike the case in the monkey, calbindin immunoreactivity gave rise to staining patterns that resembled those found with parvalbumin, especially the first two patterns described above. Calretinin, however, with the exception of the immunoreactive band in areas 29 and 30, exhibited only minor changes in neuropil labeling along the cingulate gyrus. The sources of these labeling patterns are unclear. Some is clearly due to the immunoreactive nonpyramidal neurons that populate these areas. This is most likely the explanation for the patches in layer V of areas 24 and 24', which correlate well with layer Vb pyramidal and spindle neurons in these areas (Vogt et al., 1995). The concentration of immunoreactive terminals in the immediate vicinity of a certain cell population indicates a differential distribution of certain inhibitory control. For instance, the somata and proximal dendrites of layer Vb neurons appear to be surrounded by the presumably inhibitory terminals of parvalbumin- and calbindin-immunoreactive basket and possibly double bouquet cells. Layer Va neurons in the anterior cingulate cortex, in contrast, appear to be removed from such structures. It is possible that the neurons in this sublayer, which are notable for being particularly dense in the cingulate cortex, are subject to the inhibitory control of a discrete population of neurons which do not appear with any of the antibodies used in the present study, or are modulated not by terminals surrounding their somata, but by influences more distally located on the dendritic arbor.

The dense and very intense labeling in the deep portion of layer III and of layer IV seen with antibodies to parvalbumin and calbindin, however, may be due to labeled thalamocortical terminals. The three calcium-binding proteins examined in the present study are known to be present in various thalamic projection neurons in the rodent and nonhuman primate (Stichel et al., 1987; Celio, 1990; DeFelipe and Jones, 1991;

Hashikawa et al., 1991; Jacobowitz and Winsky, 1991; Rausell et al., 1992a; Rausell et al., 1992b; Résibois and Rogers, 1992; Diamond et al., 1993; Arai et al., 1994). Since these are soluble proteins, they are distributed throughout the cytosol, and are thus present in the terminal fields of the thalamocortical neurons that contain them. This in turn gives rise to distinctive cortical labeling patterns (DeFelipe and Jones, 1991; Del Río and DeFelipe, 1994). Thus, the parvalbumin-immunoreactive band in the deep portion of layer III may derive from a projection from the mediodorsal nucleus, which projects to this layer and area in the macaque monkey (Giguère and Goldman-Rakic, 1988) and which contains numerous parvalbumin-immunoreactive neurons in the human thalamus (E. Nimchinsky, unpublished observations). The other major site of parvalbumin neuropil immunoreactivity, including all of subarea 23a and a considerable extent of subarea 23b, appears to coincide well with the regions in the monkey that receive projections from the medial pulvinar nucleus (Baleydier and Mauguière, 1985; Baleydier and Mauguière, 1987), which is also characterized in the human by parvalbumin immunoreactivity (E.A. Nimchinsky, unpublished observations). Similarly, the calretinin-immunoreactive band in areas 29 and 30, previously described in the macaque monkey (Hof and Nimchinsky, 1992) may derive from a thalamocortical projection from the laterodorsal nucleus, which projects to this layer in the macaque monkey and which, in the human, contains calretinin-immunoreactive neurons (Bentivoglio et al., 1993; Vogt et al., 1993). Of course, other structures project upon the cingulate cortex, such as the amygdala and striatum, but these projections do not have the particular regional and laminar characteristics expressed by the neuropil staining patterns described here (Porrino et al., 1981).

Functional implications

The cingulate motor areas. A recent study in the macaque monkey combining retrograde transport and immunocytochemistry demonstrated the utility of neurofilament protein as a marker for the cingulate motor areas (Nimchinsky et al., submitted). In

particular, the appearance of immunoreactive neurons in layer III and a broad layer VI indicated the presence of the rostral cingulate motor area, CMAr, the increased density of these neurons signalled the presence of the caudal cingulate motor area, CMAc, and the subsequent lower density and thinning of layer VI, in addition to the presence of layer IV on the Nissl stain, indicated area 23, which was located caudally to the motor areas. In the monkey, these changes are relatively obvious, since the density of these neurons is significantly lower than in the human. Based on overall patterns, and by analogy to the nonhuman primate, it may be possible to approximate the locations of the human cingulate motor areas, with the CMAr located where the neurofilament protein immunoreactive neurons first appear, and the CMAc located caudal to this area, and rostral to area 23. This represents a fairly large area of cortex, occupying roughly the middle third of the gyrus, but one that is proportional to the size of these areas in the macaque, and which agrees with the functional localization of these areas in the human (Grafton et al., 1993; Paus et al., 1993). Another potential clue is the calretinin-immunoreactive pyramidal neurons in layer V in the gigantopyramidal fields described by Braak (1976; Braak and Braak, 1976). The significance of calretinin immunoreactivity is that outside of the cingulate cortex, the only area in the human neocortex that has been described as containing calretinin-immunoreactive pyramidal neurons is the primary motor cortex, where a subpopulation of Betz cells contains calretinin (Nimchinsky et al., 1992). While not confirming a motor role, this represents another similarity between these neurons and the giant cells of Betz in the primary motor cortex. It is possible, then, that like the giant layer V cells of the primary motor cortex, the gigantopyramidal neurons in the cingulate cortex may represent a portion of the motor representation, such as the digital representation. It has been proposed that this area coincides, at least in part, to the cingulate motor areas described in the nonhuman primate (Dum and Strick, 1991; Dum and Strick, 1992; Dum and Strick, 1993). The present study lends further support to this hypothesis.

Other functions of the cingulate gyrus. In addition to somatomotor function, numerous functions have been ascribed, completely or in part, to the cingulate gyrus in the human. These include autonomic (Pool and Ransohoff, 1949; Pool, 1954) and oculomotor (Talairach et al., 1973; Petit et al., 1993) functions, sensory functions, including somatosensory (Richer et al., 1993) and pain processing (Foltz and White, 1962; Talbot et al., 1991; Rosen et al., 1994), the mediation of certain forms of attention (Bench et al., 1993), the selection of movement (Petersen et al., 1988) and the manifestation of emotion (Hausser-Hauw and Bancaud, 1987; Arroyo et al., 1993). The present study suggests some possible anatomic correlates for some of these functions. For instance, one feature that appears fairly specific to the cingulate cortex is calretinin-immunoreactive pyramidal neurons in layer V. These are found almost exclusively in the anterior portions of the cingulate gyrus, and are most numerous in areas 25 and 24. The region thus delineated corresponds closely to the areas whose stimulation gives rise to autonomic effects and vocalization (Smith, 1945; Pool and Ransohoff, 1949; Kaada, 1951; Pool, 1954; Showers and Crosby, 1958; Dua and MacLean, 1964; Robinson and Mishkin, 1968; Jürgens and Ploog, 1970; Jürgens, 1983). Studies that have analyzed other systems, such as selection of movement, spatial memory and selective attention have used functional imaging techniques, whose resolution limited the precise localization of function (Petersen et al., 1988; Pardo et al., 1990; Corbetta et al., 1991; Deiber et al., 1991; Bench et al., 1993; Hsieh et al., 1994; Lang et al., 1994; Larrue et al., 1994; Raichle et al., 1994). However, the consensus appears to be that these roles are subserved by cortical regions in the anterior half of the cingulate gyrus. This region is characterized by lower numbers of parvalbumin- and neurofilament protein-immunoreactive neurons, and greater numbers of calbindin-immunoreactive pyramidal neurons. While the meaning of the presence or absence of these proteins is as yet unclear, it is worth noting that hippocampal CA1 pyramidal neurons also exhibit low levels of nonphosphorylated neurofilament protein and express calbindin (Seress et al., 1991;

Seress et al., 1992; Vickers et al., 1994). It is possible that structures such as layers II and III of the anterior cingulate cortex and the hippocampus that are involved in learning and memory (Gabriel, 1993; Perani et al., 1993) may also share these neurochemical characteristics.

It is of note that none of the cytoarchitectonic regions described in the present study is delineable by sharp chemoarchitectonic borders, a fact that must be borne in mind when attempting to assign a cortical area to a region of functional activation. The anatomic boundaries in the cingulate gyrus may best be described as areas of transition from one staining pattern to another. The neuropil staining patterns, in particular, vary largely independently of cytoarchitecture. It would not be surprising, therefore, to find that the same holds true for the functional divisions of the human cingulate cortex. Further studies using markers with connectional or physiological significance may thus prove more practical than cytoarchitecture alone for the functional parcellation of this complex cortical region.

FIGURE LEGENDS

Fig. 3.1. Distribution of nonphosphorylated neurofilament protein-containing neurons in a horizontal section through the human cingulate gyrus. This section occupies the middle portion of the gyrus, and includes the border between areas 24 and 23 (arrow). Note that at this level on the medial wall of the hemisphere, neurofilament protein-containing neurons appear in layer III rostrally to the border between areas 24 and 23. Scale bar = 1 mm.

Fig. 3.2. Distribution of nonphosphorylated neurofilament protein-containing neurons in a coronal section through the human anterior cingulate cortex. Note the near absence of neurofilament protein-containing neurons in layer III in the ventral portion of the gyrus, and the increase dorsally (arrowhead). CS = cingulate sulcus. icd = intracingulate dimple. Scale bar = 1 mm.

Fig. 3.3. Distribution of nonphosphorylated neurofilament protein-containing neurons in areas 24a (A) and 24c' (B) of the human cingulate cortex. Area 24a is marked by the virtual absence of neurofilament protein-immunoreactive neurons in superficial layers, and a compact layer Va densely populated with immunoreactive neurons and their proximal dendrites. Layer Vb is markedly sparser, and contains numerous immunoreactive spindle neurons. Area 24c', in contrast, contains a substantial population of immunoreactive neurons in layer III, and has a sparser layer Va. CS, cingulate sulcus. icd, intracingulate dimple. Scale bar = 150 μ m.

Fig. 3.4. Distribution of neurofilament protein-containing neurons in areas 29 (A), 23a (B) and 23b (C) of the human cingulate cortex. In area 29, the major layers are clearly discernable, III and Va. Layer III contains scattered large neurons, which frequently appear also in layer IV. Layers Vb and VI are neuron-sparse. The star indicates the corpus callosum. Areas 23a and 23b both contain a well-defined layer IV on Nissl-stained materials, which is represented here as an immunoreactive soma-sparse zone between layers III and V. In area 23a, the large pyramidal neurons in layer III are scattered throughout the thickness of the layer, whereas in area 23b, the layer III neurons are more restricted to the deepest portion of the layer. Note the immunoreactive apical dendrites of layer V neurons that can be seen ascending through layer IV in area 23b. Scale bar = 150 μ m.

Fig. 3.5. Distribution of parvalbumin immunoreactivity in a coronal section through the human anterior cingulate cortex. Note the intensity of immunoreactivity in dorsal portions of the gyrus, and the extreme reduction of immunoreactivity ventrally. Patches of immunoreactive neuropil in layer Vb are evident even at this low magnification. Scale bar = 1 mm.

Fig. 3.6. Distribution of parvalbumin immunoreactivity in areas 25 (A), 24a (B) and 24c (C) in the human cingulate cortex. Area 25 contains the fewest immunoreactive neurons and the lightest neuropil staining in the cingulate cortex. Most immunoreactive neurons are found in layers V and VI, and most of the immunoreactive fibers are localized to layer V. Area 24a contains immunoreactive neurons from layers II through VI, but most are concentrated in layers III and Vb. Layer III is characterized by a band of neuropil staining in its deepest portion, and layer Vb contains patches of immunoreactive labeling. Layer Va is relatively lightly stained. Immunoreactive presumptive cartridges of chandelier neurons are most abundant in layer VI (curved arrows), and are shown at higher magnification in figure 3.8D. Area 24c contains a very intensely immunoreactive band of neuropil labeling that extends throughout the deeper half of layer III. Immunoreactive clusters in layer Vb are also evident in this area, and cartridges are reduced in number. Scale bar = 150 μ m.

Fig. 3.7. Distribution of parvalbumin immunoreactivity in areas 24a' (A) and 24c' (B) in the human cingulate cortex. Note the increase in immunoreactive neuropil labeling in layer III of area 24a' compared with that found in area 24a (Fig. 3B). Area 24c' has a well-defined layer Va showing a lower labeling intensity than area 24c (Fig. 3.3C), and small patches of immunoreactive labeling in layer Vb. Scale bar = 150 μ m.

Fig. 3.8. Distribution of parvalbumin immunoreactivity in areas 23a (A), 23b (B), and 29 (C) in the human cingulate cortex. Area 23a, and parts of area 23b more caudally, area characterized by moderate-to-intense, fairly homogeneous neuropil labeling in layers II through VI. No distinct band is visible (A). In contrast, much of area 23b, and all of area 23c contains an intensification in neuropil labeling in the deep portion of layer III and in layer IV (B). Area 29 (C) resembles area 23a, but is significantly thinner. Parvalbumin-immunoreactive presumptive cartridges of chandelier neurons in layer VI of area 24a' are shown in (D). These structures were most abundant in layer VI (Fig. 3B), and varied in length from less than 20 μm to over 50 μm . Scale bar = 150 μm (A-C); 25 μm (D).

Fig. 3.9. Flattened representation of the cingulate cortex in a human brain, showing (A) the changes in parvalbumin-immunoreactive neuropil labeling observed along the gyrus, and (B) the distribution of calretinin-immunoreactive pyramidal neurons in layer V. The gigantopyramidal field is outlined with dotted lines, and a vertical broken line indicates the border between areas 24 and 23 as determined using the Nissl stain. In (A), the gray scale indicates relative intensity of staining, with darker shades of gray indicating more intense immunoreactivity, and is not quantitative. Note the presence of two gradients: First, one of increasing intensity of immunoreactivity in layer III of the dorsal and posterior direction anteriorly in the gyrus; second, a pattern indicated by the dark outline on the right of the figure. This represents the labeling of cortical layers II through VI observed in areas 23a and parts of 23b (Fig 8A). In (B), note that calretinin-immunoreactive pyramidal neurons are found largely in the most anterior and ventral portions of the cingulate gyrus. Elsewhere, they are scattered, mostly in the ventral bank of the cingulate sulcus. They are also found in densities comparable to area 24 in area 25, which is not included in this representation.

Fig. 3.10. Distribution of calbindin immunoreactivity in areas 25 (A), 24a (B) and 24c (C) of the human cingulate gyrus. In area 25, calbindin immunoreactivity is mostly neuronal, and largely consists of immunoreactive nonpyramidal and pyramidal neurons in layer II and the superficial portion of layer III (A). Area 24a is characterized by the addition of a band of immunoreactive neuropil in the deep portion of layer III, which is more compact and intense dorsally in area 24c. These neuropil labeling patterns are similar to those observed with parvalbumin (Figs 6B and 6C). Scale bar = 150 μ m.

Fig. 3.11. Distribution of calbindin immunoreactivity in areas 24a' (A) and 24c' (B) of the human cingulate gyrus. Note the marked increase in neuropil labeling in layer III in the dorsal portion of the gyrus (B) compared to the ventral area (A). Some patchy neuropil labeling, similar to that observed with parvalbumin (Figs 6B, 6c, 7A, 7B) is observed in layer Vb in area 24a', but is not evident in area 24c'. The arrow indicates an immunoreactive neurogliaform neuron in layer VI. Scale bar = 150 μ m.

Fig. 3.12. Distribution of calbindin immunoreactivity in areas 23a (A), 23c (B) and 29 (C) of the human cingulate gyrus. Area 23a is characterized by neuropil labeling throughout layers II through VI, but mostly in layers II through V. In contrast, in area 23c, neuropil labeling is most intense in layer IV, and much weaker elsewhere. In both these areas, immunoreactive neurons are found in layers II through V, and to a lesser extent in layer VI. In area 29, a slightly increased density of immunoreactive neurons is observed in layer IV, but, in contrast with the pattern in the macaque monkey, no increased immunoreactive neuropil labeling characterizes area 29 or 30. Scale bar = 150 μ m.

Fig. 3.13. Distribution of calretinin immunoreactivity in areas 25 (A) and 24a (B) of the human cingulate gyrus. Immunoreactive nonpyramidal neurons are most abundant in both areas in layer II and the superficial portion of layer III. Most resemble double bouquet neurons and have dendritic arbors oriented radially in the cortex. These areas also contain the highest density of calretinin-immunoreactive pyramidal neurons, which are found largely in layer V. Examples of these neurons are shown in (C). A very large calretinin-immunoreactive neuron in area 24c'g, presumably one of the gigantopyramidal neurons that characterize this area is shown in (D). Scale bar = 150 μm (A, B), and 40 μm (C, D).

Fig. 3.14. Distribution of calretinin immunoreactivity in areas 24c (A), 24c' (B), 23b (C) and 29 (D) of the human cingulate gyrus. In all these areas, calretinin-immunoreactive pyramidal neurons are found mostly in layer II and the superficial portion of layer III. Note the presence of calretinin-immunoreactive pyramidal neurons in layer V of area 24c (A) and their absence in areas 24c' and 23b. Area 29 contains somewhat fewer immunoreactive nonpyramidal neurons than the other areas, and is distinguished by a band of immunoreactivity in layer IV. Scale bar = 150 μm .

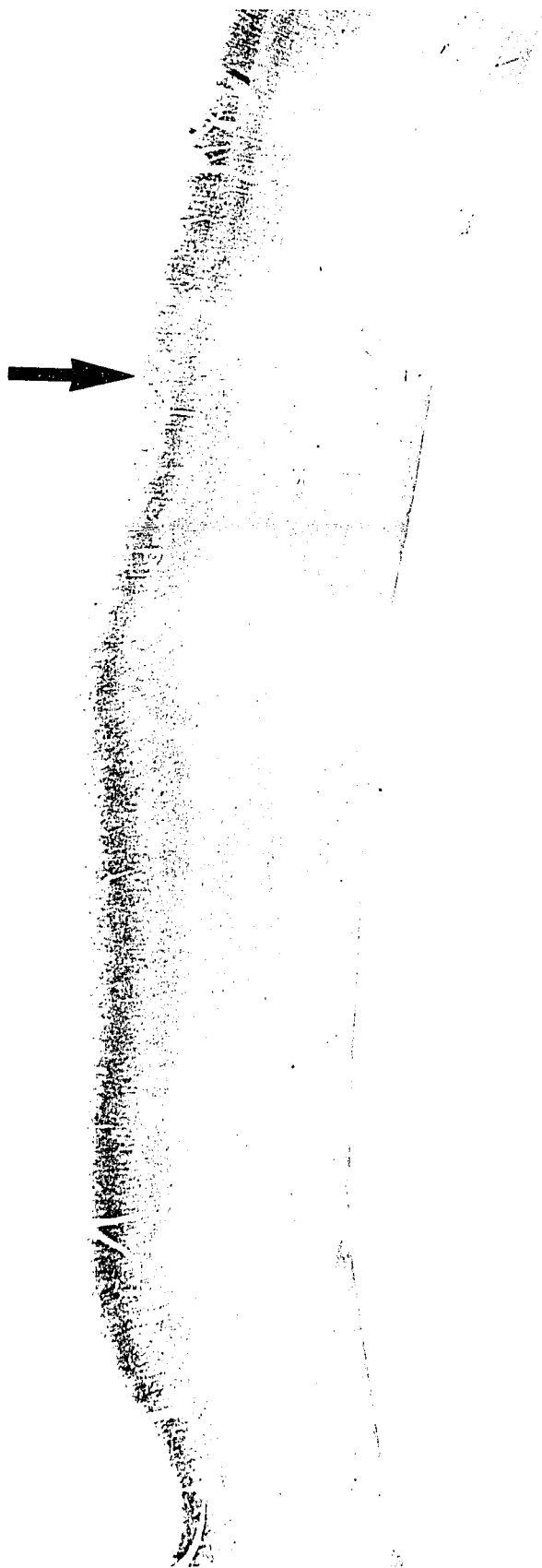


Fig. 3.1

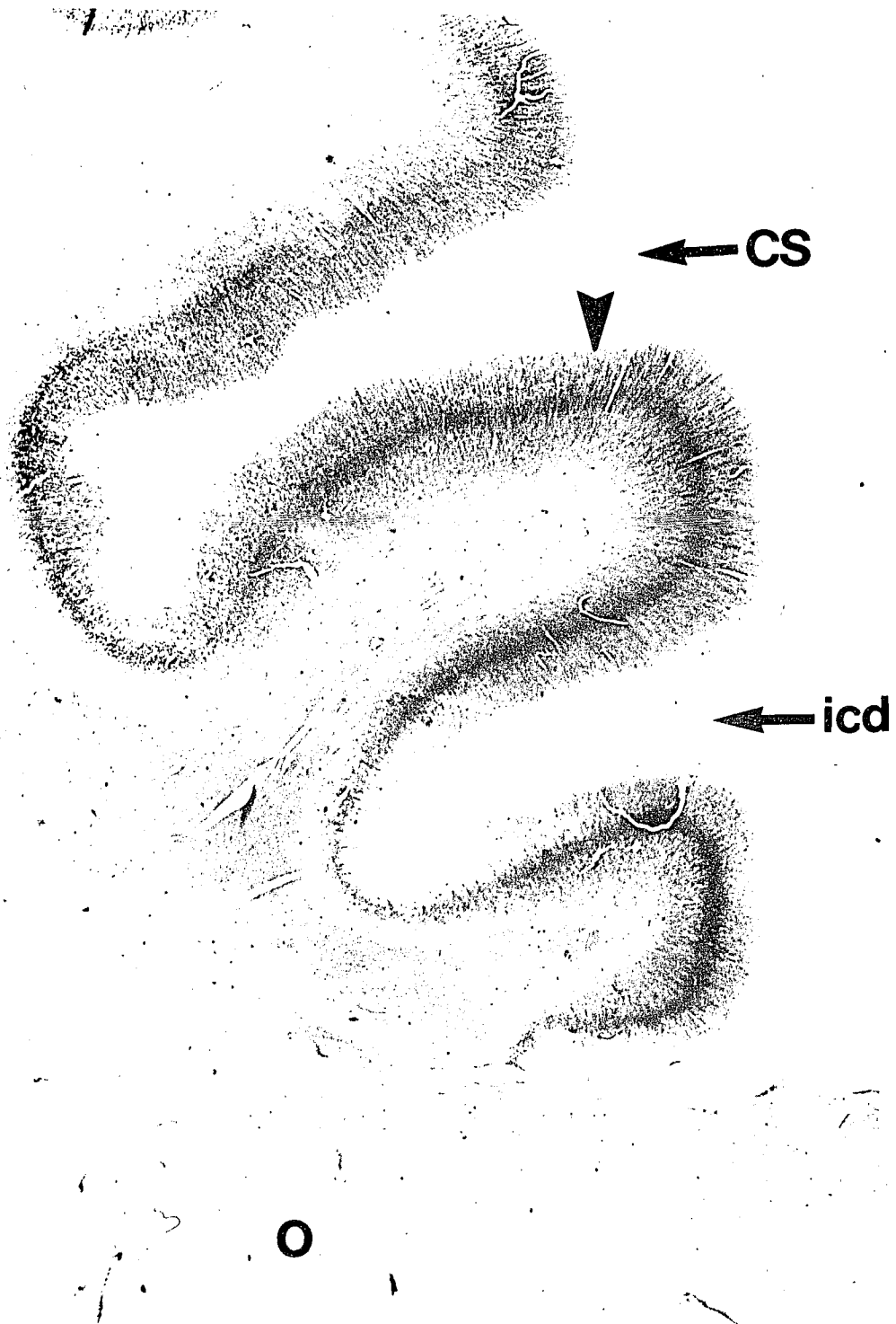


Fig. 3.2



Fig. 3.3

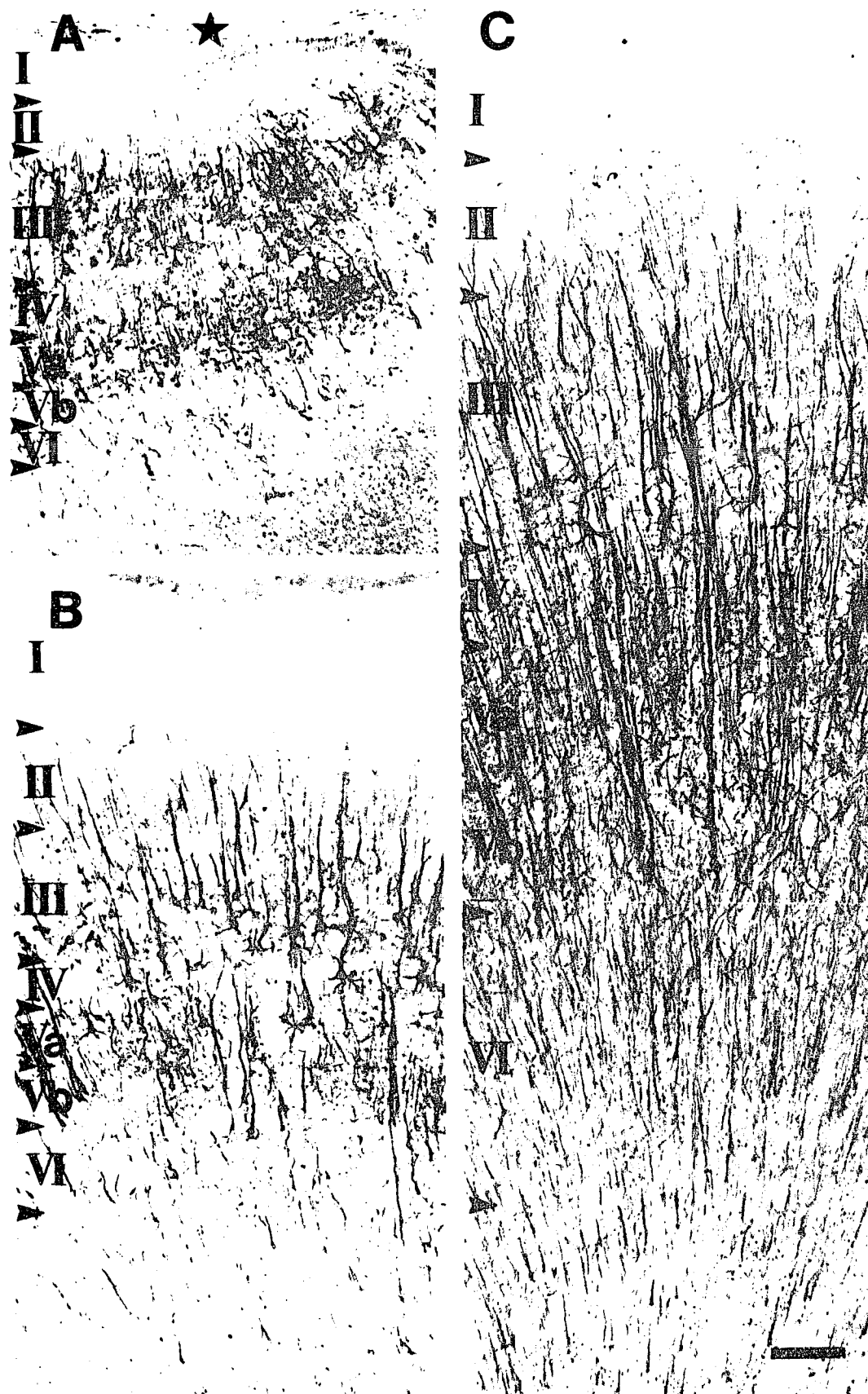


Fig. 3.4

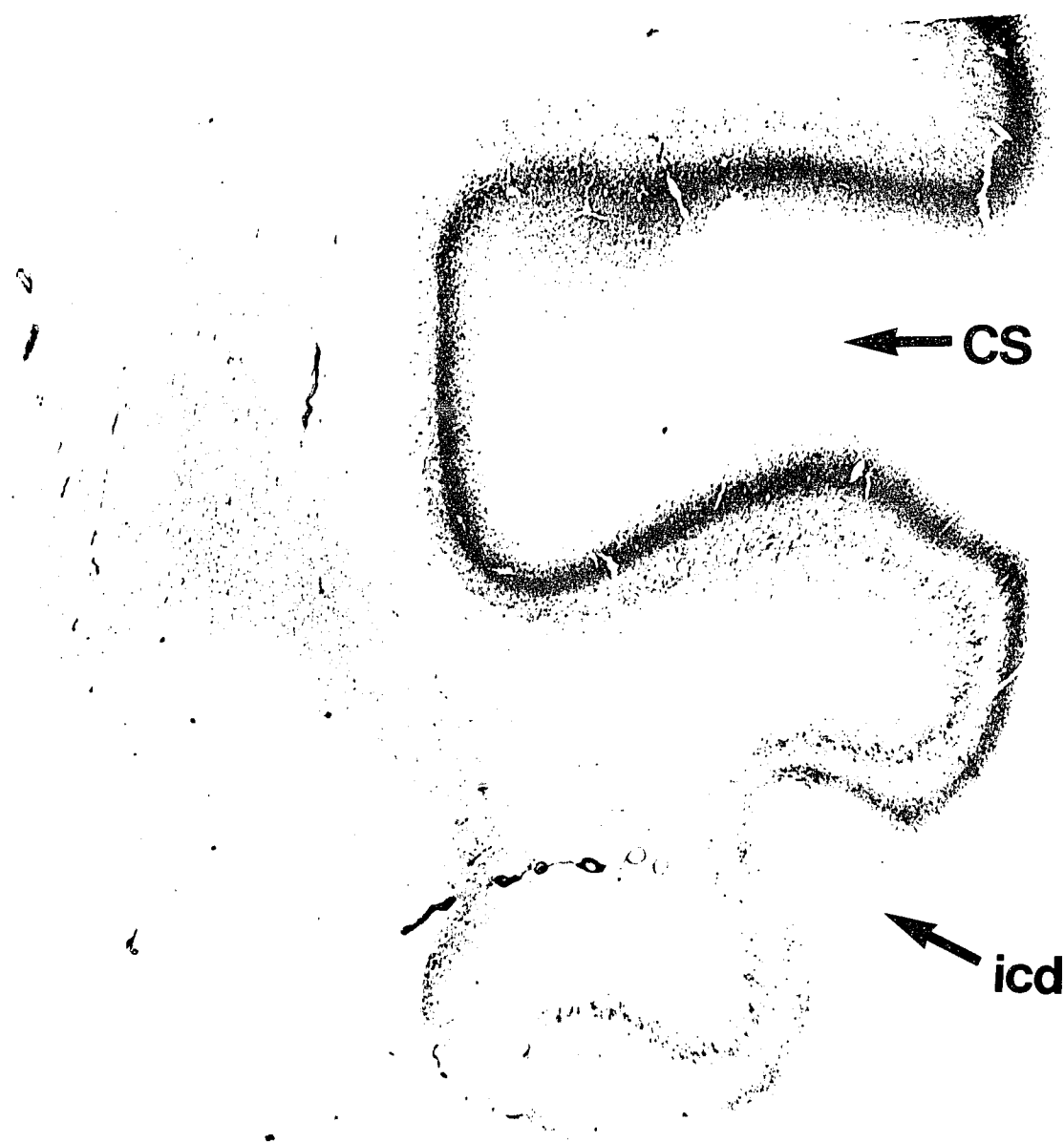


Fig. 3.5

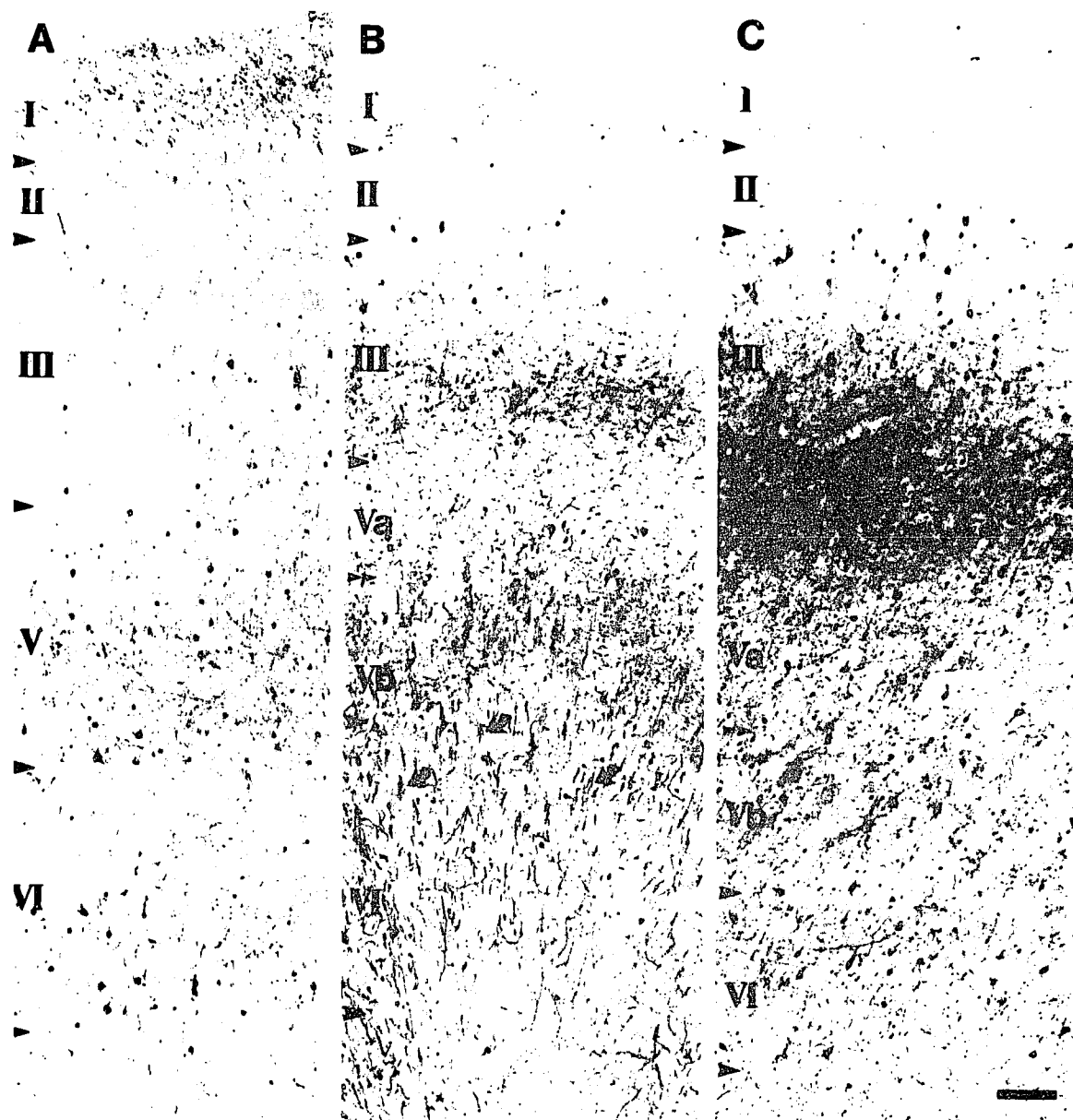


Fig. 3.6

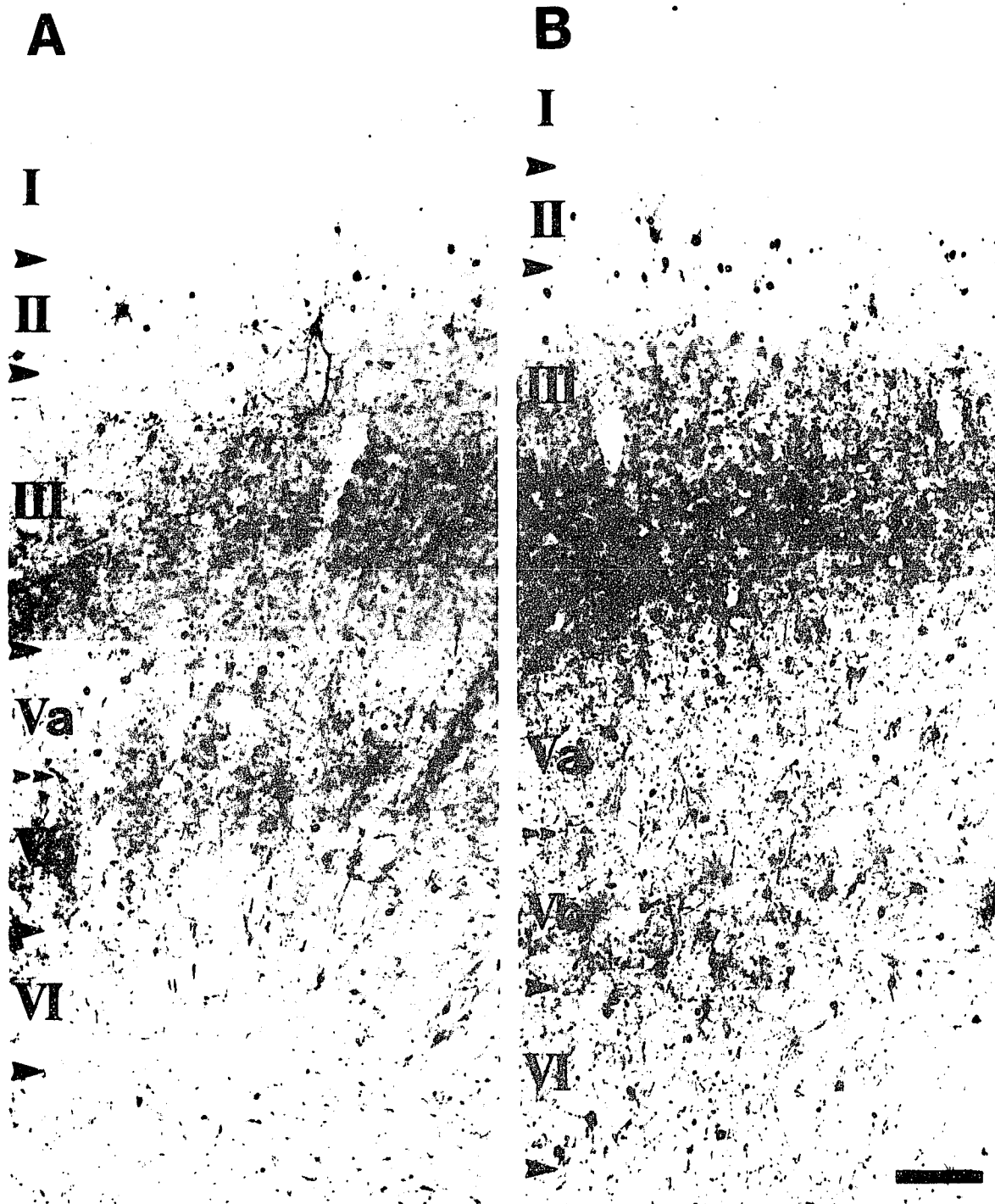


Fig. 3.7

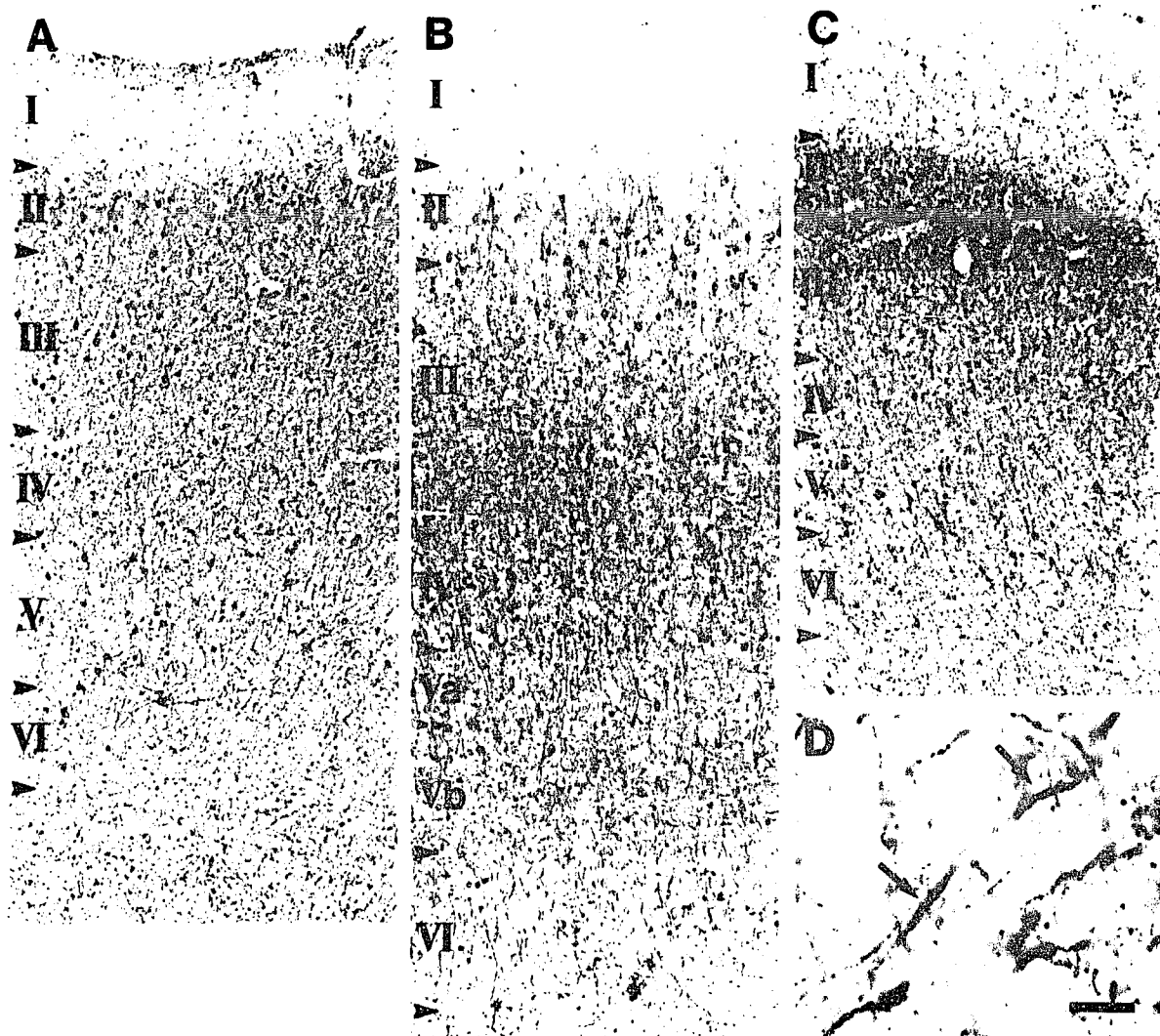


Fig. 3.8

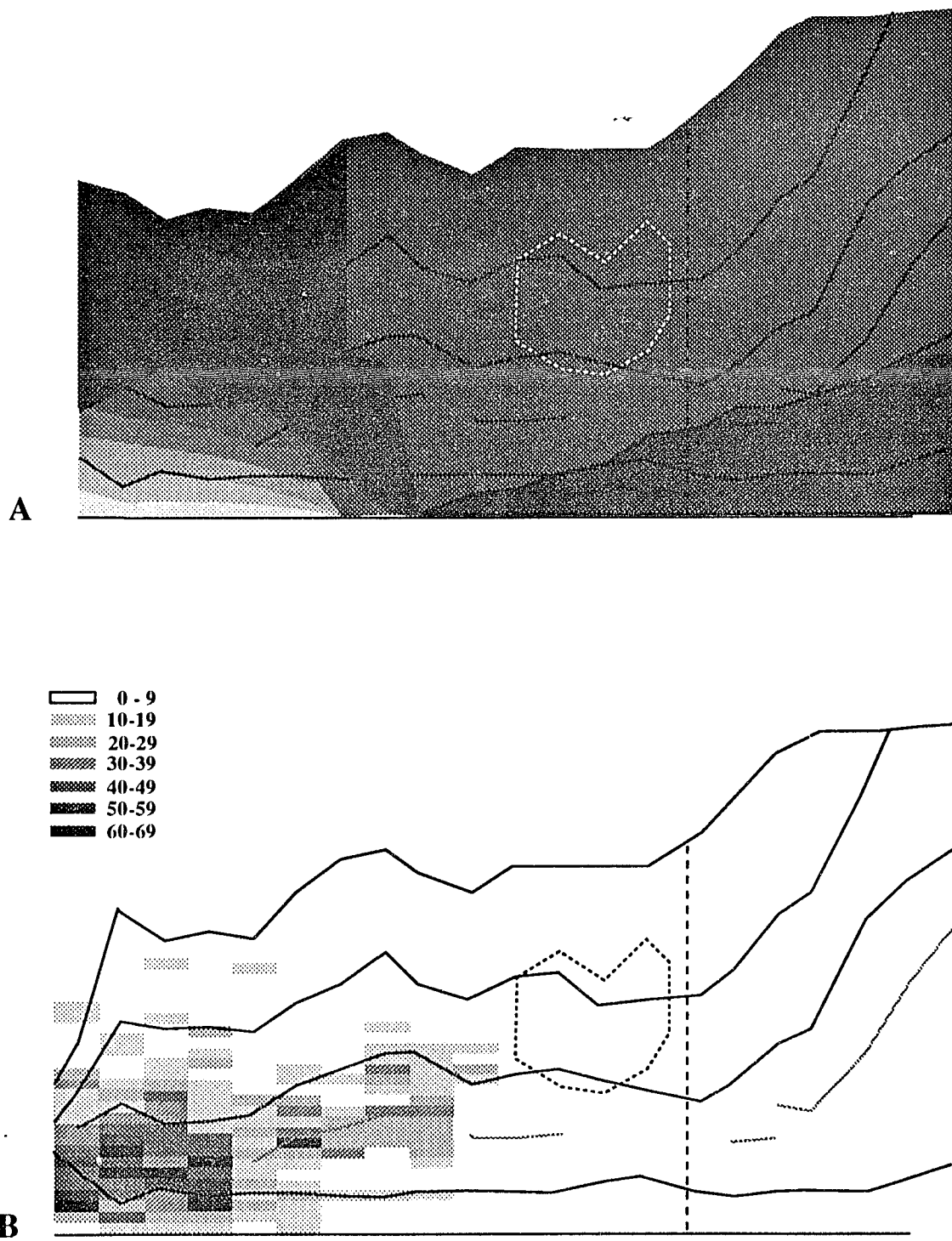


Fig. 3.9

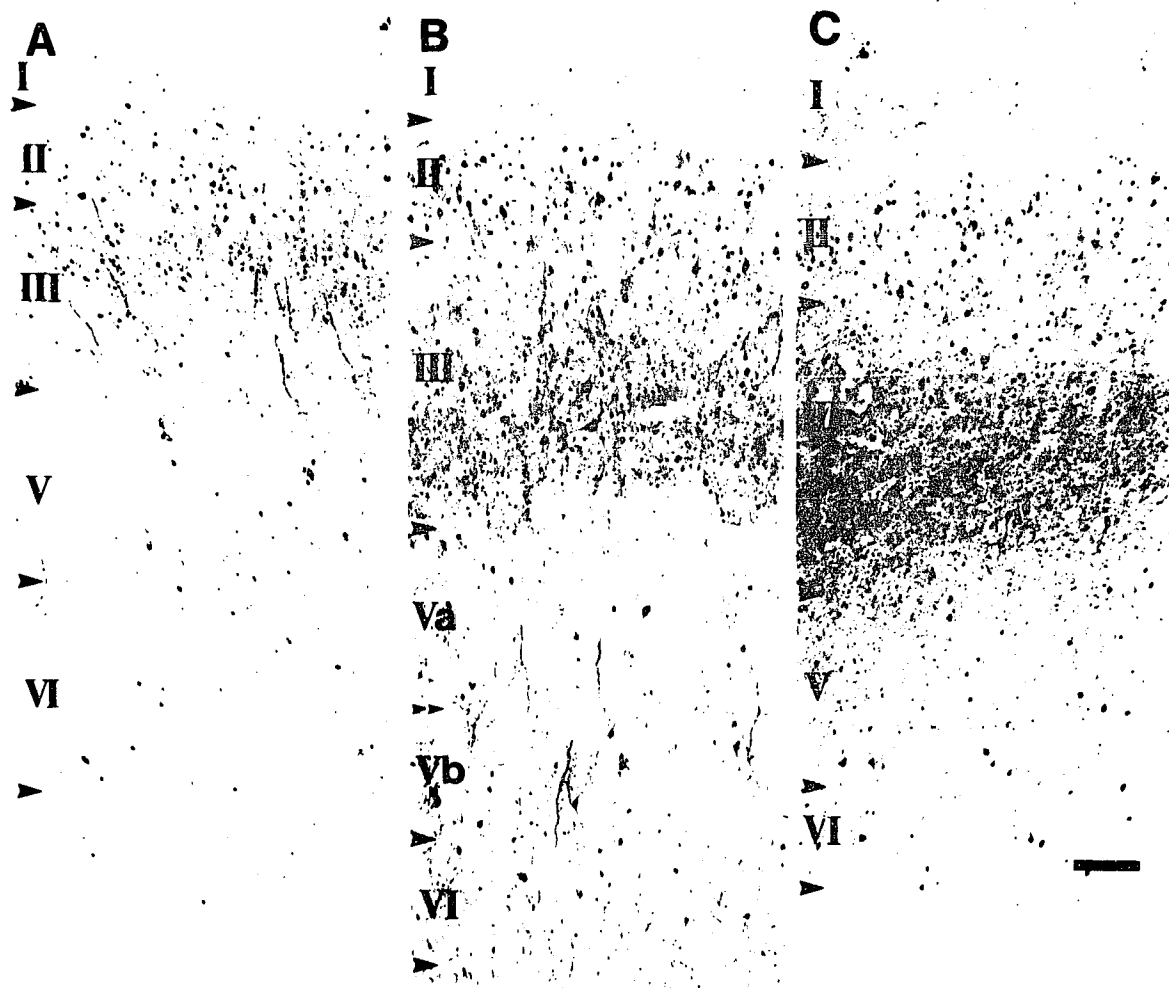


Fig. 3.10

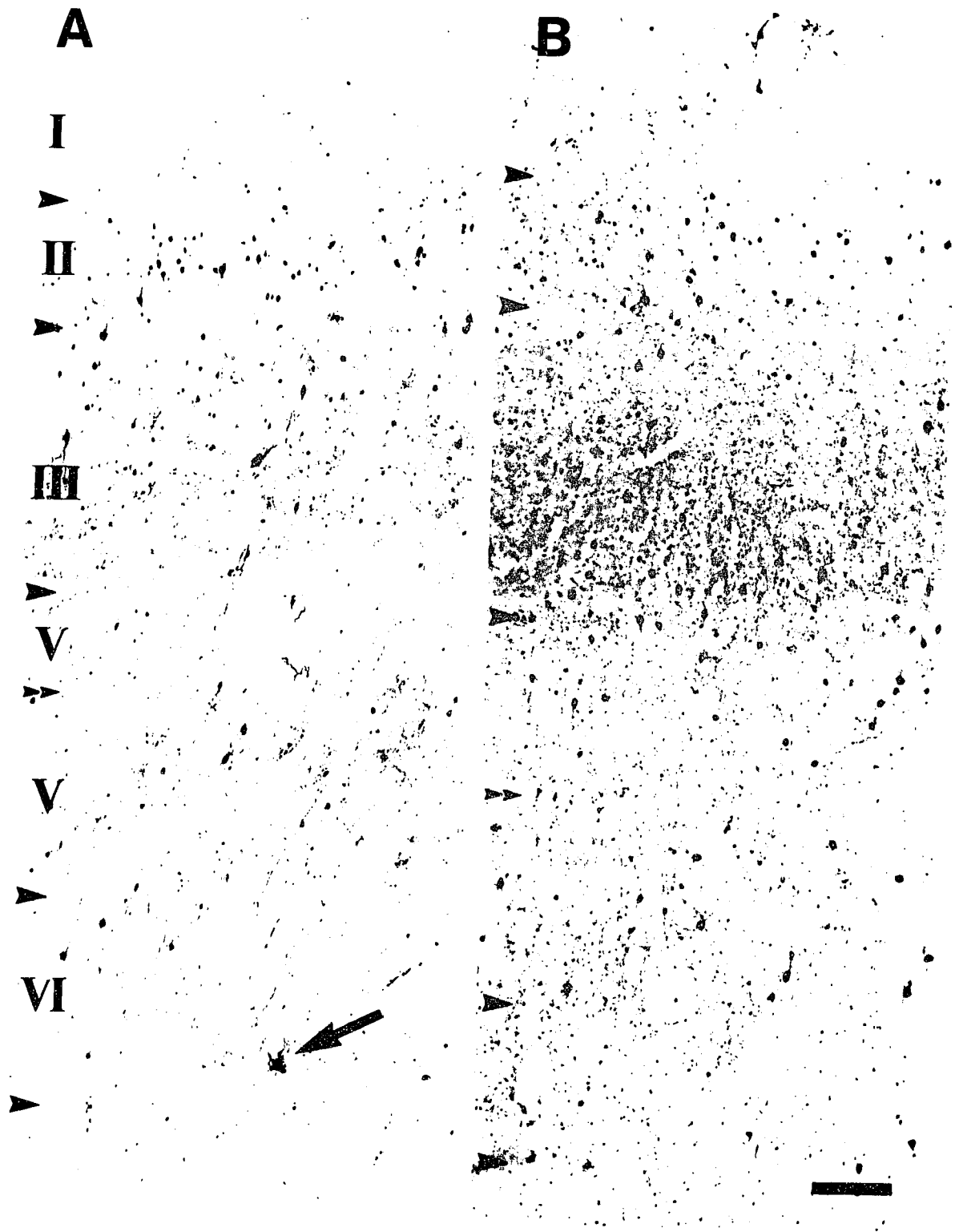


Fig. 3.11

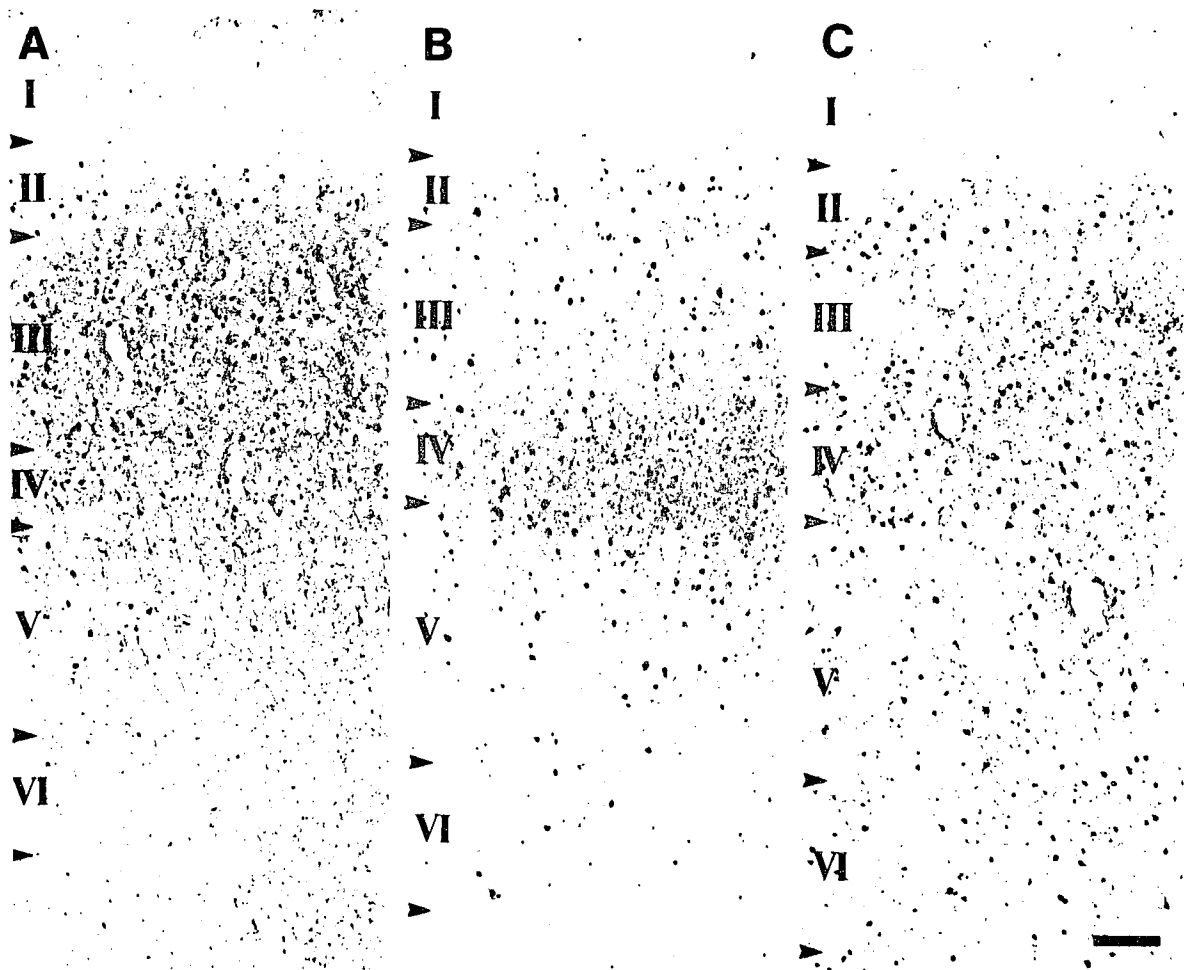


Fig. 3.12

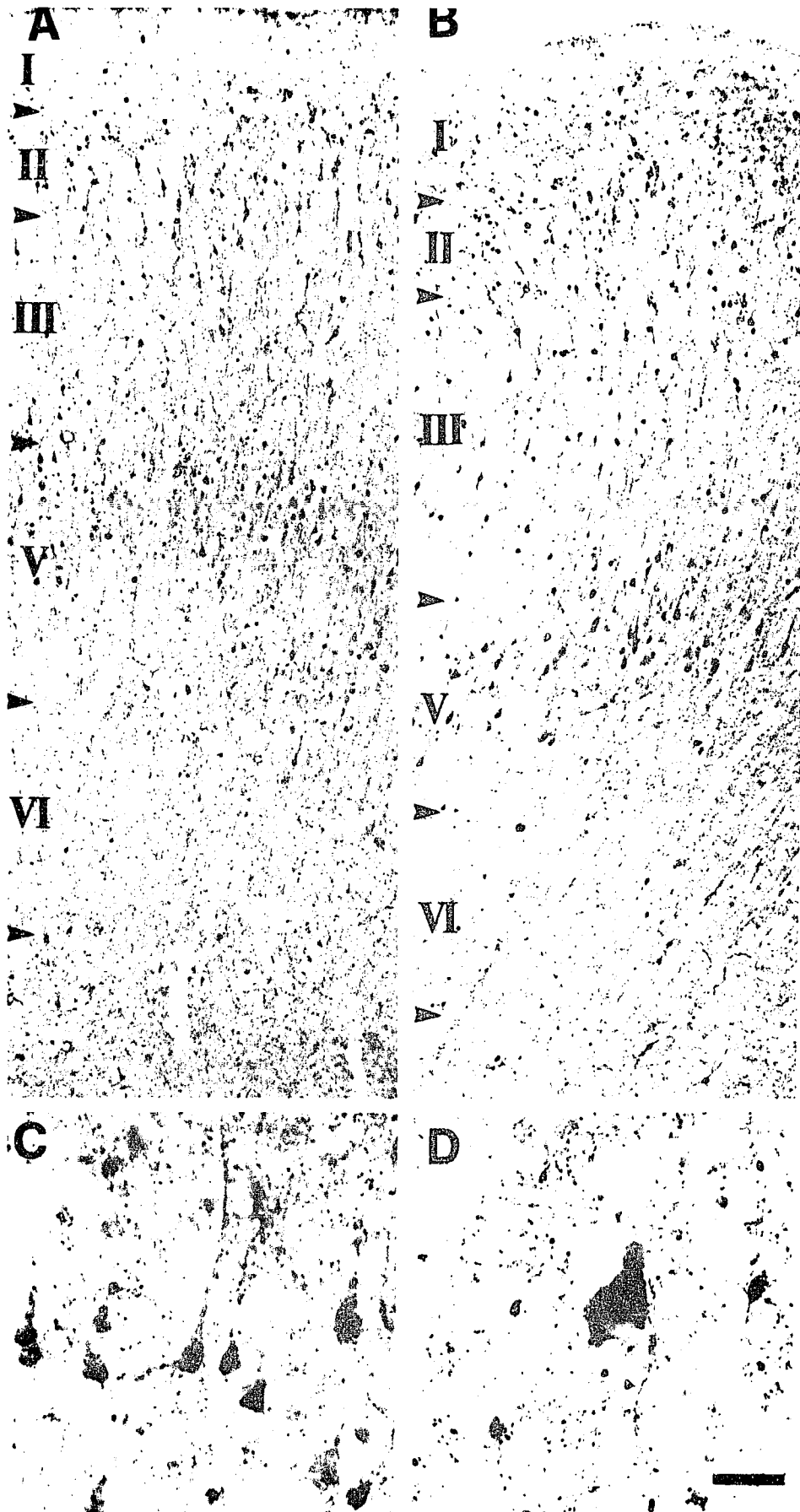


Fig. 3.13

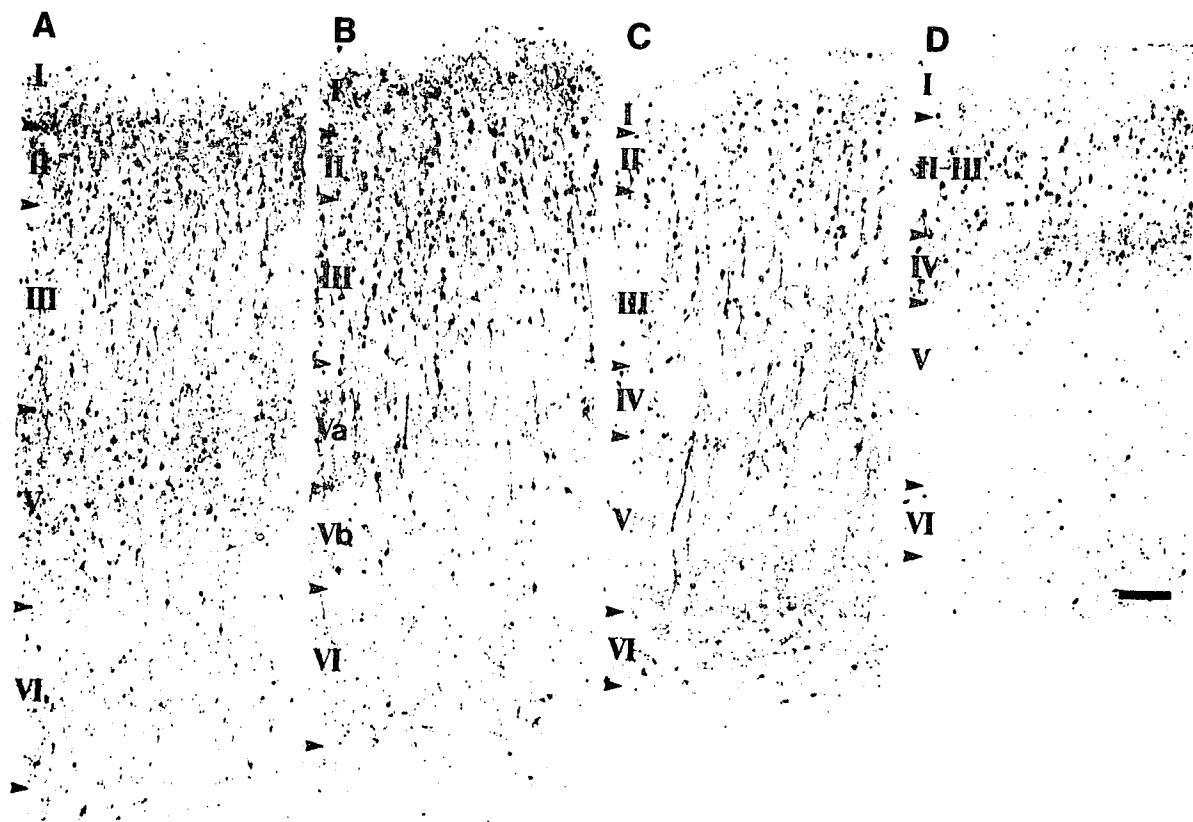


Fig. 3.14

Chapter 4

Spindle Neurons of the Human Anterior Cingulate Cortex

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SUMMARY

The human anterior cingulate cortex is distinguished by the presence of an unusual cell type, a large spindle neuron in layer Vb. This cell has been noted numerous times in the historical literature but has not been studied with modern neuroanatomic techniques. For instance, details regarding the neuronal class to which these cells belong, and their precise distribution along both ventrodorsal and anteroposterior axes of the cingulate gyrus are still lacking. In the present study, morphological features and the anatomic distribution of this cell type were studied using computer-assisted mapping and immunocytochemical techniques. Spindle neurons are restricted to the subfields of the anterior cingulate cortex (Brodmann's area 24), exhibiting a greater density in anterior portions of this area than in posterior portions, and tapering off in the transition zone between anterior and posterior cingulate cortex. Furthermore, a majority of the spindle cells at any level is located in subarea 24b, on the gyral surface. Immunocytochemical analysis revealed that the neurofilament protein triplet was present in a large percentage of these neurons, and that they did not contain calcium-binding proteins. Injections of the carbocyanine dye DiI into the cingulum bundle revealed that these cells are projection neurons. Finally, spindle cells were consistently affected in Alzheimer's disease cases, with an overall loss of about 60%. Taken together, these observations indicate that the spindle cells of the human cingulate cortex represent a morphological subpopulation of pyramidal neurons whose restricted distribution may be associated with functionally distinct areas.

INTRODUCTION

In recent years the human cingulate cortex, especially its anterior portion, has been the subject of heightened interest. This development stands in stark contrast to many decades of neglect due in large part to its inaccessibility. The relatively few physiological studies that were performed in the monkey generally focused on the role of the anterior cingulate cortex in autonomic control (Kaada, 1951; Showers and Crosby, 1958; Dua and MacLean, 1964; Robinson and Mishkin, 1968). These complemented the handful of studies performed in human subjects which involved stimulation of this area during neurosurgical procedures (Pool and Ransohoff, 1949; Pool, 1954). The results of these experiments confirmed the role of the anterior cingulate gyrus in autonomic regulation and supported the notion that the monkey anterior cingulate cortex was functionally homologous to that of the human. However, studies of patients with anterior cingulate lesions, organic or surgically induced, hinted at a broader role for this area, involving speech initiation (Nielsen and Jacobs, 1951; Barris and Schuman, 1953; Nakasu et al., 1991) attentiveness (Watson et al., 1973), and the expression of emotion (Arroyo et al., 1993). With the advent of functional imaging techniques, such as positron emission tomography, this region has been implicated in manual and oculomotor control (Petit et al., 1993), memory (Perani et al., 1993), attention (Pardo et al., 1990; Corbetta et al., 1991; Bench et al., 1993; Corbetta et al., 1993) and the experience of pain (Jones et al., 1991; Talbot et al., 1991; Pagni and Canavero, 1993). Although some of these functions, such as the processing of pain or painful stimuli, were originally described in non-primate species (Sikes and Vogt, 1992), some of them seem distinctly human, such as the expression of the emotional content of speech (Nielsen and Jacobs, 1951) and the experience of auditory hallucinations in schizophrenia (McGuire et al., 1993). Finally, recent detailed studies of the cytoarchitecture of the cingulate gyrus in the macaque monkey (Vogt, 1976; Vogt et al., 1987; Vogt, 1993) and human (Vogt et al., 1995) demonstrate that although there are many similarities, substantive differences exist

between the cingulate cortices of these two species. As the capacity for functional studies in the human increases, so does the appreciation of the physiological significance of the anterior cingulate gyrus, and the interest in identifying structural features unique to the anterior cingulate cortex of humans.

In his 1881 series of articles on the cytoarchitecture of the human cerebral cortex, Betz described a distinctive neuron in the human cingulate gyrus (Betz, 1881). What was so striking was not merely its shape — vertical spindle-shaped neurons can readily be encountered in layer VI of most cortical areas (Tömböl, 1984) — but its size, abundance, and especially its location, in layer V. Almost two decades later, Cajal, who credited both Betz and Hammarberg with their initial descriptions (Betz, 1881; Hammarberg, 1895), reported these neurons in the insular cortex of a newborn human and mentioned their presence in the cingulate gyrus, referring to their location as the "layer of spindle cells" (Cajal, 1899). He reported that he was unable to apply successfully the Golgi stain to interhemispheric cortex in any animal but rodents and other small mammals. The Golgi-impregnated spindle neurons he drew were taken from the insular cortex and showed neurons with a long, tapered soma with a basal dendrite that formed a tuft of spiny dendrites, and an apical dendrite that usually branched in layer IV but occasionally branched shortly after its origin. The axon descended toward the white matter after giving off numerous collaterals. In 1926, Von Economo undertook a study of these cells, which he first thought were pathological structures, since he saw them initially in the brains of patients with encephalitis lethargica (Von Economo, 1926). Rose made mention of them in his 1927 monograph and made the interesting observation that these neurons can also be observed in the cingulate cortex of the chimpanzee (Rose, 1927). In his 1932 description, Ngowyang mentioned that these cells frequently form clusters (Ngowyang, 1932). Most recently, Braak (1980) noted these neurons' distinctive pigment pattern.

In spite of more than a century of periodic interest, however, the above studies leave many questions unanswered. First, morphological details are lacking. All of these studies excepting Cajal's were based solely on the Nissl stain and Cajal's Golgi analysis was based on neurons in the insular, not cingulate cortex. Second, it is uncertain what type of neurons these are. Cajal's report of an axon heading toward the white matter is suggestive of a projection neuron, but again, it is from a different cortical area, and could form mainly, or only, recurrent collaterals. Third, the precise location of these neurons is unclear. In view of recent advances in the anatomic parcellation of the cingulate gyrus, and of the understanding of the connectivity of its subregions, the knowledge of exactly which areas contain distinctive cell types can contribute greatly to an understanding of how the cingulate gyrus is organized and perhaps what the functional role of these neurons might be. Finally, it is unclear what happens to these cells in disease processes. Current views of neurodegenerative disorders recognize a degree of selective neuronal vulnerability among neuronal populations (Morrison et al., 1987; Hof et al., 1990; Hof and Morrison, 1990; Hof et al., 1991; Hof and Morrison, 1991; Hof et al., 1993). The susceptibility or resistance of a defined cell type to degeneration can provide information both about the cell type and the pathological process itself. The present study constitutes a more detailed analysis of these neurons and their distribution, in order to characterize further one of the features that sets the human anterior cingulate cortex apart from other cortical areas.

MATERIALS AND METHODS

Staining procedures

The brains from three neurologically normal patients (mean age 77.3 ± 5.0 years, range 72-84) and four patients clinically and pathologically diagnosed with Alzheimer's disease (AD; 73.5 ± 10.6 years, range 60-89) were obtained at autopsy. In addition, the brain of a 72 year old patient with large striatal infarcts was obtained, and since it showed

no pathological alterations in the cingulate cortex, it was utilized as a control case. All of the AD cases had numerous senile plaques and neurofibrillary tangles throughout the cerebral cortex. The neuropathologic evaluation of the control cases revealed rare neurofibrillary tangles exclusively located in the entorhinal cortex. All brains were collected between one and ten hours of death and fixed by immersion for 48-72 hours in fresh 4% paraformaldehyde. The entire cingulate gyrus was dissected out of the hemispheres and removed intact. The brain sample was placed into fresh 4% paraformaldehyde and postfixed for up to 48 hours longer. The tissue was then dissected either into 1 cm thick coronal blocks or parasagittally, separating the medial face containing largely areas 24b and 23b from the rest of the gyrus. The blocks were then cryoprotected by immersion in solutions of increasing concentrations of sucrose, from 12 to 30% in phosphate-buffered saline (PBS). Blocks were frozen on dry ice and cut at 40 μm on a cryostat or sliding microtome. Frozen sections were utilized in preference to plastic or paraffin embedded sections because neurofilament immunoreactivity is best preserved in frozen material, especially in human brain. For the neurologically normal cases, five consecutive series were taken every 25 sections, with the result that the entire gyrus was sampled once every millimeter, and all sections stained at each level were no more than 200 μm apart. The sections stained with thionin and those processed for immunohistochemistry were always immediately adjacent to each other. In the remaining cases, the anterior cingulate gyrus was sampled at the level of the genu and sections were taken in a non-serial fashion.

After sectioning, the sections were rinsed thoroughly in PBS, treated in a solution of 0.75% hydrogen peroxide in 75% methanol for twenty minutes to eliminate endogenous peroxidase activity, rinsed again, and then placed into a solution of primary antibody in a diluent containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin. One series of sections was incubated in an antibody that recognizes a nonphosphorylated epitope on the neurofilament protein triplet (SMI-32, Sternberger

Monoclonals, Baltimore, MD; Sternberger and Sternberger, 1983; Lee et al., 1988), at a dilution of 1:10,000. In addition, series from four cases were stained with antibodies to the calcium-binding proteins parvalbumin, calbindin, and calretinin (Swant, Bellinzona, Switzerland), at dilutions of 1:3000, 1:800, and 1:3000, respectively. Tissue was incubated for a minimum of 40 hours on a rotating table at 4°C. Following incubation, sections were processed by the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and intensified in 0.0067% osmium tetroxide.

Tract tracing with the carbocyanine dye DiI was performed in accordance with previously described protocols (Mufson et al., 1990; Supprian et al., 1993). Crystals of DiI were mixed into a paste with methanol and placed in the cingulum bundle with a 23 gauge needle. The deposits were made 2-3 mm below the cortex and care was taken to prevent stray crystals from dislodging and settling on other parts of the tissue. The block was then placed in phosphate buffer with 0.1% sodium azide at room temperature, protected from light, for 2 to 5 months. One hundred μ m thick sections were cut on a vibratome and viewed under rhodamine narrow pass filters at 40x to visualize the dye.

Quantitative analysis

All quantitative analyses were performed on a computer-assisted image analysis system consisting of a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage, a high sensitivity Zeiss ZVS-47E video camera, a DEC 5000 workstation and Macintosh II microcomputer, and custom software developed in collaboration with the Scripps Research Institute, La Jolla, CA. For each section, cortical fields were captured with a Zeiss 10x PlanApochromat objective and digitized, and the cingulate cortex was mapped, using a Zeiss 20x PlanApochromat objective to confirm cellular details. All labeled cells that fit the morphologic criteria described below were mapped, as were the pial surface and the corpus callosum. Adjacent Nissl-stained sections were also mapped for areal and laminar boundaries using the criteria of

Vogt et al. (1995). For the display of the maps themselves, the Nissl-based laminar maps were superimposed on the adjacent immunostained maps.

For all the analyses in this study, due to the peculiar shape of the neurons being described, only sections that were cut perpendicularly to the pial surface were used. To determine this, SMI-32-immunostained sections were examined and the corresponding level used only if apical dendrites could be followed from layer V through layer III. A neuron was considered a spindle cell on Nissl-stained material if it had an ovoid nucleus with a visible nucleolus and if it had a basal dendrite that was at least as thick as its apical dendrite. In SMI-32-immunostained material, a neuron was counted as a spindle neuron if it had an immunoreactive ovoid soma and immunoreactive dendrites, again with the basal dendrite as thick as or thicker than the proximal dendrite. For both Nissl and SMI-32 analyses, neurons whose apical and basal dendrites were much thinner than the soma were excluded. The purpose of these requirements was to eliminate any neurons that could be defined as inverted pyramidal cells or large interneurons. As a result of these criteria, neurons whose somata were not labeled, a situation commonly encountered in SMI-32-immunostained material, were not counted. In addition, neurons not oriented precisely parallel to the angle of cut could not be considered. Therefore the numbers obtained are likely to be underestimates of the actual numbers. Optical density measurements were obtained on a single section to circumvent variability in staining intensity. The first thirty pyramidal and fusiform neurons encountered in the section were sampled using a Zeiss 63x NeoFluar objective. A box measuring 3 μm by 3 μm was generated and the mean transmittance of this area was obtained. Five to eight measurements were taken of each neuron, depending on the size of the cell. Care was taken to avoid the nucleus and to sample both polar ends of the neuron, as well as to sample equally both more and less intensely immunoreactive portions of the neuron, since the neurofilament protein triplet is not homogeneously distributed throughout the soma. A mean of these observations was obtained for each neuron. In addition, a

number of measurements were made of the unstained neuropil between immunoreactive neurons. A mean was obtained for these values, and all transmittance values were divided by this background mean to obtain a standardized transmittance value. Optical density was defined as the reciprocal of this transmittance value. Statistical analysis was performed using a two-tailed Student's *t* test.

RESULTS

Spindle cell distribution

In Nissl-stained material, spindle neurons were found almost exclusively in layer Vb, among the groups of small-to-medium sized pyramidal cells that characterize this layer (Fig. 4.1). They often occurred in clusters of two or three, separate from the pyramidal cell clusters, and were more prevalent at the edges of the gyrus. At all rostrocaudal levels examined, two-thirds of the total number of spindle cells were located in subarea 24b, on the gyral surface (Table 4.1). Most of the remaining third was located in area 24a. Although area 24b had a relatively high overall density of spindle cells, the cortex in dimples within area 24b had a low density of spindle neurons. The predominance of area 24b was not due solely to the large size of this subarea. The density of these cells was highest in area 24b, and area 24a had the second highest density (Table 4.1). Furthermore, their numbers decreased steadily from anterior to posterior portions of the anterior cingulate gyrus (Fig. 4.2a-d). This latter gradient was confirmed in a horizontal series as well. The density of fusiform neurons was greater anteriorly than posteriorly, and dropped abruptly at the transition zone between anterior and posterior cingulate cortex (Vogt's area 24'; Fig. 4.3). This distribution pattern was observed both in Nissl-stained sections and in those immunostained with SMI-32 (Fig. 4.2e-h).

Spindle cell morphology

On Nissl-stained materials, spindle neurons were readily distinguishable from pyramidal neurons and exhibited a variety of morphologies (Fig. 4.4a-d). Some were

very slender and elongate, with apical and basal dendrites nearly as thick as the soma at its widest point. Others were shorter, more stout, and usually curved. Occasionally, neurons were encountered with a bifid basal dendrite or a third major dendrite emerging from the soma. In addition, lipofuscin deposits were common, and occasionally were so large that they distorted the shape of an otherwise very slender neuron. "Corkscrew" cells as described by Von Economo (1926) were not encountered in the control brains.

Immunostaining with SMI-32 revealed further morphological details. In the most slender neurons, the shape of the neuron resembled that observed in Nissl-stained material (Fig. 4.4e-g). In many instances, additional, very thin dendrites were visible emerging from the soma, in close proximity to the apical dendrite. In others, the basal dendrite bifurcated shortly after its origin. In some examples, the soma was not immunoreactive but its apical and basal dendrites were. In others, the neurofilament protein manifested as a single thick rod coursing through the neuron, parallel to its long axis, extending outward from the soma well into the apical and basal dendrites with little loss of thickness before abruptly disappearing. A common observation was a neuron whose thick, tapered apical dendrite was intensely immunoreactive and could be followed through layer III, and whose basal dendrite, equally broad and intensely immunoreactive, extended for 25-50 μm before terminating in a spray of branches in layer VI (Fig. 4.4e, f).

In order to determine whether there was a substantive difference in the mean intensity of SMI-32 immunoreactivity in these neurons when compared with neurofilament protein-immunoreactive pyramidal neurons, optical density measurements were obtained for a sample of each population (Fig. 4.5). The mean relative optical density for the fusiform neurons (22% greater than background staining) was significantly higher than that for pyramidal neurons (19% greater than background staining; $p < 0.05$). This result suggests a slightly greater content of neurofilament protein in the somatic compartment of the fusiform neurons than of pyramidal neurons. It should be noted that the fusiform neurons demonstrated a somewhat greater degree of

intracellular heterogeneity in neurofilament protein distribution. Spindle cells constituted from 16.4% of the total neurofilament protein-immunoreactive neuronal population of layer V anteriorly to 3.9% of the total population posteriorly. This reflects the progressive overall increase of neurofilament protein-immunoreactive neurons as one proceeds posteriorly in the cingulate gyrus, as well as the progressive decrease in the number of fusiform neurons. In contrast to this steady trend, the ratio of the number of neurofilament protein-immunoreactive fusiform neurons to that of the total population of spindle cells in immediately adjacent Nissl-stained sections ranged in one serial case from 28 to 80% with no evidence of a clear anterior-to-posterior trend. Samples from other cases yielded percentages within this range. In addition, when immunostained sections counterstained with thionin were examined, the percentages obtained were also within this range, but tended to be clustered toward the higher end of this range, probably because in Nissl-stained material, faint or only dendritic immunoreactivity can more easily be identified as belonging to a cell, whereas in the absence of the Nissl stain, continuity between immunoreactive profiles can be difficult to establish.

In order to confirm that spindle cells belong to the class of cortical projection neurons, the lipophilic dye DiI was placed into the white matter. These injections into the cingulum bundle labeled some fusiform neurons as well as numerous pyramidal neurons. The proximal portions of their apical and basal dendrites lacked spines, but more distal regions were spiny. Some examples are illustrated in Fig. 4.6. Finally, immunostaining with antibodies to the calcium-binding proteins revealed that no spindle cells were immunoreactive for parvalbumin, calbindin, and calretinin.

Alzheimer's disease

Analysis of the anterior cingulate cortex from the brains of patients diagnosed with AD produced variable results. Since entire gyri were not available from these cases, serial analyses were not possible. In one of the four AD cases, there was severe, almost complete loss of spindle cells and pyramidal neurons. However, in the other three cases,

there was more than a 60% loss on the basis of the Nissl stain (27.8 ± 3.3 spindle neurons per section, compared with 72.5 ± 8.5 for control cases). In the first of these, from a patient suffering from severe AD associated with Balint's syndrome (reported in (Hof et al., 1993), the anterior cingulate cortex contained numerous neurofibrillary tangles in all cortical layers. In the other two cases, there was severe cell loss, particularly in layer III, although much fewer neurofibrillary tangles were present. In all of these cases, there were morphological alterations in the spindle neurons, such as more dendritic processes per cell than found in the normal brains, and bizarrely twisted apical and basal dendrites that often assumed a corkscrew-like appearance. It should be noted that these features were also observed in the pyramidal neurons in the same area and layer. Tissue suitable for immunocytochemistry was available from one of these brains. In this brain, 70% of the spindle neurons were neurofilament protein-immunoreactive, well within the range obtained for normal brains.

DISCUSSION

The spindle neurons appear to represent a neuronal subclass with a highly restricted distribution and considerable vulnerability in AD cases. These neurons were observed in restricted domains of the human cingulate cortex, with higher densities in the anterior area 24b, and fewer in areas 24a and 24c. They were not found in the posterior cingulate cortex. A subpopulation of spindle cells was strongly immunoreactive for the neurofilament protein triplet. While neurofilament immunostaining did not label the entire neuron, it did allow certain comparisons with Cajal's Golgi preparations in the insula. Specifically, the appearance of the neurofilament protein-immunoreactive spindle cells as elongate, tapered somata with a basal dendrite that, after emerging from the soma gives rise to a spray of dendrites closely parallels Cajal's description and drawings (1899). On this basis, it may be inferred that the spindle cells in the insula and in the anterior cingulate cortex are the same cell type.

The general cell type to which the spindle cell belongs is a difficult issue. Although every attempt was made to avoid counting large interneurons as spindle cells, it is likely that some of the smaller cells counted with the Nissl stain were in fact large bitufted or even basket cells. However, given the fact that γ -aminobutyric acid-containing (GABAergic) interneurons constitute only about 20 to 25% of cortical neurons overall, and only 15 to 17% in layer V (Hendry et al., 1987; Hornung and De Tribolet, 1994), and given the precautions taken in the counting process, the number of cells thus erroneously counted is likely to be very small. The fact that no layer Vb spindle neurons were found that contained any of the three calcium-binding proteins parvalbumin, calbindin, and calretinin suggests that these cells may be considered modified pyramidal neurons. These three calcium-binding proteins are found in separate populations of cortical GABAergic interneurons (Hendry et al., 1989; Celio, 1990; Jacobowitz and Winsky, 1991; Hof and Nimchinsky, 1992; Hof et al., 1993; Hof et al., 1993), and while it is not clear that these proteins taken together label all interneurons, the absence of immunoreactivity at the very least limits the possibility that these neurons constitute a discrete population of GABAergic cells. The probable pyramidal cell-like nature of these neurons is further evidenced by their frequent content of neurofilament protein, which is found in subpopulations of pyramidal neurons (Campbell and Morrison, 1989; Hof et al., 1990; Hof and Morrison, 1990; Campbell et al., 1991), but has not been demonstrated in local circuit neurons (Vickers and Costa, 1992). Finally, the labeling of these neurons after placement of DiI in the subcortical white matter provides the strongest evidence that of at least some these neurons project into the cingulum bundle, as suggested by Cajal for the spindle cells of the insula (Cajal, 1899).

Although the above evidence suggests that the spindle neurons are pyramidal neurons, the fairly high proportion of the total spindle cell population that contains neurofilament protein, however, sets these neurons aside from pyramidal cells as a group. Neurofilament protein is estimated to be present at immunohistochemically detectable

levels in approximately 10 to 20% of cortical pyramidal neurons (Hof et al., 1990; Hof and Morrison, 1990), while it is present in roughly half of the layer Vb spindle neurons, which may also have, on average, more neurofilament protein per cell, as indicated by their higher average optical density. This is especially intriguing in view of the degree of involvement of these cingulate neurons in the degenerative process in AD. Neurofilament protein-immunoreactive neurons have been shown to be especially vulnerable to degeneration in AD (Morrison et al., 1987; Hof et al., 1990; Hof and Morrison, 1990) and it has been postulated that there is a gradual transformation of the neurofilamentous component of the cell into neurofibrillary tangles (Vickers et al., 1992; Vickers et al., 1994). A population of pyramidal neurons with a high likelihood of neurofilament protein content would be expected to be especially vulnerable. Nevertheless, in spite of a loss of spindle neurons overall, there seems to be no preferential loss of the neurofilament-immunoreactive spindle cells in AD, since the percentage of spindle cells containing neurofilament protein is comparable in control brains and in AD. Therefore, in this particular cell population at least, the degenerative process appears to affect neurofilament-immunoreactive and non neurofilament-immunoreactive neurons to the same degree.

The degree of spindle cell loss observed is higher than the 40% pyramidal cell loss reported in the prefrontal cortex in AD (Terry et al., 1981). It is, however, considerably greater than the relatively mild degree previously described for pyramidal neurons in the anterior cingulate cortex by various authors (Brun and Englund, 1981; Mountjoy et al., 1983). These results suggest that while the anterior cingulate cortex may remain relatively unaffected in AD, at least one conspicuous cell population is affected, and may represent a more sensitive indicator of the presence of a degenerative process. An alternative interpretation is that AD cases with involvement of the anterior cingulate cortex tend to belong to a subgroup of AD cases characterized by cell loss mostly in layers V and VI (corresponding to Vogt's class 4; Vogt et al., 1990). If this is true, then

such cases might not necessarily show overall pyramidal cell loss, since the degree of cell loss, although considerable for the relatively cell-sparse layer V, would be obscured by the preservation of the very high neuronal density within layers II and III.

With respect to their possible function, Braak (1980) regards spindle cells, on the basis of the laminar position and pattern of lipofuscin pigmentation, as belonging to the same class as the giant layer V cells of Betz in the primary motor cortex. This suggestion is provocative because it allows one to begin to hypothesize a possible role for these neurons. A role in motor control would not be very surprising, in view of the numerous motor functions, autonomic and somatic, that are subserved by anterior cingulate cortex (Delgado and Livingston, 1948; Pool and Ransohoff, 1949; Kaada, 1951; Pool, 1954; Showers and Crosby, 1958; Dua and MacLean, 1964; Robinson and Mishkin, 1968; Terreberry and Neafsey, 1983; Luppino et al., 1991; Shima et al., 1991; Dum and Strick, 1992; Buchanan and Powell, 1993; Dum and Strick, 1993; Grafton et al., 1993). Furthermore, both Betz cells and spindle cells contain high levels of neurofilament protein (Nimchinsky et al., 1992). However, this is true of virtually all Betz cells, but only a subpopulation of spindle cells. In addition, a subpopulation of human Betz cells have been shown to contain the calcium-binding protein calretinin (Nimchinsky et al., 1992), and as mentioned above, no layer Vb spindle neurons were found to contain any of the three calcium-binding proteins used in the study. These facts do not rule out a motor role for these neurons, but do indicate that they are a population separate from the Betz cells. Irrespective of their functions, however, it is possible that large or otherwise unusual neuronal types in layer Vb are a consistent feature of certain agranular cortical areas such as anterior cingulate, anterior insula and primary motor cortex.

The limitation of spindle cells to the subareas of the anterior cingulate cortex is sufficiently complete as to recommend them as markers of these cortical areas (when found on the cingulate gyrus). In fact, these neurons have a regional and laminar distribution that is as restricted as that of Meynert cells of the primary visual cortex. The

presence of spindle cells in all subfields of the anterior cingulate cortex and their absence from any cortical areas lying dorsally to the cingulate sulcus reflects supports the use of this sulcus as an indicator of a cytoarchitectonic border. The marked reduction in the density of these cells from anterior to posterior cingulate cortex occurs precisely in the area of transition between anterior cingulate cortex (area 24) and posterior cingulate cortex (area 23), referred to as area 24' (Vogt, 1993). This is one of the areas on the cingulate gyrus where, in the depths of the cingulate sulcus, motor areas have been described in the macaque (Muakkassa and Strick, 1979; Hutchins et al., 1988; Luppino et al., 1991; Matelli et al., 1991; Shima et al., 1991; Dum and Strick, 1992; Morecraft and Van Hoesen, 1992; Dum and Strick, 1993). It has been suggested that the gigantopyramidal field described by Braak (1976) represents at least one or part of one of these motor areas in the human (Morecraft and Van Hoesen, 1992). Due to the decreased density of spindle cells in the dorsal- and lateral-most portions of the cingulate gyrus, and to their absence in its caudal portion, they were seldom found alongside cells belonging to the gigantopyramidal field, suggesting that these cells are separate from the cingulate motor areas, as well.

While only neurons with a truly spindle-like morphology were considered for this study, it is probable that these represent one end of a spectrum ranging in morphology from the classical pyramid to the most narrow spindle cell. Many layer Vb cells were encountered which had stout basal dendrites oriented at a sharp angle to the axis of the apical dendrite (described in layer VI throughout the cortex by both De Crinis and Braak as "a pair of compass cells" (De Crinis, 1933; Braak, 1980). In addition, a large number of pyramidal neurons were observed which, although possessing the classic pyramidal somal morphology, had one vertically oriented basal dendrite that was much thicker than all the others, and often as thick as the apical dendrite. This variation of the standard pyramidal morphology, in addition to numerous inverted pyramidal neurons, was especially common in the more dorsal portions of the anterior cingulate gyrus, and all

these alternative shapes were rarely if ever observed in layer Vb elsewhere in the cortex. In addition, such cells, in addition to a few fusiform neurons were occasionally seen in layer III in the anterior cingulate cortex, especially in its more caudal portions. Thus, the anterior cingulate cortex is an area that contains many cell types with unusual morphologies. The significance of this cellular variability is not clear, but might be related to the cytoarchitectonic variability in this region. Far from being a homogeneous structure, the cingulate gyrus has been proposed as an area whose progressive differentiation is evident, from the least differentiated anteroventral portions to the most differentiated dorsal and posterior portions (Sanides, 1962; Vogt et al., 1987; Hof and Nimchinsky, 1992). All the areas considered in the present study are agranular, lacking the internal granular layer IV, or dysgranular, possessing an incipient or poorly defined layer IV. They are described as "periallocortex" or "proisocortex" (Braak, 1980; Vogt et al., 1987), not as the homotypical isocortex found in the most caudal and dorsal portions of the gyrus and in association cortices throughout the brain. In this context, it is noteworthy that the only other cortical area that is known to contain large layer Vb fusiform neurons is the anterior insula (Cajal, 1899; Von Economo, 1926; Ngowyang, 1932). This area too, is proisocortical and ranges from agranular to dysgranular. Both these areas are considered "limbic" cortices because of their poorly differentiated cytoarchitecture and their strong connections with other components of the limbic system, most notably the amygdala (Mufson et al., 1981; Pandya et al., 1981; Barbas and Pandya, 1989; Van Hoesen et al., 1993). Large layer Vb spindle cells may, therefore, be characteristic of certain limbic cortical areas.

It is interesting that areas such as the anterior cingulate and agranular insular cortex, whose cytoarchitecture is regarded as relatively archetypal are the preferential loci for a neuronal type that appears to be found only in primates. In his comprehensive survey of the anterior cingulate and retrosplenial cortices in a wide range of mammals, Rose (1927) reports the presence of layer Vb spindle neurons in small numbers in the

ring-tailed lemur. They are much more abundant in the common chimpanzee, mostly in the area corresponding to 24b and to a lesser degree in area 24a (Rose, 1927). In *Macaca sp.* spindle neurons are occasionally encountered in anterior cingulate cortex (Nimchinsky, unpublished observations). These observations attest to the fact that the anterior cingulate cortex has continued to evolve structurally. It is likely, therefore, that this cortex has also evolved functionally, developing roles that are now emerging from functional imaging studies.

Table 4.1. Distribution and density of spindle neurons in the subareas of the human anterior cingulate cortex^a

Area	Percent of total number	Mean density (per mm ²)
24a	13.0 ± 4.4	1.4 ± 0.7
24b	68.0 ± 1.8	19.6 ± 9.9
24c	19.0 ± 5.8	5.9 ± 3.1

^aTable shows the proportion of the total number of spindle cells counted that was observed in each of the cingulate subareas ("Percent of total number"), and the areal density of spindle cells in each subarea ("Mean density") in Nissl-stained material. Values represent means ± SEM from four levels in the anterior cingulate cortex.

FIGURE LEGENDS

Fig. 4.1. Photomontage of a traverse through area 24b in the anterior cingulate cortex. Note the lack of a granular layer IV, and the spindle cells (curved arrows) limited to layer Vb. Scale bar = 50 μ m.

Fig. 4.2. Computer-generated maps showing the distribution of spindle cells in coronal sections through the anterior cingulate cortex of a representative control case. Sections are 1 cm apart. The most anterior sections are on the left. Maps showing the distribution as observed in Nissl-stained material (a-d) are of sections adjacent to those showing the locations of neurofilament protein-immunoreactive spindle cells (e-h). Borders shown indicate the pia, the layer III/V border, and the layer VI/white matter border. Arrowheads indicate the borders between (ventrally to dorsally): 24a and 24b; 24b and 24c. Scale bar = 2 mm.

Fig. 4.3. Computer-generated map showing the distribution of spindle cells in a horizontal section through ventral area 24b. Arrowheads indicate the zone of transition between anterior and posterior cingulate cortex. Anterior is to the left; the medial wall of the hemisphere is up. Arrow indicates the caudal bank of a vertically-oriented dimple. Scale bar = 2 mm.

Fig. 4.4. Examples of spindle cells as seen with the Nissl stain (a-d) and with SMI-32 immunohistochemistry (e-g). Note variations in somal thickness and in the degree of tapering toward the apical and basal dendrites. In e and f, note the abrupt branching of the basal dendrite into numerous secondary dendrites. Scale bar = 25 μ m.

Fig. 4.5. Frequency distributions of the relative optical densities (OD) of SMI-32-immunoreactive pyramidal neurons (a) and SMI-32-immunoreactive spindle cells (b). Note that the relative OD distribution approximates a normal curve for pyramidal neurons but is skewed to the right for spindle cells.

Fig. 4.6. Spindle cells (a, b) in area 24b stained after placement of crystals of the carbocyanine dye DiI into the cingulum bundle. Note the labeling of two distinct morphological types; one with a stout soma and apical and basal dendrites (a) and one with much narrower apical and basal dendrites (b). Values represent means $\pm$ SEM from four levels in the anterior cingulate cortex. A classical pyramidal neuron (c) is shown for comparison. The pial surface is up. Scale bar = 50 μ m.

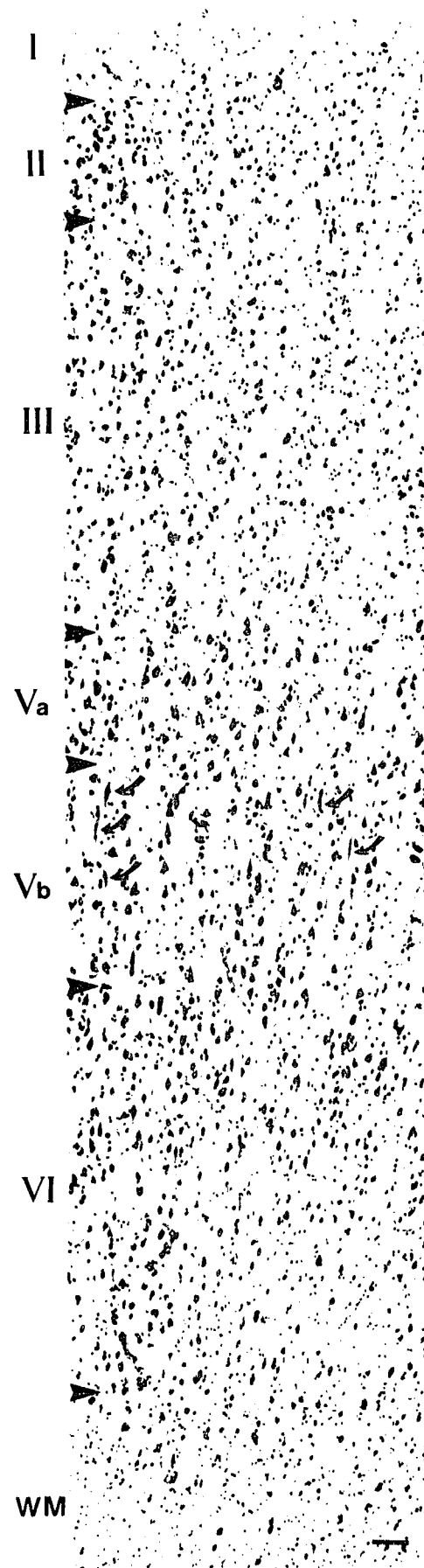


Fig. 4.1

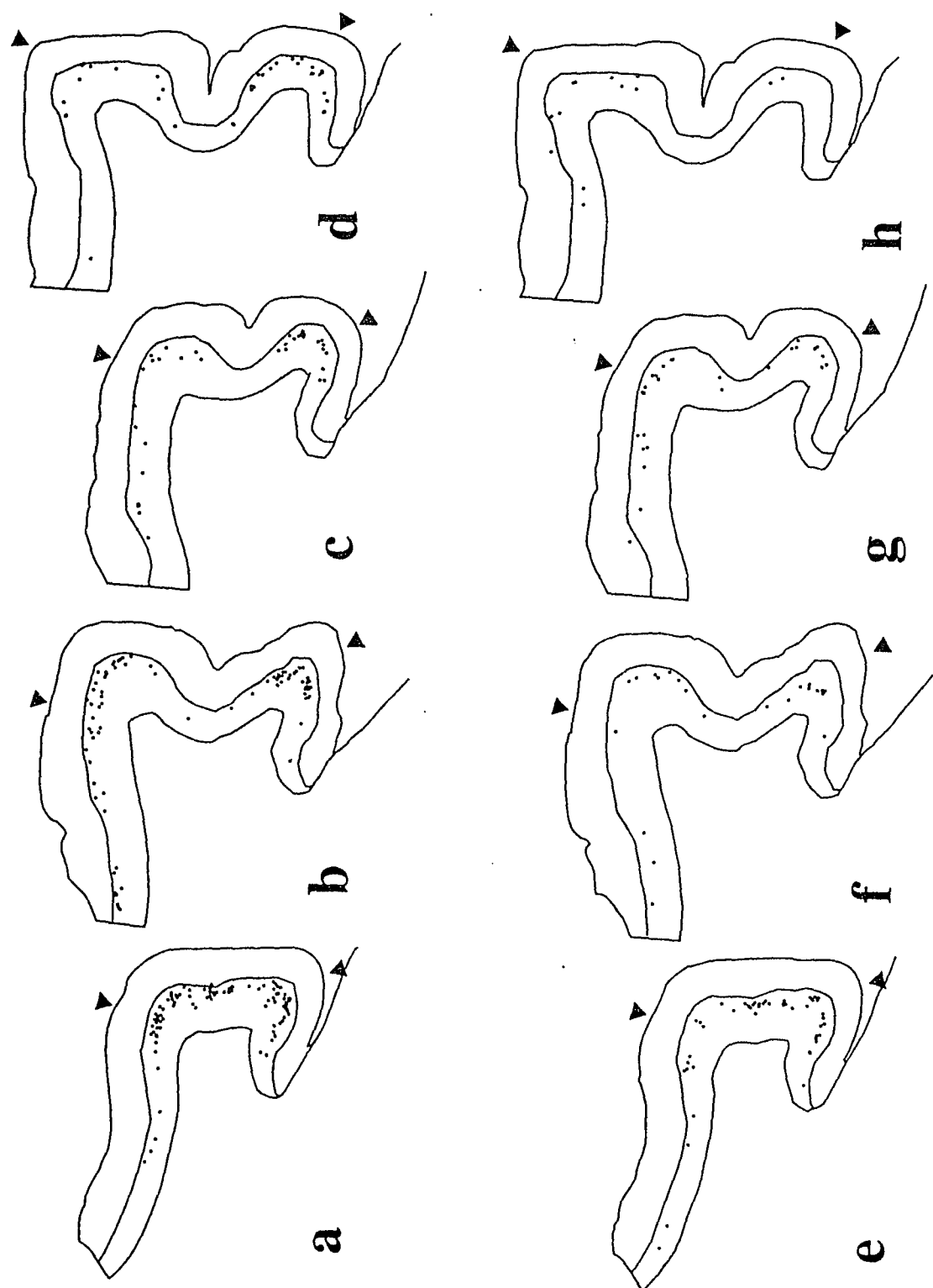


Fig. 4.2

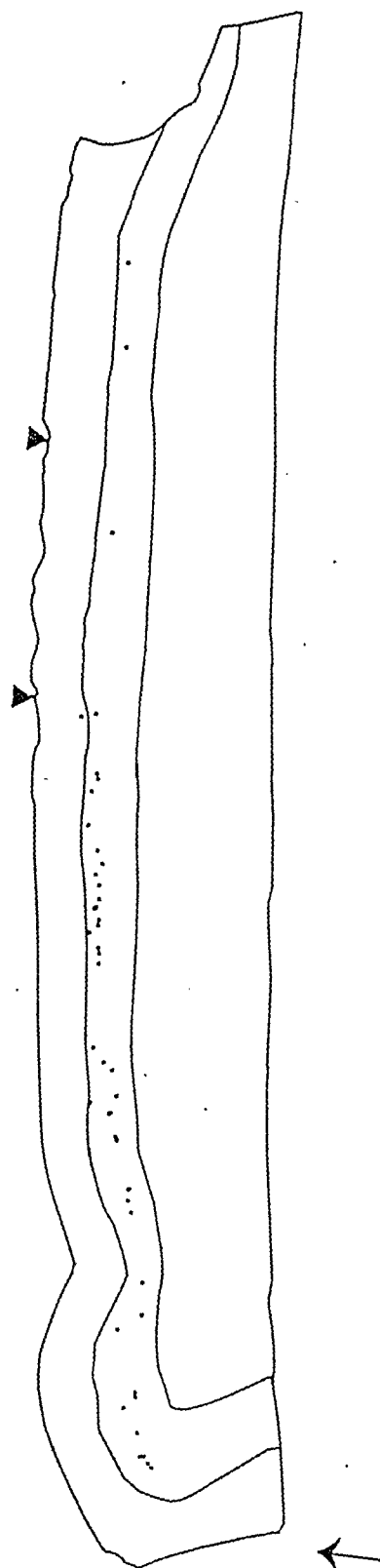
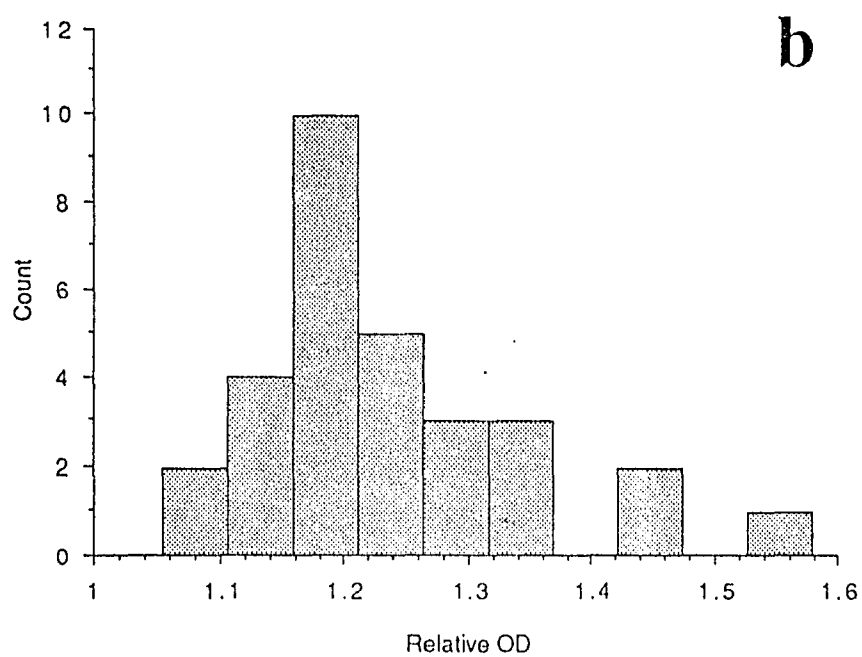
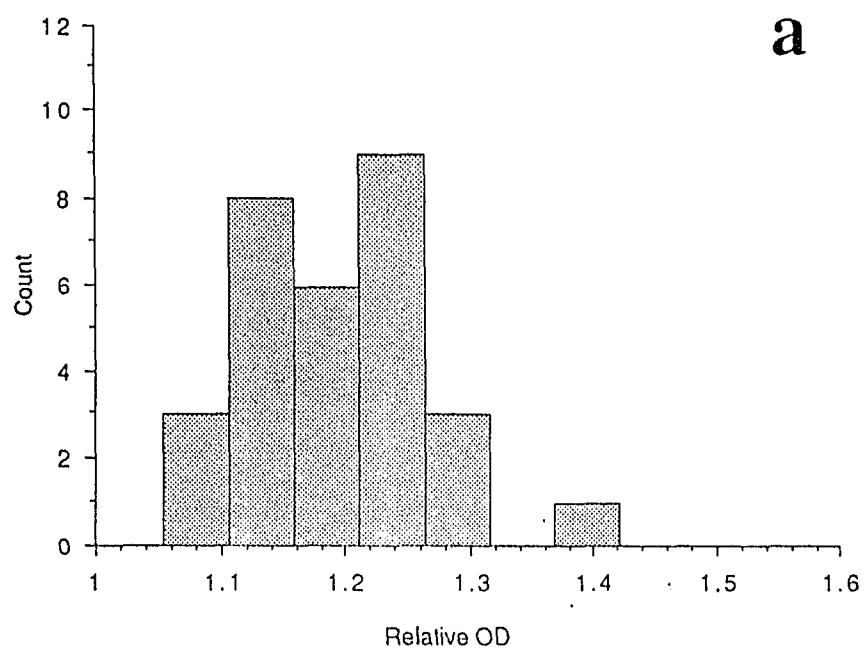


Fig. 4.3



Fig. 4.4

**Fig. 4.5**

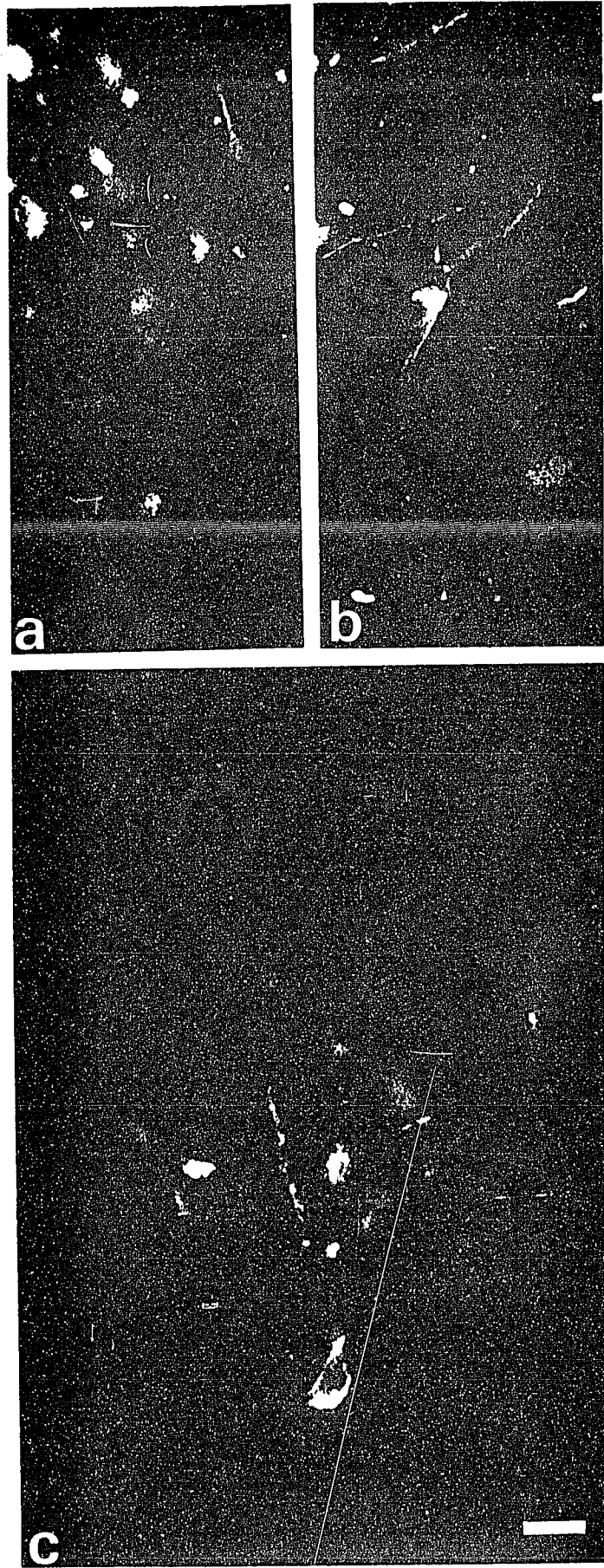


Fig. 4.6

Chapter 5

Neurochemical, Morphologic and Laminar Characterization of Cortical Projection Neurons in the Cingulate Motor Areas of the Macaque Monkey

SUMMARY

The primate cingulate gyrus contains multiple cortical areas that can be distinguished by several neurochemical features, including the distribution of neurofilament protein-enriched pyramidal neurons. In addition, connectivity and functional properties indicate that there are multiple motor areas in the cortex lining the cingulate sulcus. These motor areas were targeted for an analysis of potential interactions among regional specialization, connectivity, and cellular characteristics such as neurochemical profile and morphology. Specifically, intracortical injections of retrogradely transported dyes and intracellular injection were combined with immunocytochemistry to investigate neurons projecting from the cingulate motor areas to the putative forelimb region of the primary motor cortex, area M1. Two separate groups of neurons projecting to area M1 emanated from the cingulate sulcus, one anterior and one posterior, both of which furnished commissural as well as ipsilateral connections with area M1. The primary difference between the two populations was laminar origin, with the anterior projection originating largely in deep layers, and the posterior projection taking origin equally in superficial and deep layers. With regard to cellular morphology, the anterior projection exhibited more morphologic diversity than the posterior projection. Commissural projections from both anterior and posterior fields originated largely in layer VI. Neurofilament protein distribution was a reliable tool for localizing the two projections, and for discriminating between them. Comparable proportions of the two sets of projection neurons contained neurofilament protein, even though the density and distribution of the total population of neurofilament protein-enriched neurons was

very different in the two subareas of origin. Overall, no correlation was observed between somatodendritic morphology and neurofilament protein content. Thus, while the neurons that provide the anterior and posterior cingulate motor projections to area M1 differ morphologically and in laminar origin, their neurochemical profiles are similar, suggesting that neurochemical phenotype may be an important unifying feature of these projections.

INTRODUCTION

In recent years, investigations into the organization of the motor system have demonstrated the existence of numerous somatotopically arranged motor fields in the primate cingulate cortex (Muakkassa and Strick, 1979; Hutchins et al., 1988; Shima et al., 1991; Dum and Strick, 1992; Dum and Strick, 1993). At least two distinct motor areas have been described; one in the anterior portion of the ventral bank of the cingulate sulcus (CMAr), and one or two in the posterior portion, located in either the ventral (CMAv) or the dorsal bank (CMAd; Dum and Strick, 1991; Dum and Strick, 1992; He et al., 1995). These areas have been defined by physiological recording methods (Luppino et al., 1991; Shima et al., 1991), or by the presence of neurons projecting directly to the spinal cord (Hutchins et al., 1988; Dum and Strick, 1991; He et al., 1995) or to the primary motor cortex (M1; Muakkassa and Strick, 1979; Morecraft and Van Hoesen, 1992). Thus far, no reliable anatomic marker has been demonstrated for any of these areas.

Neurofilament protein immunoreactivity has proved to be very useful as both an indicator of regional specialization and as a mechanism to discriminate corticocortical circuits in macaque neocortex (Campbell and Morrison, 1989; Campbell et al., 1991; Hof and Morrison, 1995; Hof et al., 1995; Hof et al., 1995; Hof et al., in press; Hof et al., submitted). In the cingulate cortex, the distribution of neurons enriched in neurofilament protein changes dramatically as one proceeds along the cingulate gyrus from rostral to

caudal levels (Hof and Nimchinsky, 1992). In general, the anterior cingulate cortex, in all its subdivisions, is characterized by immunoreactive pyramidal neurons in layers V and VI, and virtually none in layers II and III. The posterior portion of the cingulate cortex has comparable labeling in the deep layers, but is characterized by the presence of numerous strongly immunoreactive neurons in layer III. The site where these layer III neurons first appear is in the most dorsal portions of the cingulate gyrus, in the cortex lining the cingulate sulcus. The region bounded anteriorly by the first appearance of these neurons and posteriorly with a well-populated layer III was described as representing a "transition zone" between the anterior cingulate area 24 and posterior cingulate area 23 (Hof and Nimchinsky, 1992). However, the transition zone thus defined has a much greater anterior-to-posterior extent than the transition as defined by the Nissl stain. Since the neurons that best characterize this region are located in the superficial layers of the cortex, which are known to give rise to corticocortical projections (Jones, 1984), and since connections with the primary motor cortex are one of the distinguishing features of the cingulate motor areas (Luppino et al., 1990; Morecraft and Van Hoesen, 1992), it is possible that rather than merely signalling a cytoarchitectonic transition between two different areas, these neurons indicate the existence of a distinct area, quite independent of either the "anterior" and "posterior" cingulate cortex.

Overall, neurofilament protein-containing pyramidal neurons are believed to constitute about 20% of the total number of pyramidal neurons; however, there is extreme regional variation in their proportion (Hof et al., 1990; Hof and Morrison, 1990; Del Río and DeFelipe, 1994). However, studies combining retrograde transport and immunocytochemistry in the macaque monkey have shown that the neurofilament protein-containing fraction of the pyramidal neurons comprising a given corticocortical projection varies with the projection (Campbell et al., 1991; Hof et al., 1995; Hof et al., in press; Hof et al., submitted). For instance, 75% of the layer II and III neurons projecting from prefrontal association area 46 to the ipsilateral superior temporal sulcus contained

neurofilament protein, but this proportion dropped to only 42% of the neurons in this area and layer in the projection to the inferior parietal cortex (Hof et al., in press). In the most extreme example, the commissural projection from the border between visual areas V1 and V2 is close to 100% neurofilament protein-immunoreactive (Hof et al., 1995). Such findings indicate that one of the key intrinsic properties of a corticocortical projection is its proportion of neurofilament protein-enriched neurons. We have been investigating the degree to which particular processing streams or functional categories of corticocortical circuits can be distinguished by their neurofilament protein content as a major reflection of neurochemical phenotype. In the present context, this characteristic might be useful in determining the degree to which the numerous cingulate motor areas provide inherently different projections with unique neurochemical properties.

Another quality that characterizes corticocortical projections is their laminar origin. Projections from cytoarchitectonically different cortical areas tend to originate in different cortical layers, with those from less differentiated cortical areas, such as area 24, originating predominantly from deep layers, and those from more differentiated areas, such as area 23, taking origin also from superficial layers (Barbas, 1986). The anterior and posterior CMAs have been reported to be located in very different cytoarchitectonic areas, with the anterior CMA in an agranular cortical field (area 24) lacking, by definition, a layer IV, and the posterior CMA in an area (area 23) that possesses a well-developed layer IV (Dum and Strick, 1991). The laminar distribution of their neurons of origin might, therefore, be another distinguishing characteristic of the CMAs, and since cortical and subcortical afferents terminate in specific laminar patterns, this property could have functional implications as well.

Another potentially distinguishing characteristic of some cortical efferent projections is the morphology of their participating neurons. Generally, the morphology of neurons providing a given corticocortical projection is diverse (De Lima et al., 1990), but there are examples where the morphology is so distinctive that it is a defining

attribute of the projection. For instance, the Meynert cell is morphologically distinctive and generally projects from the primary visual cortex to area MT (Lund et al., 1975; Tigges et al., 1981; Fries et al., 1985), and the Betz cells in the primary motor cortex uniformly contribute to corticospinal tract (Lassek, 1940; Lassek, 1941). The human cingulate cortex contains numerous distinctive and unusual cell types, most notably the gigantopyramidal cells in layer V in the cingulate sulcus (Braak, 1976), and the spindle neurons in layer Vb in area 24 (Nimchinsky et al., 1995b). The macaque cingulate cortex appears to lack these obvious examples, but more subtle differences in the morphology of pyramidal neurons in the anterior and posterior cingulate cortex have been described in the rat (Vogt and Peters, 1981) and human (Schlaug et al., 1993), and could be found in the monkey as well. Finally, in view of the consistency of the neurofilament protein-containing neuronal population within a given projection, it would be interesting to determine whether a correlation exists between the morphology of a pyramidal neuron and its content of neurofilament protein. If such correlations existed, they would suggest that functional classes of corticocortical circuits have unique and identifiable cell biological signatures. In order to further our understanding of the relationships between chemoarchitecture and the cell typology, connectivity and neurochemical characteristics of neurons furnishing corticocortical projections, we analyzed the laminar origin, morphology and neurofilament protein profile of two distinct yet related convergent projections from the cingulate cortex to area M1.

MATERIALS AND METHODS

Surgical procedures

Five cynomolgus monkeys (*Macaca fascicularis*) were used in the present study. All experimental protocols were conducted within NIH guidelines for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mount Sinai School of Medicine. The animals received injections into

the forelimb representation of the primary motor cortex. The locations of these injections were not mapped physiologically, but were determined on the basis of previous reports (Woolsey et al., 1952; Kwan et al., 1978; Muakkassa and Strick, 1979; Godschalk et al., 1984; Huntley and Jones, 1991; Lu et al., 1994). After the opening of a 3 cm-long craniotomy and reflection of the dura, the central sulcus and posterior portion of the arcuate sulcus were exposed. Aqueous solutions of Fast Blue (FB; 4%; Sigma, St. Louis, MO) and Diamidino Yellow (DY, 4%; Sigma) were injected into the left and right hemispheres, respectively, using a 5 μ l Hamilton microsyringe with a 24 gauge needle. Injections were placed at depths from 0.8 to 1.4 mm below the pial surface at the horizontal level of the genu of the arcuate sulcus, and no more than 2-3 mm anteriorly to the central sulcus, in order to avoid involving the premotor cortex. Ten to twelve 500 nl injections were placed in each site and none penetrated the underlying white matter. The locations of the injections are illustrated in Figure 5.1. Following the injection procedure, the dura was closed and covered with a layer of Gelfoam, and the muscle, galea aponeurotica and skin closed in layers.

Tissue preparation and staining procedure

After a survival time of three weeks to permit optimal retrograde transport (Campbell et al., 1991; Hof and Morrison, 1995) the animals were perfused intracardially as previously described (Hof and Nimchinsky, 1992; Hof and Morrison, 1995). Briefly, the animal was deeply anesthetized with ketamine hydrochloride (25 mg/kg) and pentobarbital sodium (20-35 mg/kg i.v., as necessary), intubated and mechanically ventilated. The chest was then opened, the heart exposed, and 1.5 ml of 0.1% sodium nitrite was injected into the left ventricle. The descending aorta was clamped and the monkeys were perfused transcardially with cold 1% paraformaldehyde in phosphate-buffered saline (PBS) and then for 8-9 minutes with cold 4% paraformaldehyde in PBS. Following perfusion, the brain was removed from the skull, photographed from dorsal, medial and lateral aspects. The temporal lobe was removed for use in other studies. The

brain was blocked in the following manner: the hemisphere that received FB injections was cut coronally caudally to the splenium, and the anterior block containing the frontal and parietal lobes was cut in two parts midway through the cingulate gyrus. These two blocks were postfixed for 6 hours in 4% paraformaldehyde, then immersed in increasing concentrations of sucrose in PBS (12, 16, 18, 30%), remaining in each solution until they sunk. They were then frozen on dry ice and cut at 40 μ m on a freezing microtome. Four consecutive 1:10 section series were collected: the first was mounted immediately for analysis of cell distribution, the second and fourth were placed in PBS and stained immunocytochemically as described below with fluorescence or 3,3'-diaminobenzidine (DAB), respectively, and the third was mounted and stained with thionin. In addition, at levels containing the injection sites, a 1:5 section series was taken and stained with thionin. Five sections for the thionin series were first processed for immunocytochemistry but without primary antibody, to serve as negative controls, and to allow for the quantification of tissue shrinkage due to immunocytochemistry.

Immunocytochemistry was performed using antibody SMI-32 (Sternberger Monoclonals, Baltimore, MD). This antibody recognizes a nonphosphorylated epitope on the medium (168 kDa) and heavy (200 kDa) molecular weight subunits of the neurofilament triplet protein (Sternberger and Sternberger, 1983; Lee et al., 1988). Sections were immersed for two days in a primary antibody solution of SMI-32 at a 1:10,000 dilution for brightfield microscopy, or 1:5000 for fluorescence in a diluent containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin in PBS. They were then rinsed thoroughly, and for brightfield analysis, were processed with the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine as a chromogen. Sections were then mounted, intensified with 0.067% osmium tetroxide, dehydrated, and coverslipped with DPX mounting medium. For fluorescence microscopy, after rinsing following the incubation in primary antibody, the sections were immersed in a solution of biotinylated anti-mouse IgG secondary antibody

(Vector Laboratories; 1:200) in the same diluent for two hours, rinsed again, and then placed into a solution containing FITC-avidin D (Vector Laboratories; 1:200) for two hours. They were then rinsed again and mounted, and coverslipped with Permafluor.

Quantitative regional and laminar analysis

Quantitative analyses were performed using a computer-assisted morphometry system consisting of a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage (Zeiss, Oberkochen, Germany), a Zeiss ZVS-47E video camera system (Zeiss, Thornwood, NY), a Macintosh 840 AV microcomputer, and custom designed morphometry software developed in collaboration with the Scripps Research Institute (La Jolla, CA; Young et al., 1995). This software (NeuroZoom) was designed to permit mapping and quantitative analysis of neurons and neuronal populations at different levels of resolution. Injection sites were recorded in two ways. First, scaled photographic slides of the whole brains were mapped using NeuroZoom with a Zeiss NeoFluar 1.25x objective and the locations of the injections were drawn (Fig. 1A). Then, thionin-stained sections through the injections themselves were plotted using a Zeiss NeoFluar 1.25x objective, and the extent of the injections were indicated on these coronal maps (Fig. 5.1B,C). For quantitative maps of retrogradely labeled neurons, sections were analyzed using epifluorescence with a Zeiss Plan Neofluar 40x objective and a Zeiss UV filter to detect retrogradely labeled neurons, and an FITC narrow-pass filter to determine whether they were neurofilament protein-immunoreactive. A Zeiss Fluar 10x objective was used to outline the pial surface, the white matter border, and the border between layers III and V. A red filter was used to discriminate between lipofuscin, which emits also in the red range, and fluorescein, which does not. A neuron was considered to contain DY if its nucleus was clearly fluorescent and it was considered neurofilament protein-immunoreactive if its perikaryon was immunoreactive. Neurons whose perikarya were non-immunoreactive but whose proximal dendrites appeared to be so were not considered immunoreactive if it were not possible to establish continuity

between the soma and the labeled dendrites. The same criteria were used for FB-containing neurons, and although in this case, neurons with immunoreactive dendrites could be clearly attributed to a non-immunoreactive soma, these were not considered to contain neurofilament protein since this would inflate the number of immunoreactive FB-containing neurons relative to the DY-containing neurons. However, they were counted separately, and constituted only 3.5% of the total number of FB-containing neurons.

In each animal, all sections stained with antibody SMI-32 were systematically evaluated. All retrogradely labeled neurons were quantified and evaluated for neurofilament protein immunoreactivity. Since the cingulate motor areas often reside in part in the depths of the cingulate sulcus, where layers are compressed, no attempt was made quantitatively to distinguish between layers V and VI. A live RGB image of the microscopic field was ported to the computer screen and frozen to prevent undue bleaching of the fluorescent signal. Retrogradely labeled neurons were mapped with symbols that indicated their retrograde label (FB, DY or both), whether they were neurofilament protein-immunoreactive, and whether the immunoreactivity was restricted to the dendrites. After mapping all points detectable at 40x, the pial and white matter borders, as well as the border between layers III and V were mapped at 10x. It was possible to move between objectives, keeping complete fidelity of cell and contour spatial locations (Young et al., 1995). Upon completion of the map, areas of interest, such as individual layers in cingulate subareas, could be outlined and the number of points of each structure type reported. Numbers were expressed as the total number of retrogradely labeled neurons in the anterior or posterior projection for each animal. Ipsilaterally and contralaterally projecting neurons were quantified and analyzed separately. Previously published criteria for marking the boundary between the rostral and caudal cingulate motor areas, for instance the posterior end of the arcuate spur (Morecraft and Van Hoesen, 1992), were not always useful, as animals varied considerably in their sulcal patterns. However, the anterior commissure proved to be a reliable landmark, less

affected by slightly different angles of section, and corresponded well stereotactically to the level of the arcuate spur (Szabo and Cowan, 1984). Such landmarks were rarely necessary, however, since the retrogradely labeled neurons generally had a bimodal spatial distribution, and the anterior and posterior projections were clearly separable. Since the underlying statistical distribution was unknown, a chi-square test was used to analyze the difference in neurofilament protein immunoreactivity between anterior and posterior projections in layers III and V-VI in each animal.

Cell loading, reconstruction and confocal analysis

In three cases (CYN2-4), the cingulate gyrus was dissected out and cut into approximately 5 mm-thick coronal blocks and postfixed for two hours in 4% paraformaldehyde. Then the blocks were placed into PBS and cut at 300 μm on a Vibratome. For intracellular injection, the sections were mounted on nitrocellulose filter paper and immersed in PBS. Fast Blue or DY-containing neurons were identified under epifluorescence with a UV filter, impaled, and loaded with 5% Lucifer Yellow (Molecular Probes, Eugene OR) in distilled water under a DC current of 3-8 nA for 10-12 minutes, or until the dye had filled distal processes and no further loading was observed (De Lima et al., 1990). Sections containing filled cells were then coverslipped with Vectashield mounting medium and reconstructed using the microscopy system and NeuroZoom software described above. Cells were located using a Zeiss Fluar 10x objective and drawn using a Zeiss Apochromat 100x objective with a numerical aperture of 1.4. A live RGB image was ported to the computer screen and mapping was performed by moving the stage in 1 μm steps through the z axis along the length of each dendrite. Spines were plotted at the same time. Then the section itself was drawn at 10x. In this manner, the x, y, and z coordinates of each dendritic segment of spine was recorded to enable later three-dimensional representation and rotation of the reconstructed neuron, as well as rendering of the neuron in a macroscopic context. In order to compare the spine densities of the reconstructed neurons, an oval was drawn, centered on the

soma, which excluded the most distal portions of the dendritic tree. This was necessary because since many dendrites extended deep into the section, and while the dendritic shaft could be resolved at depths in excess of 150 μm into the section, Lucifer Yellow-filled dendritic spines became difficult to resolve. Thus the apparent low spine density at the tips of dendritic branches could not be relied upon (see, for instance, the terminal branches of the apical dendrite of neuron D in Figure 5.12). The total dendritic length and number of spines enclosed in the oval was obtained, and the number of spines per length of dendrite calculated. After reconstruction, the section was rinsed, cryoprotected in 25% sucrose, and subsectioned on a cryostat at 30 μm . Immunocytochemistry was performed as described above, using a secondary antibody conjugated to the cyanine dye Cy-5 (Jackson ImmunoResearch, West grove, PA), and the sections were mounted and coverslipped. The labeled neurons were located again using conventional fluorescence microscopy, and scanned on a confocal laser scanning microscope (Zeiss LSM 410) using a 63x or 40x objective. For visualization of Lucifer Yellow, an ArKr 488/568 laser and a 515-565 bandpass emission filter were used. For visualization of immunoreactivity with Cy-5, a HeNe 647 nm laser was used with a 670-810 bandpass emission filter. Cy-5 was used because lower wavelength dyes, such as those that emit in the visible red range, cannot be adequately separated from Lucifer Yellow emission, which is very bright and extends well above 600 nm (Stewart, 1981; Haugland, 1992). The cells were scanned sequentially with the two lasers, and immunoreactivity, or the lack thereof, was thus determined. Somatic staining was determined, as well as the presence or absence of immunoreactivity in individual dendrites or dendritic segments. Lack of staining of the neuron due to incomplete penetration could be confirmed by observing staining in the surrounding neurons.

RESULTS

Chemoarchitecture of the dorsal cingulate gyrus

At least four separate areas could be readily distinguished in the cortex lining the ventral bank of the cingulate sulcus on neurofilament protein-immunostained material, whereas such differences were less obvious in Nissl-stained sections (Fig. 5.2). Most anteriorly, beginning rostral to the genu of the corpus callosum, area 24c was characterized by virtually no staining in layers I to III, and relatively lightly immunoreactive neurons in layers V and VI, whose well-stained apical dendrites coursed through the cortex in large bundles (Fig. 5.2B). Moving caudally, occasional intensely immunoreactive neurons appeared in the deep portion of layer III. These defined another area, which in the present article is referred to as rostral area 24c' (Fig. 5.2D), using the nomenclature of Vogt et al. (1993). The density of these deep layer III neurons remained fairly constant until more caudally, at approximately the level of the anterior commissure their frequency abruptly increased, marking the beginning of the caudal portion of area 24c' (Fig. 5.2F). This area was characterized by the highest density of these layer III neurons in the cingulate gyrus. They often occurred in clusters, and were frequently very large. There was a marked increase in the staining intensity of the neuropil in layer III, as large, well ramified neurons were labeled. There was also increased neurofilament protein immunoreactivity in the neuropil in layer V, which contained few intensely immunoreactive somata. Layer VI was broad and readily distinguishable from layer V, and it contained numerous intensely immunoreactive neurons and extensive dendritic labeling. Finally, in area 23c, the density and size of the deep layer III neurons decreased to a pattern of more regularly spaced, medium-to-large pyramidal neurons (Fig. 5.2H). Strongly immunoreactive neurons were present in layer V, and immunoreactivity in layer VI was greatly reduced in quantity and intensity. Although certain differences could be resolved using the Nissl stain, as between 24c and 23c (Figs 5.2A and 5.2G, respectively), the distinctions among areas 24c and rostral and caudal 24c' were much

more difficult to discern. In contrast, the cortex lining the medial surface of the hemisphere above the cingulate sulcus, in particular the region defined by retrogradely labeled neurons as the SMA (see below) was distinguishable from all these areas (Fig. 3). With neurofilament protein immunoreactivity, this area was characterized by a much greater population of large, intensely immunoreactive neurons in layer III than in the cingulate gyrus. These neurons were not restricted only to the deepest portion of layer III, but could be found throughout the layer. Layer V was remarkable for periodic large immunoreactive neurons, and layer VI was very broad and with many well stained multipolar neurons and dendrites (Fig. 5.3B). In Nissl-stained material, this area had no layer IV, and differed most from the cingulate areas in its absence of a prominent, compact layer V (Fig. 5.3A). Occasional very large neurons were found in layer V, but these were far less frequent than those in the primary motor cortex.

Regional and laminar distribution of retrogradely labeled neurons

Ipsilaterally projecting neurons constituted by far the majority of retrogradely labeled neurons. They were found in a number of locations in the cingulate sulcus. In general, there was a sparse rostral projection originating overwhelmingly from deep layers, and a much more sizable caudal projection that took origin from superficial and deep layers (Fig. 5.4A, B). The rostral projection (rostral cingulate motor area, CMAr) was found in area 24 at levels anterior to the tip of the rostrum of the corpus callosum, mostly in the depth of the cingulate sulcus, and shifting dorsally into the dorsal bank, eventually meeting the forelimb projection of the supplementary motor area (SMA) dorsally (Fig. 5.5). The caudal projection (caudal cingulate motor area, CMAc) began in the ventral bank of the cingulate sulcus, and as described (Morecraft and Van Hoesen, 1992), spiraled through the depth of the cingulate sulcus, ending in the dorsal bank. The shift in the location of this projection was continuous and gradual, and no objective demarcation was able to separate the projection in the ventral from that in the dorsal bank (Fig. 5.6). At some levels the SMA would extend past the lip and into the cingulate

sulcus with no apparent discontinuity (Fig. 5.5F). However, the posterior CMA was in most cases clearly distinct from the SMA. Occasionally the CMA and SMA seemed to merge for a few sections, but a dividing line could be drawn based on more rostral and caudal sections. The contralateral projections, while much sparser and with different laminar distributions, had locations much like those of the ipsilateral projections, and usually overlapped with them considerably (Fig. 5.7).

As a result of individual differences among animals, and the variability among injection sites (Fig. 5.1), the precise localization of the rostral and caudal cingulate motor areas varied greatly among animals (Figs 5.8, 5.9). However, in all cases, the locations of the projections corresponded to chemoarchitectural features, with the anterior projection located in rostral 24c', and the posterior projection in caudal 24c'. No retrogradely labeled cells were found at levels that had no neurofilament protein-immunoreactive neurons in layer III, or at levels containing a clear layer IV or other characteristics of area 23c (Fig. 5.2 G, H).

Case CYN3 had virtually no retrogradely labeled neurons projecting ipsilaterally to area M1 that would correspond to an anterior projection (Fig. 5.8). This was not due to lack of transport, since the posterior projection was amply represented, or to some anomalous interruption of fibers leading into this area, since there was a contralateral anterior projection. In all other cases, the retrogradely labeled neurons located anteriorly originated largely in layer V and, to a lesser extent, in layer VI. The proportion of projecting neurons found in layers V and VI varied from 77 to 95% in the anterior projection (mean = 88%; Table 5.1). The location of the posterior projection was somewhat more variable. It was much larger in rostrocaudal extent than the anterior projection, and its cells far more numerous (Fig. 5.9). Retrogradely labeled neurons were evenly distributed in superficial and deep layers (51% and 49%, respectively; Table 5.1). In case CYN1 both anterior and posterior projections to ipsilateral area M1 were very sparse. The anterior projection was especially small, but, in contrast to that of CYN3, it

was clearly defined and spanned several consecutive sections (Fig. 5.8). The posterior projection had a bimodal distribution, with a trough of low numbers of cells between two larger projections (Fig. 5.8). Cases CYN2, CYN4 and CYN5 were generally comparable in the size and distribution of their anterior projections, although in CYN2, both rostral and caudal projections were shifted anteriorly (Fig. 5.8).

Neurons projecting to the contralateral forelimb region of area M1 were less numerous, but also showed anterior and posterior distributions, even in case CYN3, which lacked an anterior ipsilateral projection (Fig. 5.9). As in the ipsilateral anterior projection, the contralateral anterior projection arose mostly from deep layers (89%), and the posterior projection had a contribution from layer III (Table 5.1). However, even in the posterior projection, retrogradely labeled neurons were more numerous in deep than in superficial layers (72 vs. 28%, respectively). In addition, they were very frequently located in layer VI, not V, as were the ipsilaterally projecting neurons.

Neurons containing both retrograde labels, and that therefore projected to area M1 in both hemispheres were encountered, albeit infrequently. Of the 75 such neurons counted, 81% were in layers V and VI. The FB- and DY-containing neurons found in layer III almost always belonged to the group of large, intensely immunoreactive neurons along the border between layers III and V.

Neurofilament protein content

When retrogradely labeled neurons were analyzed for the presence of neurofilament protein, laminar differences arose (Table 5.2; Figs 5.5-5.7). In the posterior projection, ipsilaterally projecting neurons in layer III were much less likely to contain neurofilament protein in their somata than those in layers V and VI (38 vs. 61% overall, respectively; Fig. 5.6). However, these layer III neurons were just as likely to contain neurofilament protein as the far scarcer layer III neurons in the anterior projection (28 vs. 38% overall, respectively; $p > 0.05$, Chi square test). Layer V and VI neurons in general were also equally likely to contain neurofilament protein anteriorly as posteriorly

(61% overall for both, $p > 0.05$), although in two animals, significant differences were observed, with layer V and VI neurons in the anterior projection more likely than those in the posterior projection to contain neurofilament protein (Table 5.2).

As in the ipsilateral projections, contralaterally projecting neurons in layer III were less enriched in neurofilament protein than those in layers V and VI, but the proportion was higher than for the ipsilaterally projecting neurons. In addition, their numbers were very low overall, making comparisons difficult to interpret (Fig. 5.7). The proportion of immunoreactive layer V and VI neurons in the contralateral projections was comparable to that of the ipsilaterally projecting neurons in the same layer (Table 5.2). As was the case with the ipsilateral projections, there was generally no difference in the proportion of neurons containing neurofilament protein between the anterior and posterior projections for layer III or for layers V and VI. In one animal (CYN5), layer V and VI neurons in the posterior projection were significantly more likely to contain neurofilament protein than cells in the anterior projection in the same layers ($p = 0.02$), but this was the only instance of such a difference. Neurons containing both FB and DY, while scarce, were the population most likely to be neurofilament protein-immunoreactive, with 86% in layer III and 84% in layers V and VI containing neurofilament protein.

Analysis of retrogradely labeled neurons in the SMA in two animals indicated that the proportion of neurofilament protein-containing neurons in this projection was comparable to the proportions found in anterior and posterior CMAs (39% for layers II and III; 64% for layers V and VI).

Neuronal morphology

Morphologic studies of Lucifer Yellow-loaded retrogradely labeled neurons projecting from anterior and posterior CMA to the forelimb region of area M1 revealed heterogeneity, with certain recurring features (Table 5.3; Fig. 5.10). Virtually all layer III neurons, whether located anteriorly or posteriorly, were typical pyramidal neurons. In

layers V and VI, however, atypical pyramidal cells were often encountered, more frequently in anterior than in posterior cingulate cortex. For instance, pyramidal cells with one major basal dendrite which was strongly oriented in one direction, either toward the midline or the lateral aspect of the brain, constituted 39% of the layer V and VI cells studied in anterior cingulate, and of these, 71% had their major basal dendrite directed laterally (Fig. 5.11G-J). In posterior cingulate, 11% had major basal dendrites with a preferred orientation, in all cases laterally. Fusiform cells constituted 17% of the deep layer cells in CMAR, but only 7% in CMAc (for example, see Fig. 5.11C). Following reconstruction, confocal microscopic analysis of immunocytochemically stained cells revealed no clear correlation between somatodendritic morphology and neurofilament protein content. For instance, the two layer III neurons shown in Figure 5.13 (reconstructed in Figs 5.12A and C), both projecting ipsilaterally, and with extensive dendritic arborization differed markedly in their neurofilament protein content, one of them having a strongly immunoreactive soma and apical dendrite and non-immunoreactive basal dendrites, and the other with a non-immunoreactive soma and apical dendrite, but with a few very lightly immunoreactive secondary dendrites. The presence or absence of immunoreactivity bore no apparent relationship with the order or caliber of the dendrite in question (Fig. 5.13G-L). Furthermore, analysis of 17 reconstructed neurons revealed no correlation between spine density and neurofilament content (Table 5.4). For instance, the neurons described above (5.12A and 5.12C) had comparable spine densities (0.439 and 0.460 spines per μm of dendritic length, respectively). Conversely, there was no clear morphologic feature common to all neurofilament protein-enriched neurons, or to all those which lacked neurofilament protein. Neurons in both groups included typical pyramids, fusiform neurons, and asymmetric forms (Figs 5.11, 5.12).

DISCUSSION

Location and laminar origin of the cingulate projections to area M1

The present study reveals certain differences and similarities between the projections from CMAr and CMAc to the forelimb region of the primary motor cortex. The primary differences are in overall size, laminar origin and the somatic morphology of the constituent neurons. However, the neurochemical phenotype as reflected in neurofilament immunoreactivity is similar in both cingulate motor projections and in the SMA. Finally, this study describes the existence of a reliable marker for the cingulate motor areas, namely its distinctive pattern of neurofilament protein distribution.

One feature of the cingulate motor areas is the variability of their placement along the cingulate gyrus. In general, the anterior edge of the anterior commissure tended to fall between the anterior and posterior projections, and being in a line with the projection in question, it was less susceptible to variability in the angle of section than a landmark such as the caudal end of the arcuate spur. Furthermore, the extreme degree of variability in sulcal patterns renders this latter guide much less reliable. However, these landmarks, reliant as they are on features unrelated to the cingulate motor projections, were not as useful or consistent as the chemoarchitectonic ones described in the present study. It was clear, though, that the posterior projection, at least for the forelimb representation, resides not in area 23c as previously reported (Dum and Strick, 1991; Shima et al., 1991; Morecraft and Van Hoesen, 1992), but in an agranular or dysgranular area here referred to as 24c'. This is in agreement with the conclusions of Luppino et al. (1991), who referred to this area as area 24d.

Features of the ipsilateral anterior projection consistent across all animals but one (CYN3) were its sparseness, its preferential location in the depths of the cingulate sulcus, and its origin almost exclusively in deep layers. It is notable that in case CYN3, no anterior ipsilateral projection was observed. Examination of the injection site lends support to an earlier finding that the anterior wall of the central sulcus in the forelimb

region, to which this injection site was virtually restricted, does not project to CMAR (Holsapple and Strick, 1991).

The absence of a separate, dorsal posterior projection corresponding to the CMAd earlier described was the only significant departure in the present data from earlier reports of these projections (Hutchins et al., 1988; Dum and Strick, 1991; He et al., 1995). However, other descriptions have also been unable to make an anatomic distinction between projections in the ventral and dorsal banks of the cingulate sulcus (Morecraft and Van Hoesen, 1992; Galea and Darian-Smith, 1994; Rouiller et al., 1994). It is possible that this discrepancy is due to alternative interpretations of the cortical distribution patterns, and differences arising from the use of coronal sections as opposed to flat maps to illustrate neuronal distributions (Hutchins et al., 1988; Dum and Strick, 1991; He et al., 1995). Thus the present study recognizes only two cingulate motor areas: a sparse anterior one originating largely from deep layers, and a much larger posterior one spanning both banks of the cingulate sulcus, that originates from superficial and deep layers, and comes close to, but is completely separate from, the SMA.

The ipsilateral posterior projection differed most from the anterior projection in that it took origin equally from superficial and deep layers. Part of the reason for this may be that the posterior CMA is closer than the anterior CMA is to the primary motor cortex, and therefore to the injection site, and many pyramidal neurons in superficial layers, especially in layer II and the superficial portion of layer III, make short corticocortical connections (Jones and Wise, 1977). However, many, and at some levels most of the retrogradely labeled layer III neurons were located in the deeper portions of layer III, so while these neurons project the same distance as the layer II neurons, they may be of a different type, more specialized than those that connect all cortical areas with their immediate neighbors. It may also be a result of the fact that the anterior-most portion of the cingulate cortex is somewhat less differentiated than slightly more caudal levels, in that the former has no granular layer IV, whereas as one proceeds caudally,

small granular neurons begin to appear between layers III and V constituting an incipient layer IV, which becomes more developed in the isocortical posterior cingulate area 23. While both motor projections in the present study were located in area 24c', the caudal one may have more of the characteristics of more differentiated cortex, namely a tendency for its corticocortical projections to originate in superficial and deep layers, in contrast to less differentiated cortex, whose projections originate mostly from deep layers (Barbas, 1986). This conclusion supports the contention that significant changes in the laminar origin of corticocortical connections can occur even with subtle cytoarchitectonic changes (Barbas, 1986).

The contralateral projection originated preferentially in deep layers, whether as part of the anterior or the posterior projection. In fact, these neurons were most frequently found in layer VI, and often abutted the white matter border. This is in agreement with another account of this projection (Rouiller et al., 1994), and is in marked contrast to most other callosal projections in the macaque, which tend to come from neurons in layer III (Innocenti, 1986; Hof et al., in press). The only other callosal projections in the primate described as originating significantly in layer VI are the homotopic projection to M1 (Zant and Strick, 1978) and the homotopic commissural projection from the hand representation of the SMA, which, however, also originates from superficial layers (Rouiller et al., 1994). Thus a large contribution from layer VI to commissural projections may be a common feature of motor areas in general.

It is important to note that the present study made use only of the putative forelimb representation to define motor areas. This fact does not affect the preceding interpretation, however, since after the face representation, the forelimb representation in the anterior CMA is the most rostral, followed by the hindlimb, then in the posterior CMA by forelimb and hindlimb representations as well (Dum and Strick, 1991; Luppino et al., 1991; Morecraft and Van Hoesen, 1992; He et al., 1995). Thus, the first appearance of retrogradely labeled neurons in this study marks the onset of the

representation of the body below the neck, the low density area in between the two projections probably accommodates the anterior hindlimb representation, and their disappearance caudally represents the end of the caudal forelimb representation. This would explain why retrogradely labeled neurons disappear before the beginning of area 23c, since it would make sense for the posterior hindlimb projection to be in an area cyto- and chemoarchitectonically similar to the one that contains the posterior forelimb projection. In fact, in case CYN4, whose contralateral injection extended dorsally well beyond the forelimb region, the distribution of retrogradely labeled neurons in the contralateral cingulate gyrus was very extensive in the rostrocaudal axis for both anterior and posterior projections. In this case, the involvement of hindlimb projections partially filled in the gaps seen with the other injections, further supporting previous reports of the somatotopy of this region.

Differential neuronal morphology in the cingulate motor areas

The greater morphologic diversity observed in the anterior projection than in the posterior projection is another feature distinguishing the two. It is possible that the greater variability in somal shape could be explained by a preferential localization in layer VI, where atypical cell types predominate (Tömböl, 1984). However, there was no greater tendency for the cells in question to be located in layer VI anteriorly than posteriorly. The spine density reported here for layer III neurons is on the order of earlier estimates (Globus and Scheibel, 1967; Kemper et al., 1973; Parnavelas et al., 1973). The slightly lower figures in this study may be the result of the inclusion of the initial segments of the dendrites, which lack spines, in the calculations. The lower spine density for neurons in layers V and VI than in layers II and III is in agreement with other studies (Kemper et al., 1973), although the occasionally very low densities reported in the present study may point to a distinctive feature of these projections. The finding of more unusual pyramidal cell forms anteriorly is in agreement with Vogt (1976), who described trends in morphologic types in the posterior cingulate cortex in the macaque. In that study,

cortical areas such as areas 29 and 30, which are considered to be less differentiated, in that they lack many of the features of isocortex, are characterized by pyramidal neurons with less elaborate basal dendritic trees. In fact, many have fusiform somata. As the cortex in area 23 approaches isocortical cytoarchitecture, basal dendritic trees become more extensive, and neuronal packing density is decreased as a result. The present study does not describe such anatomic differences in the ventral-to-dorsal direction, since the cingulate motor areas exist only in the dorsalmost part of the cingulate gyrus. However, similar arguments for progressive cortical elaboration have been made for the cortical changes in the anterior-to-posterior direction as well, with the anterior cingulate cortex representing less differentiated cortex in contrast to the isocortical posterior cingulate cortex (Vogt et al., 1987; Hof and Nimchinsky, 1992). The present study suggests that a neurocytological trend evident in the progression from ventral to dorsal regions of the gyrus also exists in the similar anterior-to-posterior progression toward isocortex. Finally, in the depths of the human cingulate sulcus, there is a cortical field characterized by the presence of giant layer V pyramidal neurons which are remarkable for their dense lipofuscin deposits (Braak and Braak, 1976). Golgi-stained examples of these neurons resemble certain of the neurons described in the present study in that their apical dendrites bifurcate repeatedly, beginning not far from the soma. However, unlike the neurons of the human cingulate cortex, those in the present study have apical dendrites with numerous secondary branches, and lack spine-like appendages on the soma. It is also of note that distinctive spindle-shaped neurons are conspicuous in layer Vb of the human anterior cingulate cortex (Nimchinsky et al., 1995b). Whatever the role of these neurons, these studies in the human indicate that the presence of atypical cell types is a characteristic feature of the anterior portion of the cingulate cortex in general and the cortex of the cingulate sulcus in particular, something the present study suggests is true in the macaque for even a restricted set of projections.

Neurofilament protein represents a reliable marker for cingulate motor areas

The only truly reliable indicators of the presence of the cingulate motor areas were chemoarchitectonic, specifically the presence of large, wide and clustered neurofilament protein-containing pyramidal neurons in the deep portion of layer III, and intensely immunoreactive neurons in a broad layer VI. Although cytoarchitectonic differences may be detectable with the Nissl stain, they are subtle at best. The presence of large neurofilament protein-containing neurons in layer III had earlier been recognized as a feature distinguishing the caudal portions of the cingulate gyrus from the most rostral part (Hof and Nimchinsky, 1992). These cells were observed to appear at first gradually, and increase in density as one proceeded from the rostral to the caudal parts of the gyrus. This seemed to correlate with the approximate position of the cingulate motor areas. The present study confirms this finding, with the following observations: first, no retrogradely labeled neurons were observed in areas devoid of layer III neurofilament protein-containing neurons. Second, in all five animals analyzed in the present study, the first increase in the density of layer III neurofilament protein-containing neurons correlated with the first appearance of retrogradely labeled neurons, and the further, almost stepwise increase in their density corresponded with the beginning of the caudal projection. The presence of the distinctive chemoarchitecture of area 23c was a reliable indicator that the caudal end of the projection had been reached. Third, deep layer III neurofilament protein-containing neurons were themselves occasionally retrogradely labeled, suggesting that these neurons play a direct role in the motor projection itself, and were not merely cytoarchitectonic markers.

Similarly, large, intensely immunoreactive neurons in layer VI were frequently retrogradely labeled, very often from the contralateral hemisphere. This fact, and the marked diminution of layer VI in area 23c as seen in neurofilament protein-stained material, suggest that this chemoarchitectonic feature, like the layer III neurons, indicates the presence of the motor area, and perhaps more specifically a contralateral contribution. Finally, the present study demonstrates that neurofilament protein immunoreactivity is a

useful marker not only for the cingulate motor areas in general as distinct from the rest of the cingulate cortex, but is also able to distinguish between the anterior and posterior projection areas. This is an unusually fine regional subdivision, made possible by the fact that throughout its extent, the cingulate gyrus exhibits fewer neurofilament protein-immunoreactive neurons than other cortical areas, even those just across the cingulate sulcus, such as the SMA (Fig. 3). As a result, the superimposition of the CMAs with their large layer III and layer VI neurofilament protein-containing neurons is conspicuous and clearly delineable.

Consistency of neurofilament protein content in the cingulate motor areas

On the population level, the observation that neurons projecting to the same cortical site (i.e., area M1) from the anterior and posterior areas were equally likely to contain neurofilament protein points to a unifying feature in these two projections. Although in two animals there was a greater proportion of neurofilament protein-containing neurons in layers V and VI in the anterior than in the posterior projection, this was not a consistent trend in the other animals, in two of which the proportions, although not significantly different, showed a trend in the opposite direction. Thus, neurofilament protein content was not found to be a factor that distinguishes between the two projections. This result was surprising, given the marked difference in the layer III neurofilament protein-containing population between the two areas. The fact that the same proportion of this projection contains neurofilament protein, irrespective of whether it originates in a neurofilament protein-rich or -poor area, suggests that neurofilament protein content is a factor related to the projection itself, and is not merely an artifact of the chemoarchitecture of its area of origin. A possibility that presents itself as a result is that the characteristic neurofilament protein-based chemoarchitecture of an area represents the sum total of the neurofilament protein complements of its constituent projections. In other words, the chemoarchitecture of an area may be the result, not the determinant, of the neurochemical profile of its efferent projections.

The importance of this commonality in neurochemical phenotype is further reinforced by the fact that the laminar origin of the projection to area M1 and the morphology of the projection neurons are fundamentally different for these two areas and projections. The results of the present study suggest that certain characteristics of a projection, such as its laminar distribution and the somatodendritic morphology of its neurons, may be less related to its function than to the constraints imposed by the cytoarchitectonic properties of its area of origin. A study of callosal neurons in the visual cortex of the cat similarly concluded that differences in somatodendritic morphology are more closely related to local influences than target area (Vercelli and Innocenti, 1993). Thus the differences between the two projections considered here may be attributed to their slightly different positions along the cingulate gyrus and the resultant differences in their immediate milieu. However, although one physiological study has been able to attribute slightly different properties to these projections, with neurons active in self-paced movements, and those that discharge well in advance of the onset of movement represented more heavily in the CMAR than in CMAc, the same study pointed to the prevalent similarities between them (Shima et al., 1991). This essential functional similarity is best reflected in their similar neurochemical profiles. Comparable results have been obtained in studies of other corticocortical connections in the macaque monkey, where functionally similar projections tend to have comparable neurofilament profiles (Hof et al., in press; Hof et al., submitted). Thus, neurofilament protein content appears to be a more salient property of a projection than laminar distribution or neuronal morphology.

The fact that very few of the corticocortical connections studied thus far are composed entirely or almost entirely of neurofilament protein-containing neurons (Campbell et al., 1991; Hof et al., 1995; Hof et al., in press; Hof et al., submitted) indicates that it is this property of the population as a whole, not of the individual neuron, that is critical for the projection. Neurofilament protein-containing neurons may

represent a pool of highly specialized neurons with as yet uncharacterized functional properties that participate in a given corticocortical circuit to varying degrees. The proportions of neurofilament protein-immunoreactive neurons reported here for the projections from area 24c' to the primary motor cortex are in striking contrast to those reported for projections from area 24 to association areas in the prefrontal and superior temporal cortex (7.7 and 10.2%, respectively, for layers II and III, and 14.5 and 12.9%, respectively, for layers V and VI; Hof et al., in press). This suggests that neurofilament protein content may represent a more important feature in the cingulate cortex for its cortical motor circuits than its connections with association areas.

It has been proposed that projections enriched in neurofilament protein content are involved in transmitting highly precise maps across cortical areas and through hierarchies (Hof et al., in press). In the context of a motor projection, this could be understood in the need to maintain precise control of body movements. It should be noted that recent studies (Cadoret and Smith, 1995; He et al., 1995) have supported a role of the cingulate motor areas in fine limb movements. In addition, numerous studies have implicated the anterior cingulate cortex of the monkey (Shima et al., 1991) and of the human (Decety et al., 1994; Lang et al., 1994) in the planning and pacing of movements. This role could be very well subserved by highly ordered projections from the cingulate cortex to the primary motor cortex, possibly those analyzed in the present study.

The factors determining neurofilament protein content in individual neurons are not known. One possibility, given the somatodendritic localization of the nonphosphorylated form of the protein, is somal size or extent of dendritic arbor. However, the present study demonstrates that neurons that are comparably sized have comparable dendritic extents do not necessarily share this neurochemical feature. Another candidate characteristic is the distance of the projection (Campbell and Morrison, 1989; Hof et al., 1990; Hof and Morrison, 1990; Campbell et al., 1991). However, layer II and superficial layer III neurons are known to participate in

feedforward and feedback local connections (Jones and Wise, 1977). Since these neurons, which virtually never contain neurofilament protein, and the intensely immunoreactive deep layer III neurons in the same patch of cortex project the same distance to M1, it is unlikely that the distance of this projection alone determines the presence of neurofilament protein. It is possible, though, that the neurofilament protein-containing neurons in the deep portion of layer III have additional, more distant target sites, and in this respect total axonal length, whether influenced by the distance from a target, or collateralization, or both, could be a determinant of neurofilament protein content in a projecting neuron (Morris and Lasek, 1982; Hoffman et al., 1987; Nixon et al., 1994). In this context it is of interest that neurofilament protein-enriched neurons, particularly those in a position to furnish long association projections, have been demonstrated to be especially vulnerable to neurodegeneration in Alzheimer's disease (Morrison et al., 1987; Hof et al., 1990; Hof and Morrison, 1990). However, these issues apply to populations of neurons. At present, it is not clear what other differences might exist between two otherwise identical neurons participating in the same projection, one of which contains neurofilament protein, and one that does not. Perhaps the only well-documented difference is the link to neurodegeneration. The present study does, however, argue against somatodendritic morphology as a correlate of neurofilament protein content, and at least for cingulate cortex, does not support the notion that the neurons providing a given corticocortical projection express a coherent phenotype that incorporates morphologic and neurochemical attributes. Further evidence against this notion is the finding that neurofilament protein appears to be segregated within the neuron, since immunoreactivity in the soma and some dendrites may be associated with a lack of immunoreactivity in other dendrites in the same neuron, even though they may be morphologically indistinguishable from the neurofilament protein-containing ones. This also argues for the possibility of a more dynamic role for neurofilament protein, perhaps linked to the nature, disposition or abundance of afferent input. Other methods will be

necessary to elucidate what anatomic features of a pyramidal neuron predispose it to contain neurofilament protein, and what other intrinsic or extrinsic characteristics determine in which subcellular compartment within the neuron it exists.

If neurofilament protein content is considered to be a neuronal marker, the present data may also provide clues to the evolution of these cortical areas. The low density of neurofilament protein-containing neurons from anterior cingulate cortex except in regions known to participate in motor circuits, and the neurochemical similarity of the SMA and CMA projections to M1 suggest that features such as the large layer III neurofilament protein-containing neurons represent the intrusion into the anterior cingulate cortex by the SMA, a mechanism that has been postulated for other cortical areas in other species (Krubitzer and Calford, 1992; Krubitzer et al., 1993). The CMAs in general could have evolved from the SMA, and a duplication event within the CMA could have given rise to the two areas. In this context, it is notable that some of the functions ascribed to the cingulate motor areas, specifically in the planning of movements, are roles associated with the SMA (Deiber et al., 1991; Tanji and Shima, 1994). Thus, the laminar distribution of the projecting neurons may have been determined by the constraints of the area they occupied, but their neurochemical characteristics, along with their presumed function, could have remained intact and the projection would have carried with it neurochemically defined cell populations that would alter the neurochemical phenotype of the invaded area. In this context, it is noteworthy that aside from the human, the only other species in which a well-defined somatomotor area in the cingulate gyrus has been described is the macaque monkey. The cingulate motor areas may, therefore, be the result of an event that occurred in the evolution of the motor system in primates.

The primate cingulate cortex is the site of numerous different functional, cyto- and chemoarchitectonic areas (Vogt and Gabriel, 1993). It is evident from the present study that the heterogeneity in this cortical region extends from the regional level to the level of the projection, and even to the constituent neurons. Neurochemical phenotype,

however, provides a unifying characteristic for these convergent projections. Neurofilament protein content appears to be a meaningful feature of this region and its constituent projections. However, it ceases to be informative at the cellular and subcellular level, at least regarding the somatodendritic morphology, spine density and compartmental disposition of neurofilament protein. It is possible that other neurochemical markers, such as neurotransmitter receptor or ion channel proteins may prove more informative at these higher levels of resolution. However, the fact that certain properties do appear to be inherent to particular projections may ultimately permit the interpretation of neurochemical patterns in cortical areas of the monkey, and eventually, the human brain, within the context of functional and physiological differences among corticocortical connections.

TABLE 5.3. Numbers of Lucifer Yellow-loaded neurons of different morphologic types in CMAr and CMAc¹

	CMAr		CMAc	
	II-III	V-VI	II-III	V-VI
Typical pyramid	7 (100)	11 (31)	19 (90)	15 (54)
Large pyramid	0 (0)	1 (3)	2 (10)	5 (18)
Asymmetric, lateral	0 (0)	10 (28)	0 (0)	3 (11)
Asymmetric, medial	0 (0)	4 (11)	0 (0)	0 (0)
Fusiform	0 (0)	6 (17)	0 (0)	2 (7)
Other	0 (0)	4 (11)	0 (0)	3 (11)

¹CMAr and CMAc refer, respectively, to the anterior and posterior cingulate motor areas. The percentage of the total number counted is indicated in parentheses. "Large pyramids" are neurons with a very large soma and a slightly tapering, stout apical dendrite. Neurons categorized as "asymmetric, lateral" are neurons with a single major basal dendrite, which was oriented toward the interior of the hemisphere. "Asymmetric, medial" neurons have their major basal dendrite oriented toward the medial surface of the hemisphere. These counts represent the combined results of three animals. For the topographic distribution of these cell types, see Figure 10.

TABLE 5.4. Spine density of reconstructed neurons¹

	Cell	Area analyzed	Spines/length	Length/spine	NFP
<i>Layers II-III</i>	11A	25000	0.253	3.95	+
	11B	13000	0.171	5.85	+
	12A	50000	0.439	2.28	+
	12B	20000	0.249	4.01	-
	12C	70000	0.460	2.17	-
<i>Layers V-VI</i>	11C	35000	0.133	7.54	-
	11D	35000	0.275	3.64	+
	11E	40000	0.110	9.07	+
	11F	35000	0.0940	10.6	-
	11G	45000	0.107	9.35	n.a.
	11H	35000	0.107	9.33	+
	11I	15000	0.0678	14.75	-
	11J	19000	0.0872	11.5	+
	12D	10000	0.238	4.20	+
	12E	20000	0.137	7.29	+
	12F	20000	0.101	9.94	-
	12G	25000	0.218	4.59	-
	12H	30000	0.050	20.2	-

¹Neurons are identified by their label in Figures 11 and 12. For each neuron, a circle was drawn, centered around the soma, such that the most distant ends were not included (see text). Area analyzed refers to the area of the circle in which spine density was analyzed. Dendritic length is expressed in μm ; area in μm^2 . The rightmost column indicates, where available, the presence or absence of neurofilament protein (NFP) in each neuron. "n.a." indicates that the neuron was not available for confocal analysis.

FIGURE LEGENDS

Fig. 5.1. Injection sites. (A) Composite of injection sites for the five monkeys used in the present study. In animal CYN3, the right hemisphere received injections of FB, and the left received DY. In all others, the right hemisphere received DY, and the left received FB. Representative sections through the injection sites of animal CYN4 (B) and CYN 2 (C). (D) and (E), photomicrographs of boxed areas in panels (B) and (C), respectively, showing depth of injection and the presence of giant cells of Betz, confirming localization in primary motor cortex. The gray-scale code applies only to panel (A). CS, central sulcus; CingS, cingulate sulcus; IPS, intraparietal sulcus; LS, lateral sulcus.

Fig. 5.2. Cyto- and chemoarchitecture at selected levels of the cingulate gyrus: Nissl stain (A, C, E, G) and neurofilament protein immunoreactivity (B, D, F, H). (A, B) Area 24c, rostral to the anterior projection. (C, D) Rostral portion of area 24c', at the level of the anterior projection. (E, F) Caudal portion of area 24c', at a level of the posterior projection. (G, H) Area 23c, caudal to the posterior projection. All pairs of photomontages represent equivalent fields from immediately adjacent sections. For the exact locations of these photomontages, see Fig. 8. Note the absence of layer IV at all levels except G and H. Also note the progressive increase in the density of neurofilament protein-immunoreactive neurons in the deep portion of layer III, which peaks at the level of the caudal cingulate motor area (E, F). Scale bar = 100 μ m.

Fig. 5.3. Cyto- and chemoarchitecture of the forelimb representation of the supplementary motor area, SMA. Nissl stain (A) and neurofilament protein immunoreactivity (B). Note the large population of neurofilament protein-containing neurons in layer III, and the absence in Nissl-stained material of layer IV, as well as of a compact layer V as seen in the cingulate cortex. Scale bar = 100 μ m.

Fig. 5.4. Low magnification photomicrographs of representative sections through the anterior (A) and posterior (B) projections in monkey CYN4. Photomicrograph (A) was taken in the fundus of the cingulate sulcus. For the exact locations of these photomicrographs, see Figure 5.8. Neuron in the deep portion of layer III in the posterior projection that is retrogradely FB-labeled (C) and neurofilament protein-immunoreactive (D). Note the relatively small number of immunoreactive neurons in this layer. Scale bar = 100 μm (A, B), and 25 μm (C, D).

Fig. 5.5. Representative maps (A-F) showing the disposition and neurofilament protein content of retrogradely labeled neurons in the anterior ipsilateral projection. Non-immunoreactive retrogradely labeled neurons are indicated by open circles, and neurofilament protein-immunoreactive neurons are indicated by filled circles. The gray line marks the border between layers III and V. The region represented by this set of maps is indicated in Figure 5.8. Note the predominant localization in deep cortical layers. The SMA is present throughout this series, in the cortex of the medial wall above the cingulate sulcus. The CMAr shifts from the depths of the cingulate sulcus dorsally, approaching the SMA. The strip of neurons in the ventral bank of the cingulate sulcus proved to be the anterior tip of the posterior projection.

Fig. 5.6. Representative maps (A-G) showing the disposition and neurofilament protein content of retrogradely labeled neurons in the posterior ipsilateral projection. Conventions are the same as for Figure 5.5. The region represented by this set of maps is indicated in Figure 8. Note the greater density of this projection than the CMAr, and its location in superficial as well as deep layers. Note also the greater density of immunoreactive neurons in deep than in superficial layers. The CMAc begins anteriorly in the ventral bank of the cingulate sulcus and shifts dorsally, eventually ending in the dorsal bank of the cingulate sulcus.

Fig. 5.7. Representative maps showing the disposition and neurofilament protein content of retrogradely labeled neurons in the anterior **(A)** and posterior **(B)** contralateral projections. Conventions are the same as for Figure 5.5. The regions represented by these sets of maps are indicated in Figure 5.9. Both of these projections occupy positions comparable to those of the analogous ipsilateral projections, but they tend to originate in the deeper layers of the cortex.

Fig. 5.8. Distribution and laminar origin of retrogradely labeled neurons following injections into the ipsilateral M1. The total number of ipsilaterally projecting neurons is indicated for each section between the genu and the splenium of the corpus callosum. Note the differences in the scales of the y-axis, due to the varying number of neurons counted in the five monkeys. Since the angle of section varied from animal to animal, the location of the anterior commissure depicted in the figure is approximate, and not representative of all monkeys. The lines marked **(a)** and **(b)** indicate the region spanned by the series of maps shown in Figures 5.5 **(a)** and 5.6 **(b)**. The open arrows indicate the levels of photomicrographs 5.4A **(C)** and 5.4B **(d)**. The closed arrows indicate the levels of the photomontages shown in Figures 5.2A-B **(e)**, 5.2C-D **(f)**, 5.2E-F **(g)** and 5.2G-H **(h)**.

Fig. 5.9. Distribution and laminar origin of retrogradely labeled neurons following injections into the contralateral M1. Conventions are the same as for Figure 5.8. Note the differences in the scales of the y-axis, due to the varying number of neurons counted in the five monkeys. The lines marked **(a)** and **(b)** indicate the region spanned by the series of maps shown in Figure 5.6A-B **(a)** and 5.6C-D **(b)**.

Fig. 5.10. Distribution of Lucifer Yellow-loaded neurons of various morphologic types in layers II-III (top) and layers V-VI (bottom). These data represent a composite of the neurons loaded in three monkeys. No attempt is made to indicate the relative sublamina positions of the neurons within the cortex. "Asymmetric, lateral" and "asymmetric, medial" refer, respectively, to neurons whose single major basal dendrite is oriented laterally, toward the interior of the hemisphere, and medially, toward the medial surface of the hemisphere. A quantitative analysis of these neurons and their distribution is presented in Table 5.3.

Fig. 5.11. Reconstructions of Lucifer Yellow-loaded neurons participating in the anterior projection to the ipsilateral M1. Cells (A) and (B) were located in layer III, and the truncation of their apical dendrites was a result of sectioning. The most commonly encountered morphologic cell types in deep cortical layers were fusiform neurons (B) and asymmetric pyramidal neurons with one dominant basal dendrite which was usually oriented laterally, toward the interior of the hemisphere (G-J). More rare were typical (F) and inverted (D) pyramids, or neurons with a bifid basal dendrite (E). Inset shows the positions of the reconstructed neurons. Only neurons A, B, D, E, H and J were neurofilament protein-immunoreactive. Neuron G could not be recovered for confocal analysis. Scale bar = 100 μ m.

Fig. 5.12. Reconstructions of Lucifer Yellow-loaded neurons participating in the posterior projection to the ipsilateral M1. Cells (A), (B) and (C) were located in layer III, and cells (D-H) were in layers V and VI. Morphologic diversity was much more limited than anteriorly. Typical pyramidal neurons predominated in superficial (A-C) and even in deep layers (D, E, F). Unusual shapes were largely restricted to layer VI (H). Neurons (G) and (H) recall the asymmetric neurons found in the anterior projection (Fig. 5.11), and both have their dominant basal dendrite oriented toward the interior of the

hemisphere, although (G) is located in the dorsal bank of the cingulate sulcus. Neurons with bifurcating apical dendrites (C, D) were not uncommon. Only neurons A, D and E were neurofilament protein-immunoreactive. Scale bar = 100 μ m.

Fig. 5.13. Confocal images of two neurons that were retrogradely labeled following injections into the ipsilateral M1 and loaded with Lucifer Yellow. The leftmost panel in each row is an image of neurofilament protein immunoreactivity, using Cy-5 as a fluorophor, and pseudocolored red. The middle panel is the image of Lucifer Yellow, pseudocolored green, in the same frame. An overlay of the two, with yellow indicating colocalization, is shown in the rightmost panel. These two neurons, which were reconstructed and appear in Figure 5.12 as cells A (A-C) and C (D-F), are of comparable laminar position, size, degree of dendritic arborization, and spine density, yet the first (A-C) is immunoreactive for neurofilament protein, and the second (D-F) is not. Panels (G-I) are higher power images of the apical dendrite and a secondary branch of the neuron shown in panels A-C, and panels (J-L) are images at the same magnification showing another secondary dendrite from the same neuron. Note that despite comparable caliber and spine density, the dendrites in panels G-I are immunoreactive, while that in panels J-L is not. Scale bar = 60 μ m (A-F), and 10 μ m (G-L).

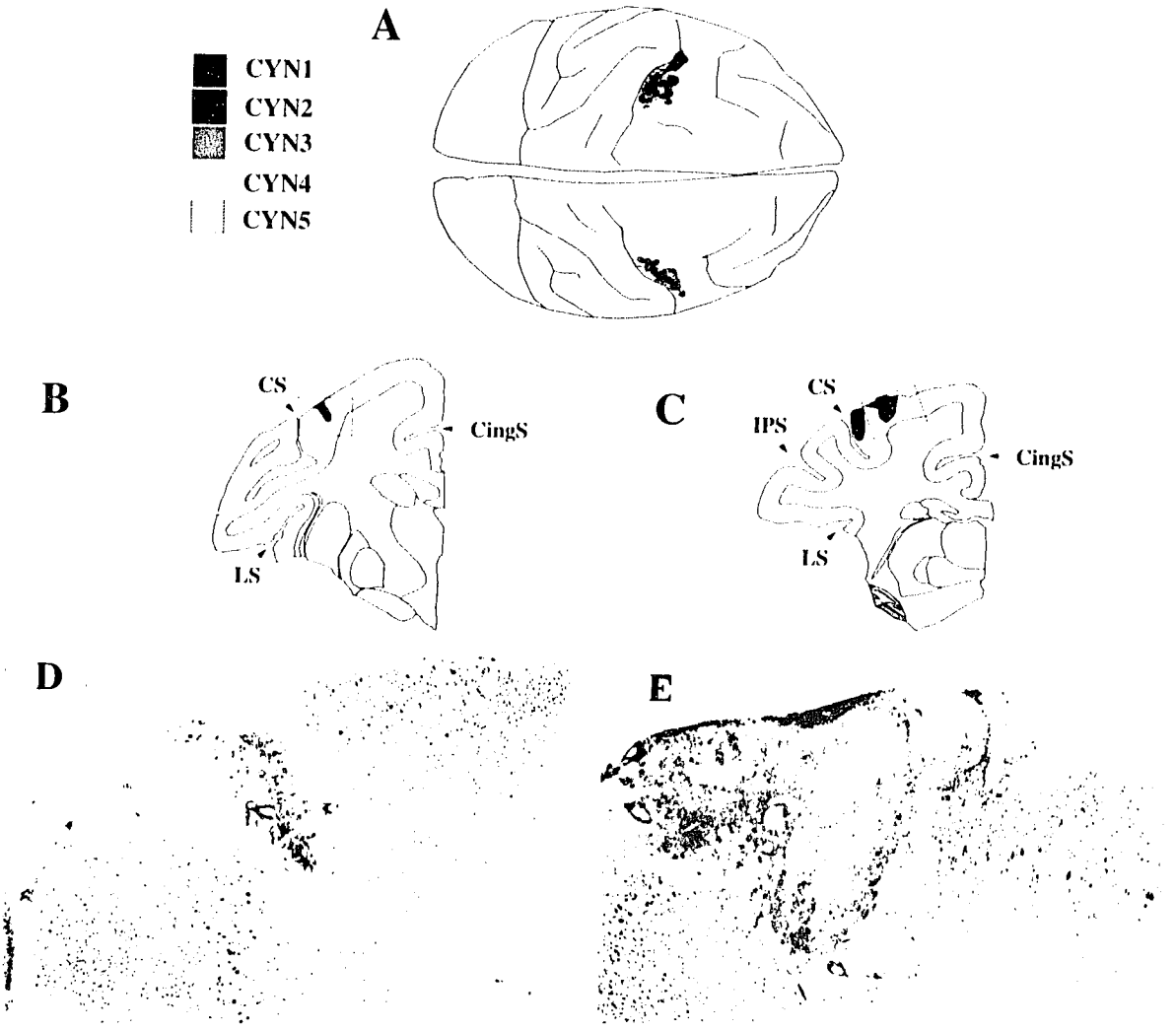


Fig. 5.1

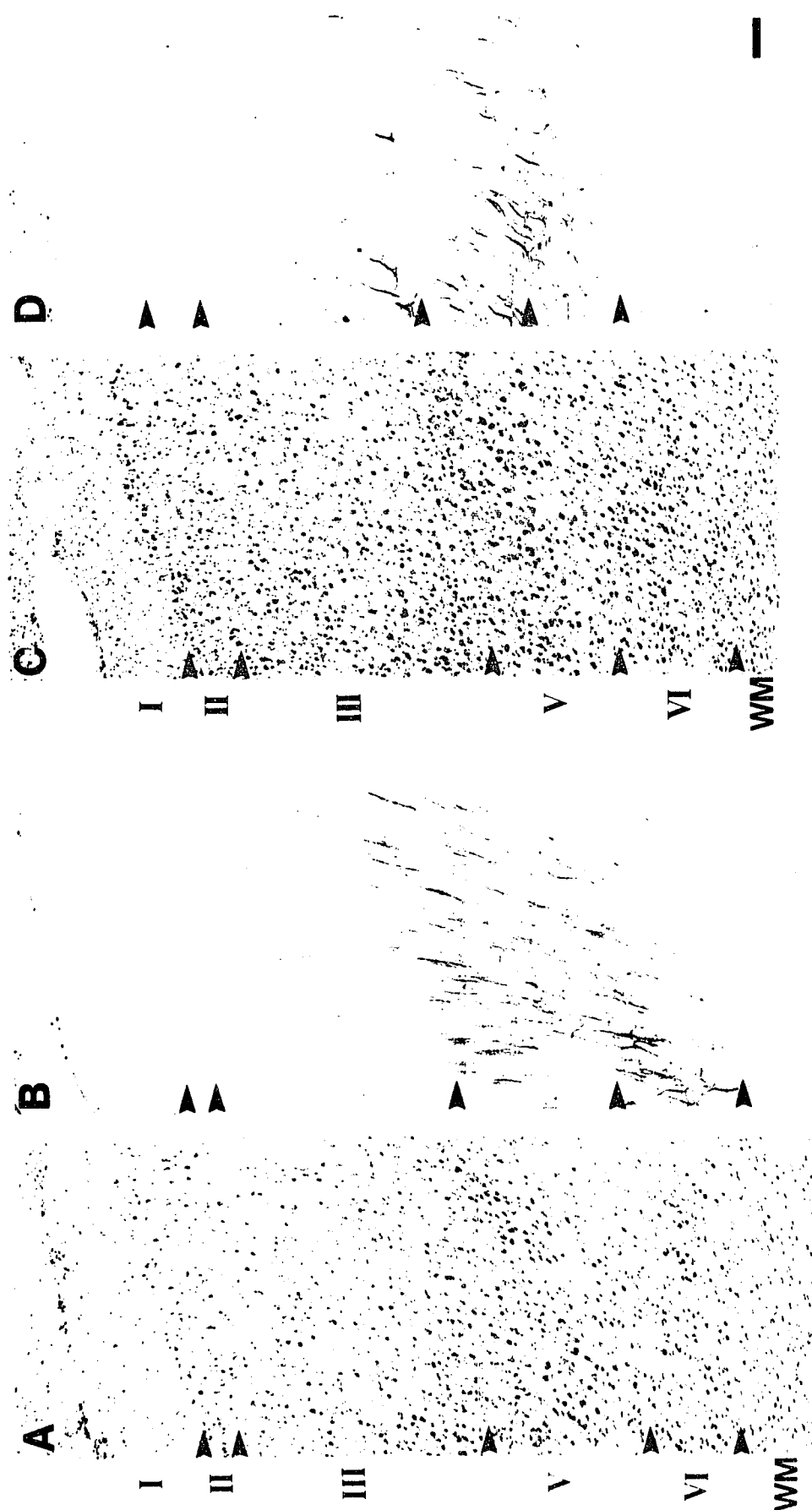


Fig. 5.2



Fig. 5.2 (cont'd)

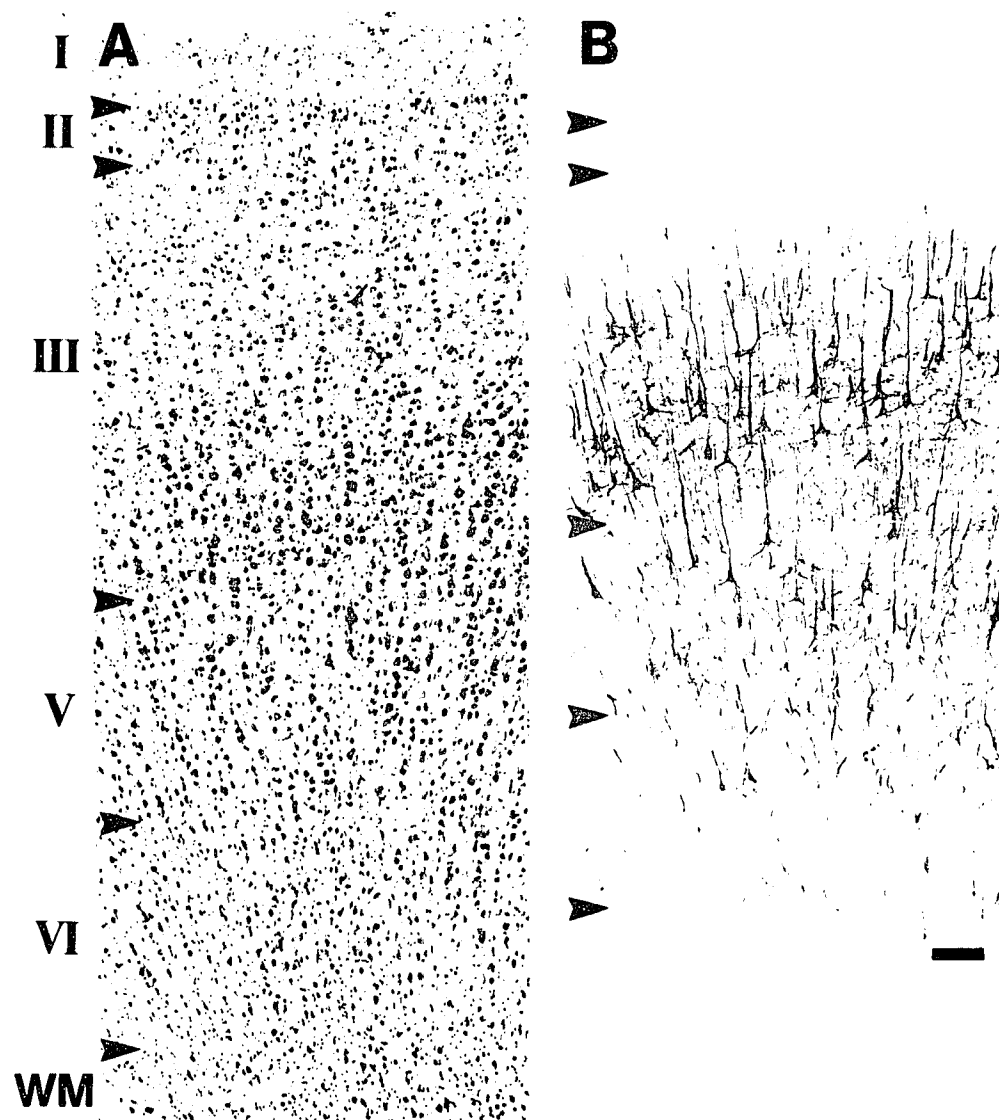


Fig. 5.3

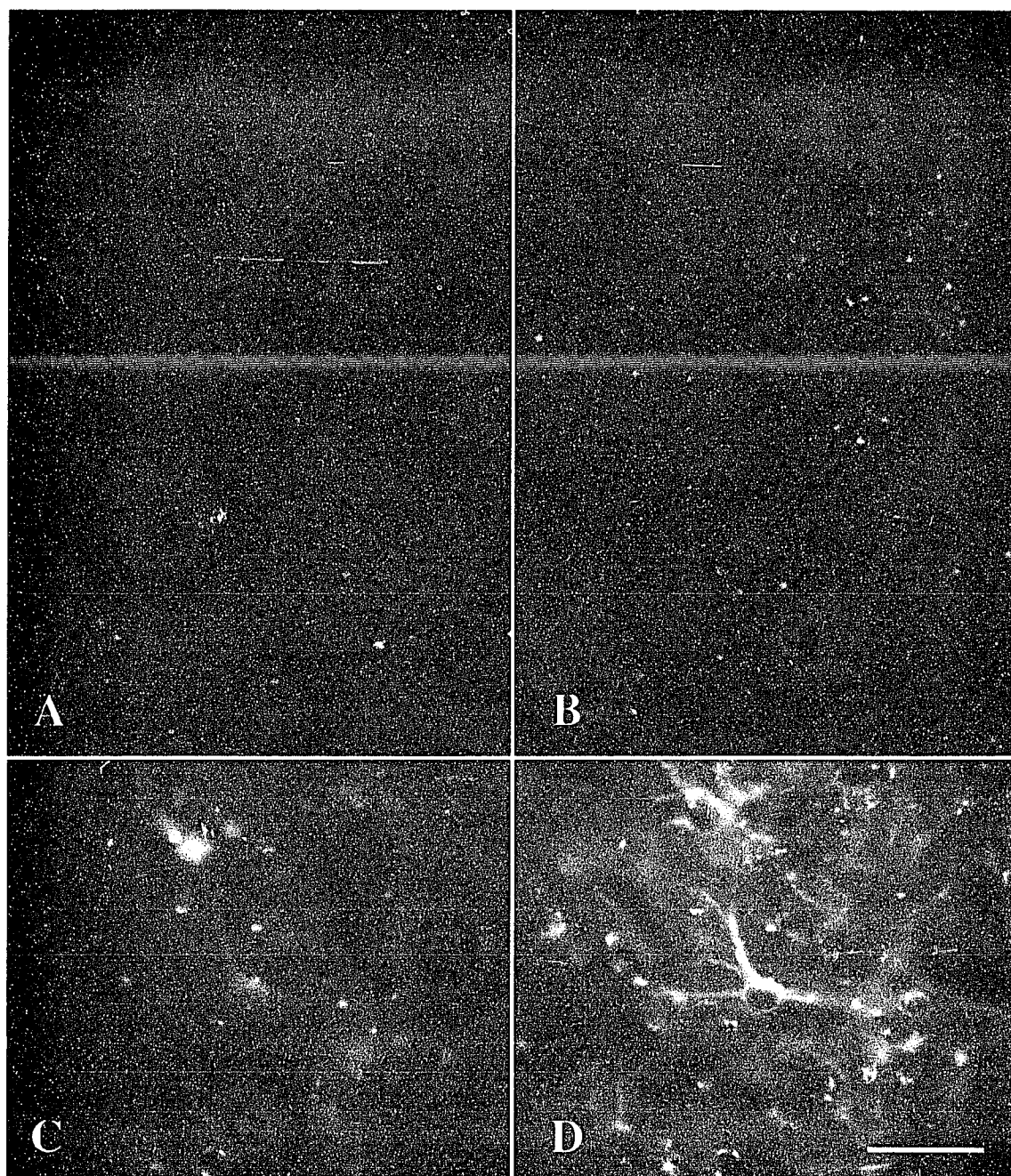


Fig. 5.4

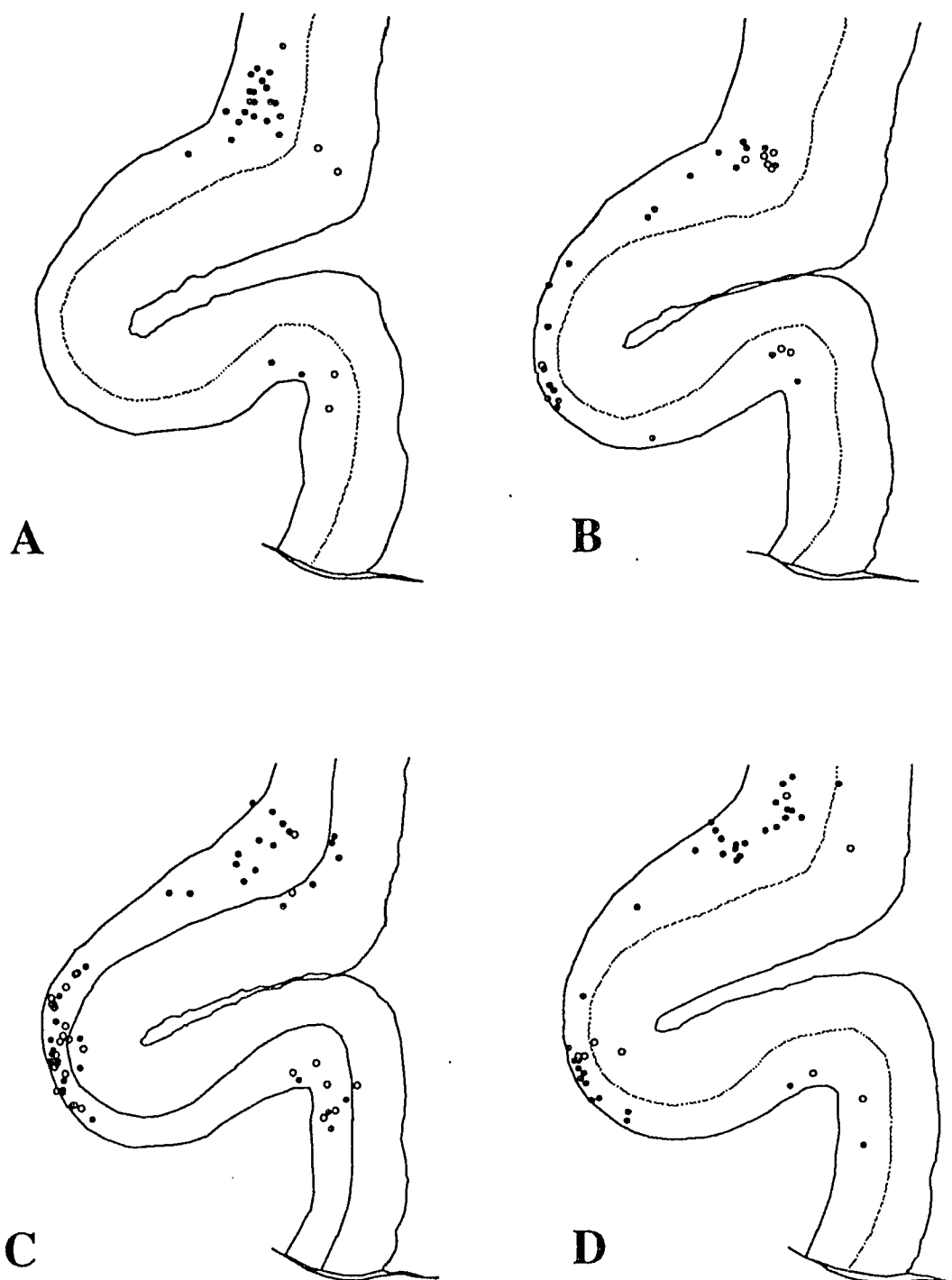


Fig. 5.5

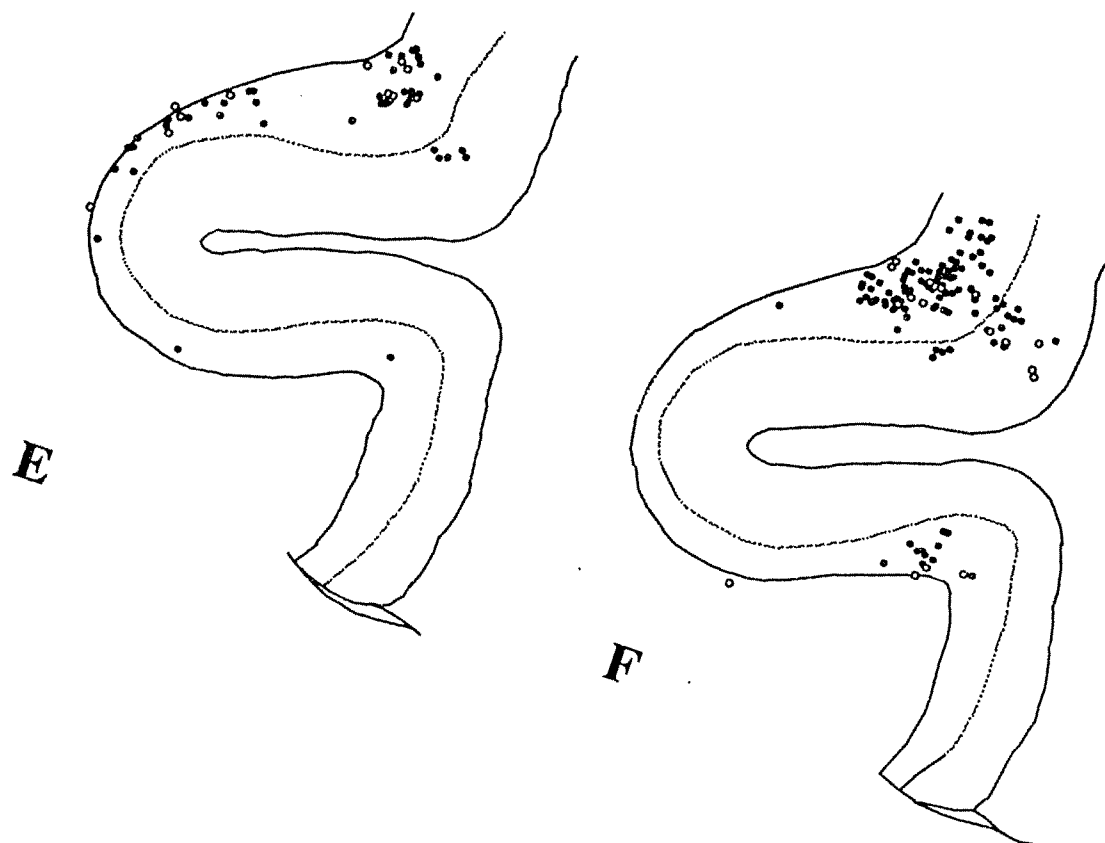


Fig. 5.5 (cont'd)

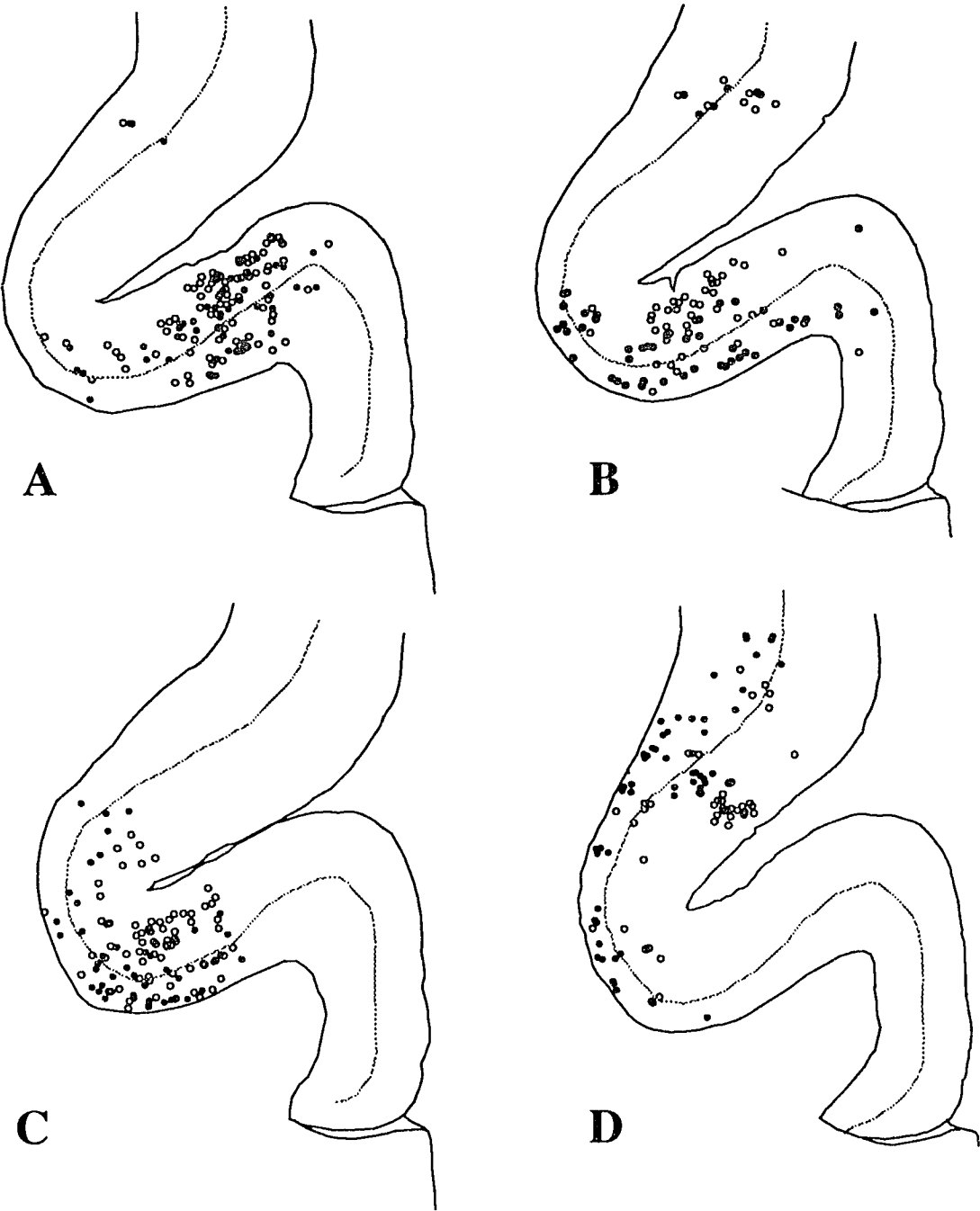
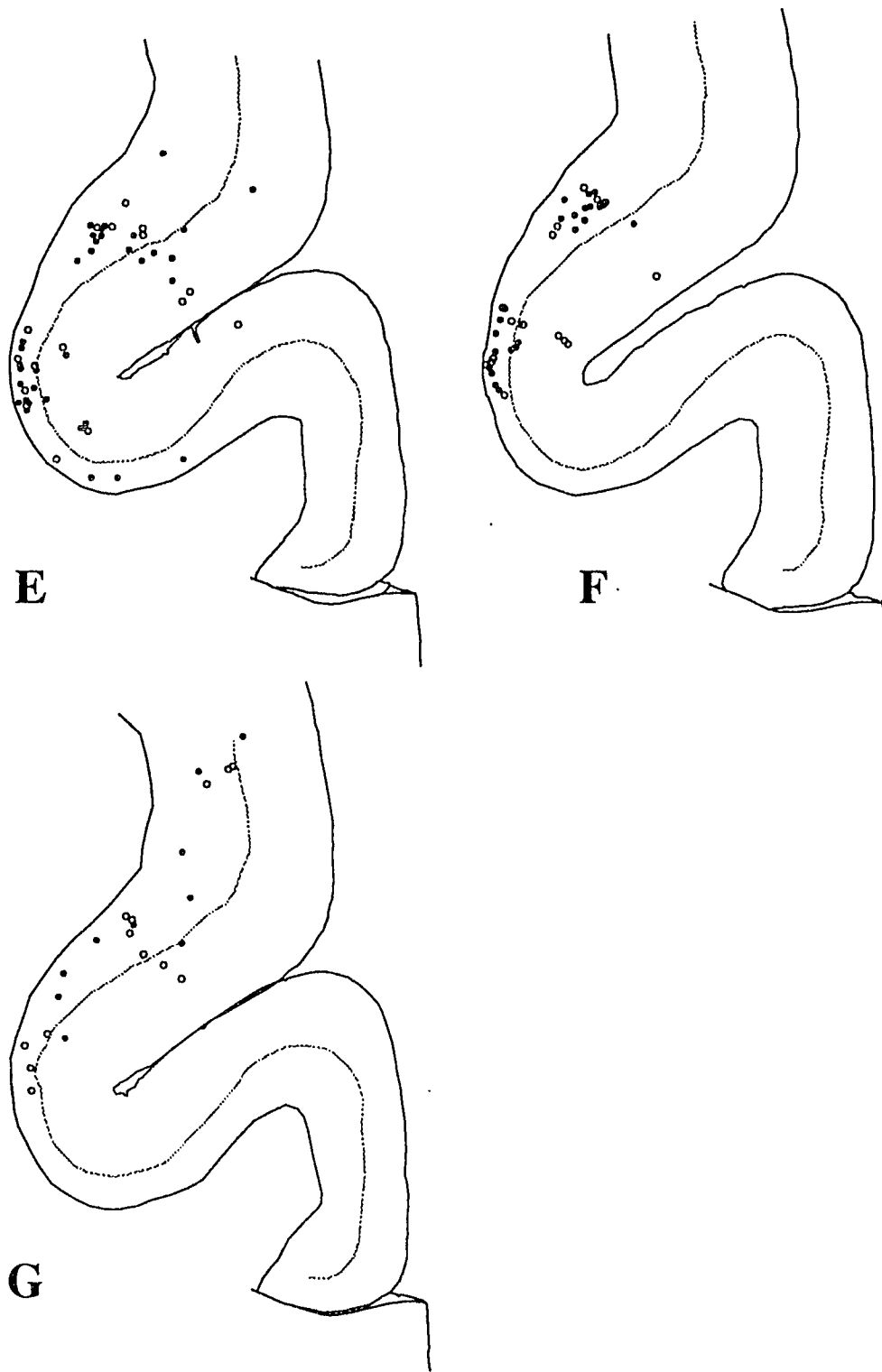


Fig. 5.6

**Fig. 5.6 (cont'd)**

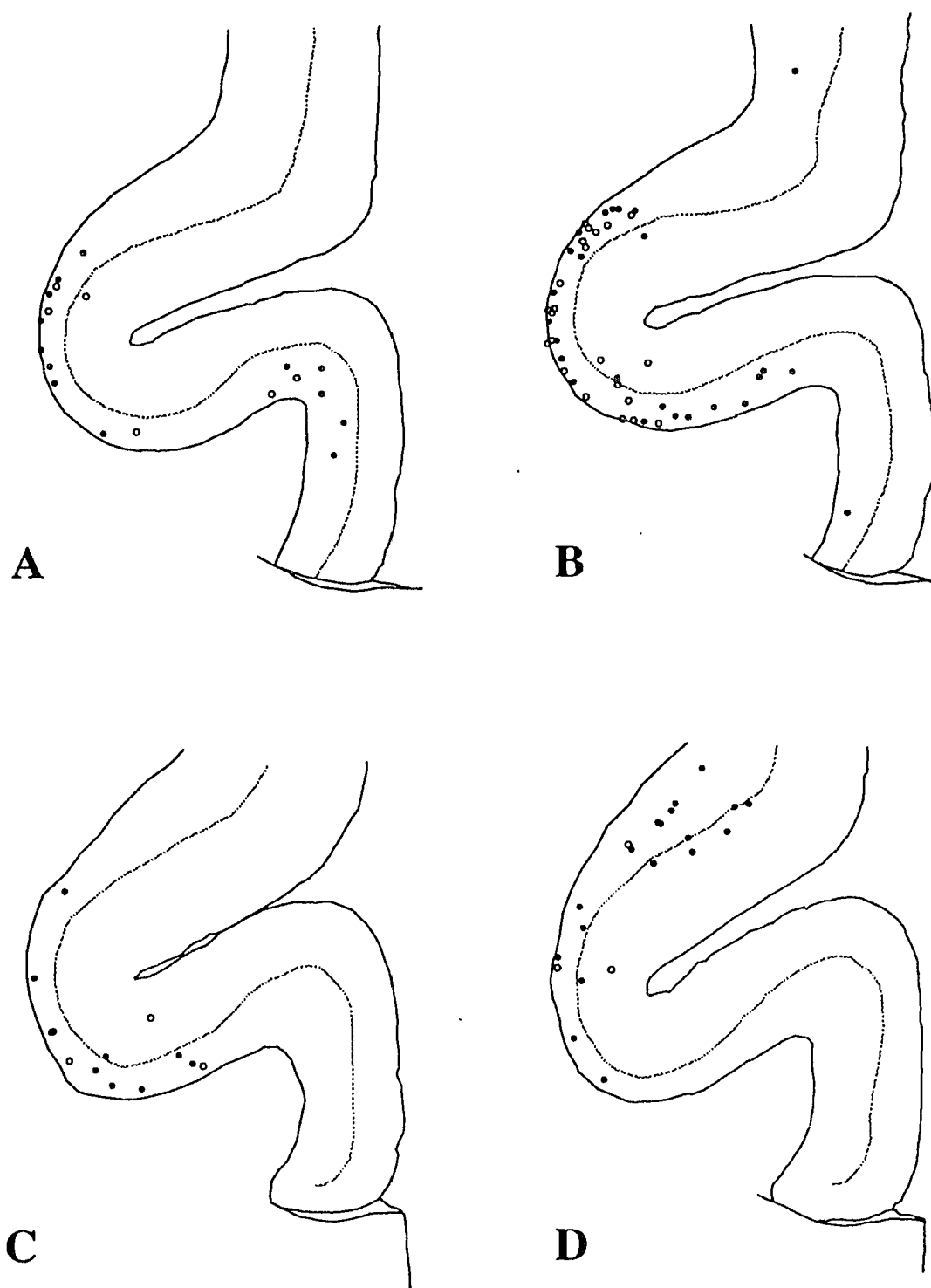


Fig. 5.7

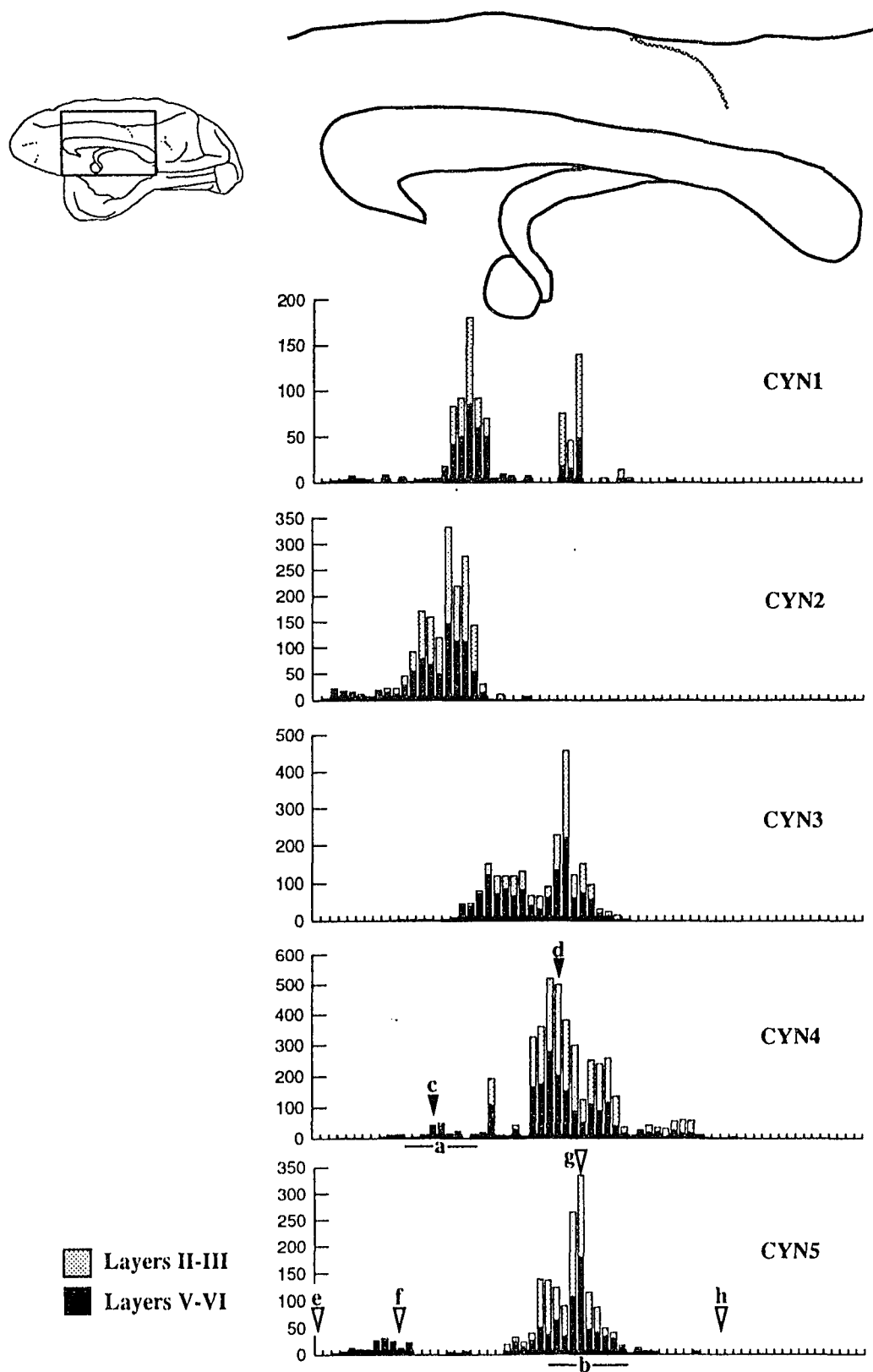


Fig. 5.8

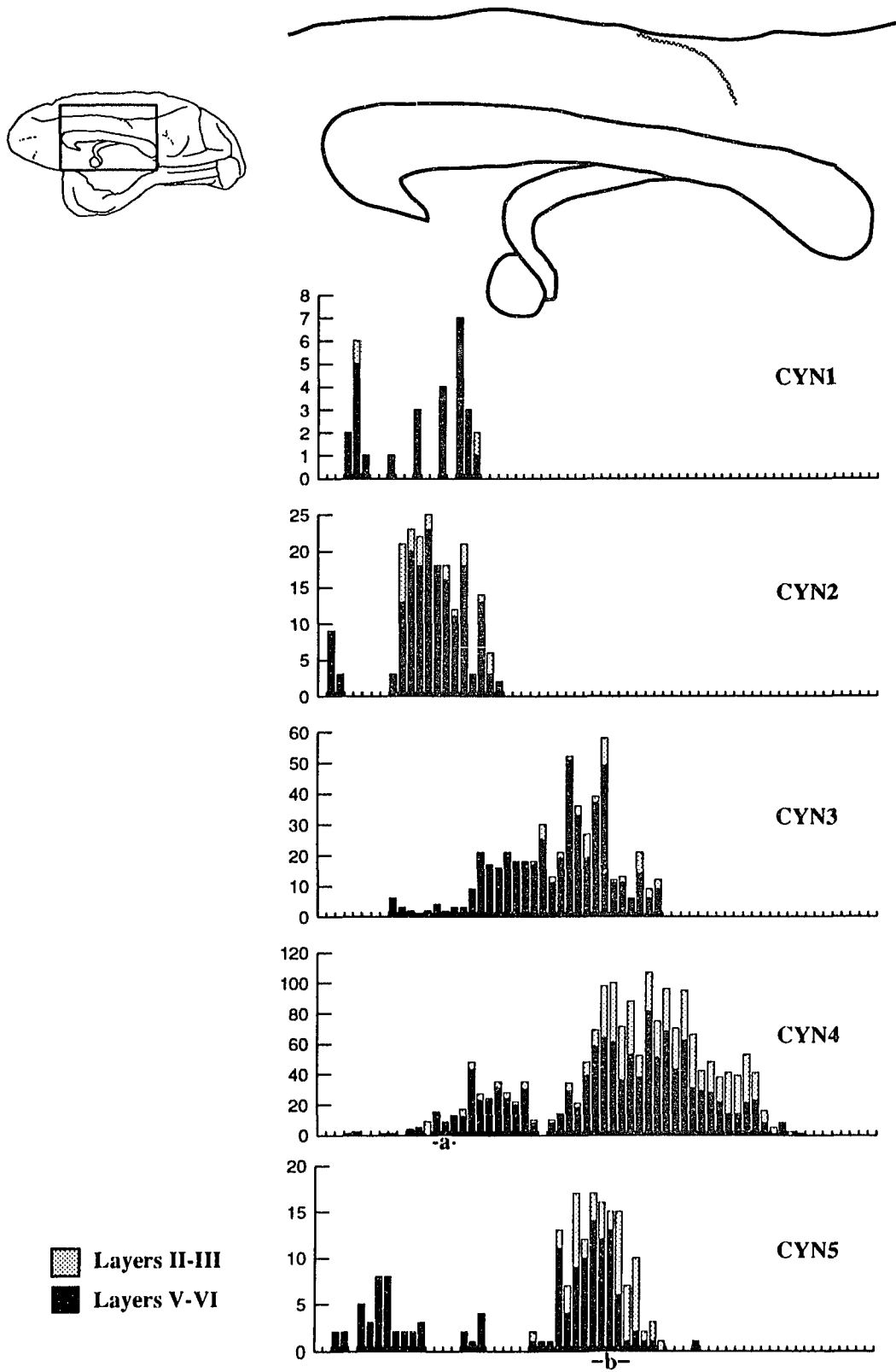
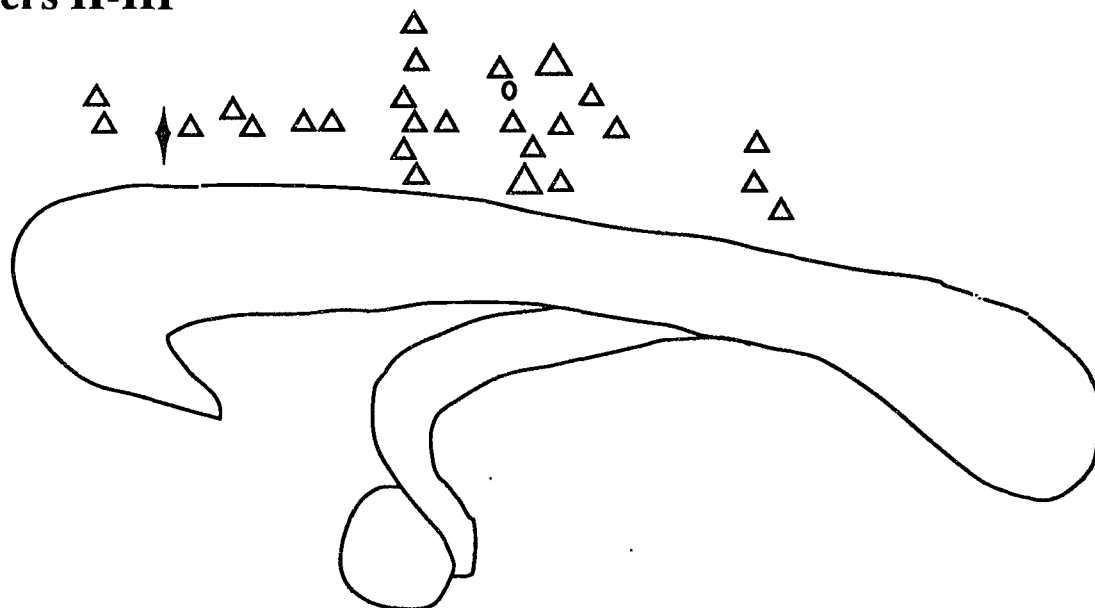
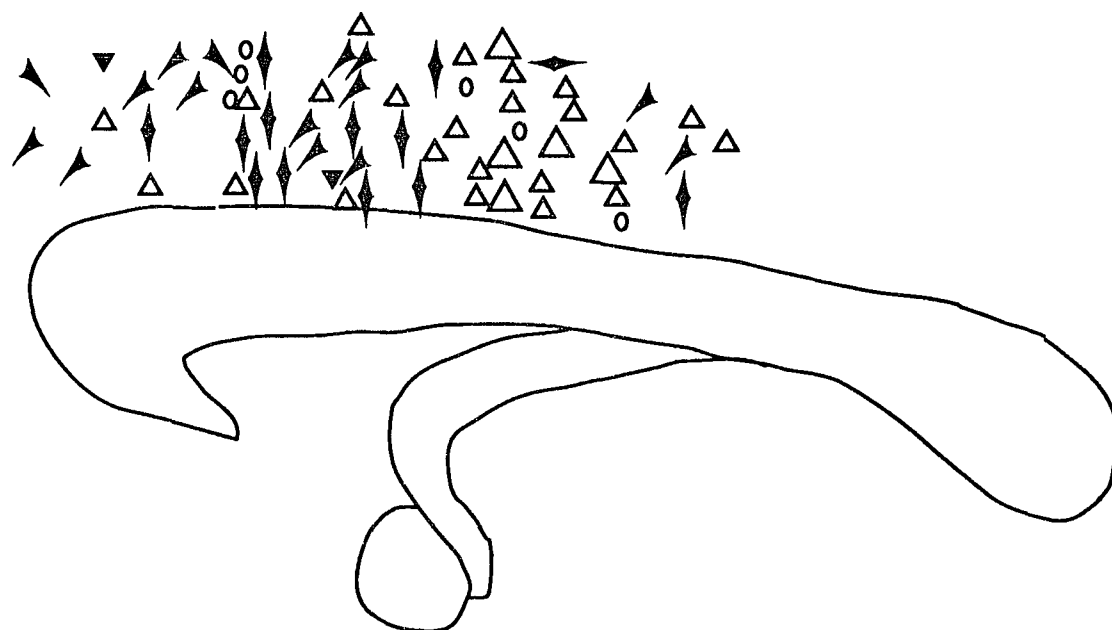


Fig. 5.9

Layers II-III



Layers V-VI



- △ typical pyramid
- △ large typical pyramid
- pyramid, thin apical dendrite
- ▼ inverted pyramid

- ▲ asymmetric, lateral
- ▲ asymmetric, medial
- ◆ fusiform
- ◆ horizontal

Fig. 5.10

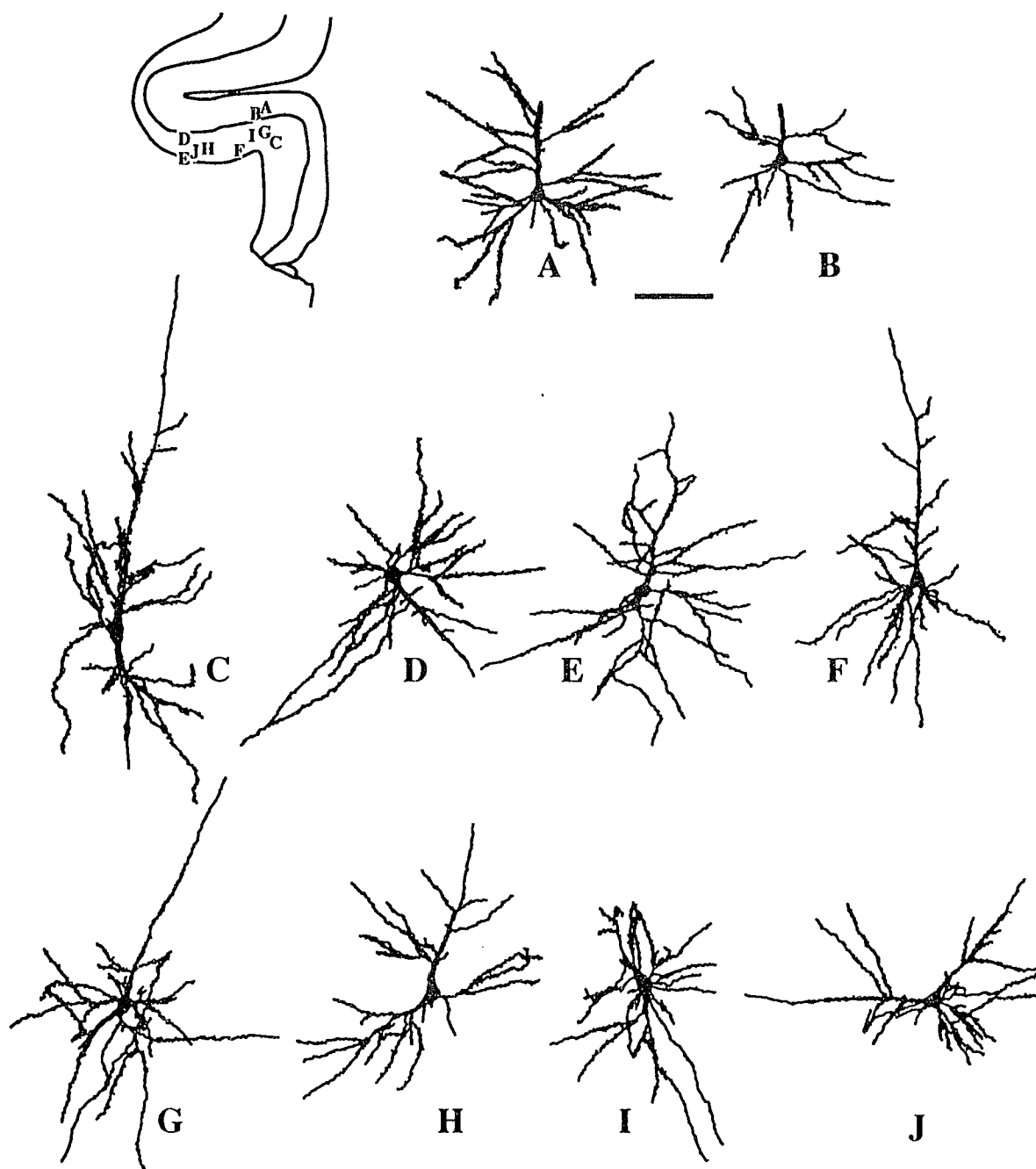


Fig. 5.11

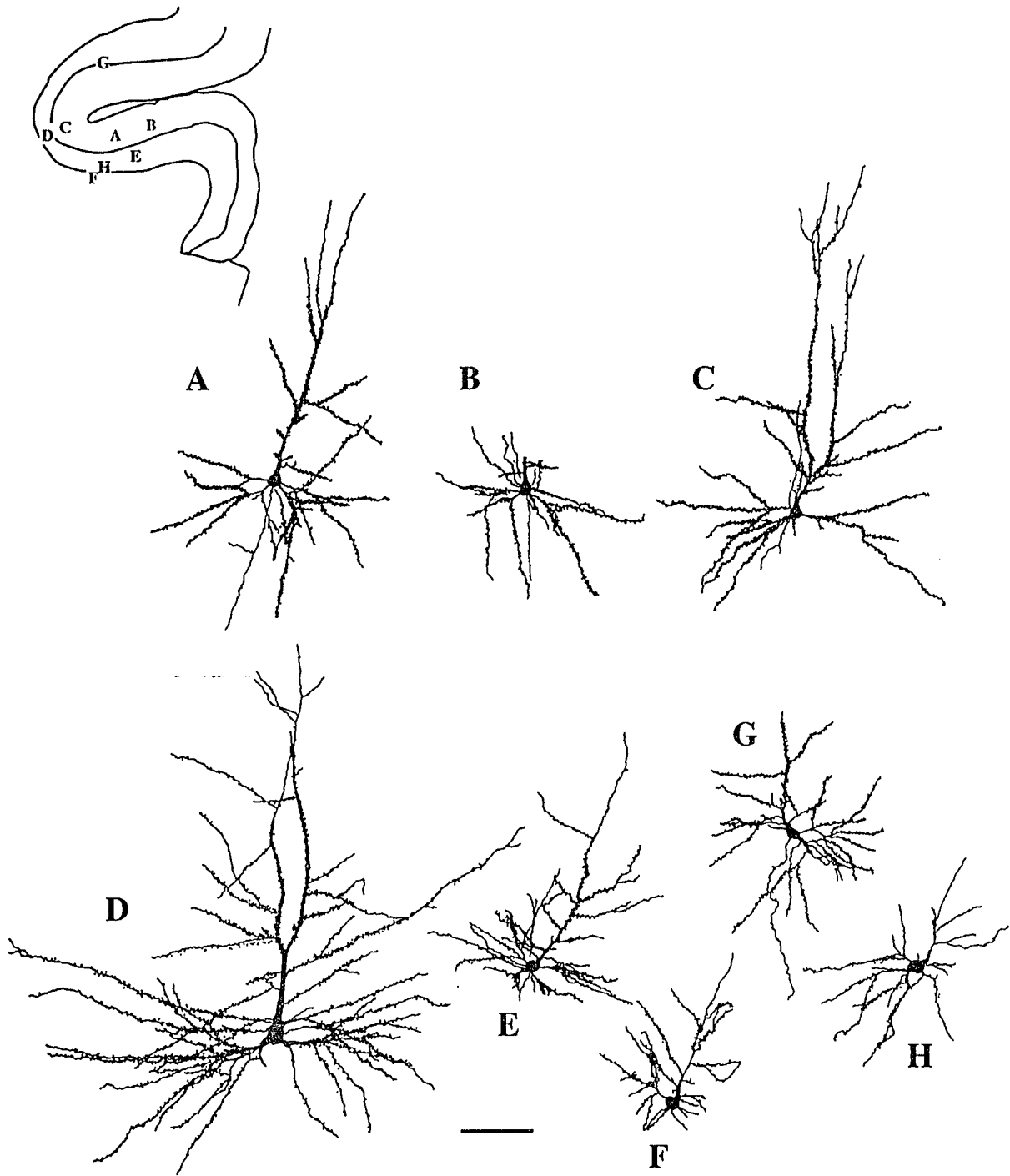
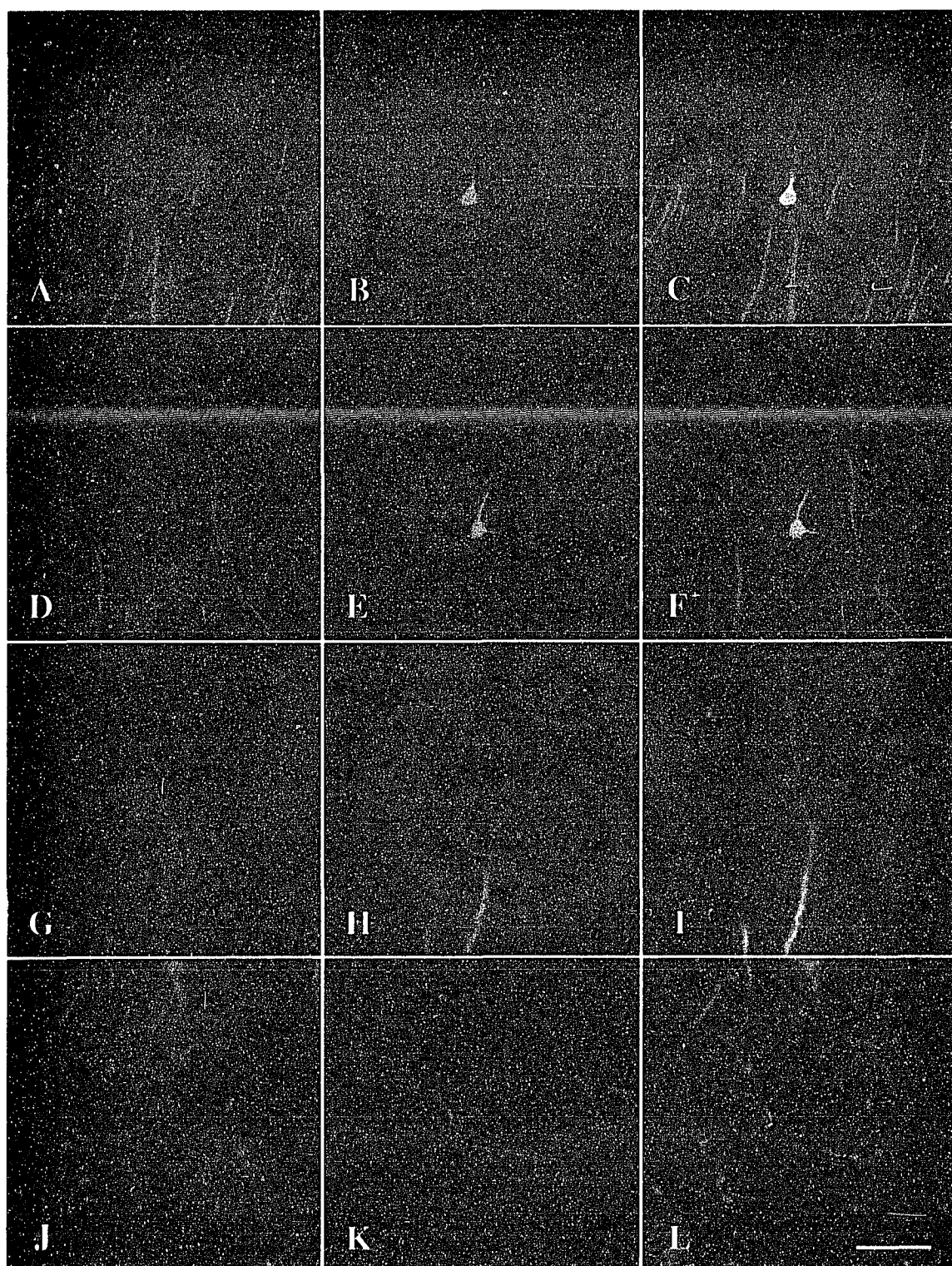


Fig. 5.12

**Fig. 5.13**

Chapter 6

Regional and Cellular Distribution of the Dopamine D3 Receptor in the Macaque Monkey and Rat Brain: an Immunocytochemical Analysis

SUMMARY

The dopamine D3 receptor has been described as having a very restricted anatomic distribution, limited to limbic areas in the ventral striatum and the archicerebellum. In the present study, a novel monoclonal antibody generated against the D3 receptor was used to investigate the regional and cellular distribution of the D3 receptor protein in the rodent and primate cerebral cortex and in select subcortical structures. Antibody specificity was demonstrated by radioimmunoassay and immunoblot analyses of proteins extracted from macaque monkey cortical tissue and from stably D3-expressing rat GH3 cells. In both the rat and monkey, the D3 receptor protein immunoreactivity was observed in every examined structure. Structures previously described as being rich in D3 receptors contained immunoreactive neurons, as did all lobules of the cerebellum, dorsal striatum, and neocortex. In the cerebellum, Purkinje cell somata were unstained, but their dendrites and spines were labeled. D3 receptor immunoreactivity was found in cortical pyramidal neuron somata and apical dendrites throughout the neocortex, and in neuropil in most cortical areas, with the exception of deep layers of the primary visual cortex. Ultrastructural analysis revealed immunoreactive dendritic shafts which received frequent symmetric synapses, and demonstrated that pyramidal cell dendritic spines were not labeled. In both species, immunoreactive glial cells were encountered throughout the brain. These results indicate that the D3 receptor is more widely distributed in the central nervous system than previously thought, and may achieve specificity in part by anatomic compartmentalization at the cellular level. As a result, D3-selective ligands may have more global neurologic and psychiatric effects than earlier believed.

INTRODUCTION

The dopaminergic innervation of the rodent and primate brain is characterized by species differences, as well as by considerable regional heterogeneity within a species. For instance, the rodent cortex has a very restricted dopaminergic innervation, largely in frontal cortical areas, whereas the primate cortex receives a much more extensive innervation that exhibits considerable regional heterogeneity (Lewis et al., 1986; Lewis et al., 1987; Lewis et al., 1988; Berger et al., 1991). A further level of complexity arises from the fact that dopamine acts through the activation of several different receptor subtypes that fall into two functionally distinct families with differing anatomic distributions, classified as D1-like and D2-like (Lidow et al., 1991; Huntley et al., 1992; Levey et al., 1993; Vincent et al., 1993; Yung et al., 1995). Dopamine receptors are members of a large superfamily of receptors that contain seven hydrophobic transmembrane domains alternating with hydrophilic stretches. They are linked to G proteins and are presumed to modulate the fast activity of ionotropic receptors (Cooper et al., 1991). The dopamine receptors are organized into two families: the D1-like family and the D2-like family (Gingrich and Caron, 1993). The D1-like family includes the D1A and D5/D1B receptors. The D2-like family includes the D2, D3 and D4 receptors. Members of the D1-like family stimulate adenylate cyclase. D2 receptors inhibit adenylate cyclase and stimulate potassium channel activity, whereas the second messengers used by the other members of the D2-like family are still unclear (Gingrich and Caron, 1993). In the rodent, D1A receptors are widespread in the brain, especially in the striatum, nucleus accumbens and olfactory tubercle (Sibley et al., 1993). whereas D5/D1B receptor mRNA is found almost exclusively in limbic structures (Sunahara et al., 1991). D2 receptors have a distribution similar to that of D1A receptors (Sibley et al., 1993), and the distribution of the D4 receptor is not well described, but seems to be found mostly in amygdala, frontal cortex, striatum, medulla and midbrain (Van Tol et al., 1991). D3 receptors, in contrast, seem to be confined to structures innervated by the

mesolimbic dopaminergic projections (Sokoloff et al., 1990). Both D2 and D3 receptors are thought to be located presynaptically, and thus to act as autoreceptors to regulate dopamine release (Sokoloff et al., 1990). On the cellular level one study suggests that D1 receptors are localized on interneurons, while D2 receptors are found on pyramidal cells (Vincent et al., 1993). The relevance of this information to studies of the primate cortex is questionable, for the primate cortex receives a much more extensive dopaminergic input than the rodent cortex, and if separate dopaminergic projections may be associated with specific receptor subtypes, as may be the case in the D3 receptor, then the rodent model may not be applicable to the primate. For instance, in the primate brain, D1, D2 and D5 mRNA were found to be present at high levels in all cellular layers of primary motor cortex (Huntley et al., 1992). In contrast, only D1 receptor mRNA is present at high levels in the rodent neocortex.

The D3 receptor has been described as having a very restricted distribution in both the rodent and the human, based on the results of numerous ligand binding and *in situ* hybridization studies which reported a high concentration of this receptor or its mRNA in certain regions, such as the ventral striatum and archicerebellum, and barely detectable amounts elsewhere (Sokoloff et al., 1990; Bouthenet et al., 1991; Lévesque et al., 1992; Murray et al., 1992; Sokoloff et al., 1992; Landwehrmeyer et al., 1993a; Landwehrmeyer et al., 1993b; Parsons et al., 1993; Herroelen et al., 1994; Murray et al., 1994; Wallace and Booze, 1995). Since most of the structures described as containing the D3 receptor are associated with the limbic system, and are thought to participate in emotive processes, and since the motor system and neocortex were thought to be devoid of this subtype, the D3 receptor was considered an ideal target for neuroleptics (Sokoloff et al., 1990; Sokoloff et al., 1992; Schwartz et al., 1993). However, a number of more recent studies have indicated that the expression of D3 receptor protein is more widespread than the original studies showed. In fact, new studies suggest that the expression of the D3 receptor protein may not be limited to subcortical sites. For example, two

immunocytochemical studies have reported the presence of this receptor in rat neocortex (Ariano and Sibley, 1994; Larson and Ariano, 1995). Furthermore, a ligand binding study described D3 receptor binding in human cortex (Herroelen et al., 1994). In addition, RNA analysis of human neocortical tissue yielded consistent levels of D3 mRNA (Schmauss et al., 1993; Liu et al., 1994), and a recent *in situ* hybridization study detected D3 receptor message in the human medial temporal lobe (Meador-Woodruff et al., 1994). This is to be expected if, as has been proposed, the D3 receptor is found preferentially in brain regions innervated by the A10 group of dopaminergic neurons in the ventral mesencephalon (Sokoloff et al., 1992), since this group of dopaminergic neurons also innervates the primate neocortex to a considerable extent (Lewis et al., 1988; Febvret et al., 1991; Gaspar et al., 1992). The difficulty in identifying cortical D3 receptors might be due to the fact that many studies utilize ligand binding techniques, which are relatively insensitive since a truly D3 receptor-selective ligand has not been available (Chumpradit et al., 1994; Liu et al., 1994; Burris et al., 1995). Others use *in situ* hybridization to localize mRNA, which by definition does not provide information about its translation into protein. It also does not reflect the true distribution of the protein within the cell (Larson and Ariano, 1995). Immunocytochemistry performed with a subtype-specific antibody enables the determination, with high sensitivity and specificity, of the localization of the D3 receptor protein, and its cellular and subcellular distribution. Moreover, in view of the regional variability of the dopaminergic innervation of the neocortex of the rat and primate, immunocytochemistry may be used to investigate whether the distribution of the D3 receptor shows complementary heterogeneities as well. Finally, the expression of neocortical D3 receptor mRNA has recently been described as being altered in schizophrenia (Schmauss et al., 1993). Dopamine D3 receptor immunocytochemistry may thus be instrumental in determining any alterations in this receptor protein in psychotic disorders.

In order to characterize further the distribution of the D3 receptor in the rodent and primate brain, we analyzed selected cortical areas in the rodent and primate cortex and subcortical structures that have been reported to contain high densities of this receptor, using a novel subtype-specific monoclonal antibody. Some of the data contained in this report have been presented in abstract form (Nimchinsky et al., 1994).

MATERIALS AND METHODS

Immunocytochemistry on rat and monkey tissue

Eighteen macaque monkeys (4 *Macaca fascicularis* and 14 *Macaca mulatta*), and four rats were used in the present study. No qualitative differences in staining were observed between the two monkey species. Materials from these animals were used for other studies as well (Hof et al., 1995; Nimchinsky et al., 1995a; Hof et al., submitted). All experimental protocols were conducted according to NIH guidelines for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mount Sinai School of Medicine. The animals were perfused intracardially as previously described (Hof and Nimchinsky, 1992; Hof and Morrison, 1995). Briefly, the animals were deeply anesthetized with ketamine hydrochloride (25 mg/kg) and pentobarbital sodium (20-35 mg/kg i.v.), intubated and mechanically ventilated. The chest was then opened, the heart exposed, and 1.5 ml of 0.1% sodium nitrite was injected into the left ventricle. The descending aorta was clamped and the monkeys were perfused transcardially with cold 1% paraformaldehyde in phosphate-buffered saline (PBS) and then for 8-9 minutes with cold 4% paraformaldehyde in PBS. Following perfusion, the brain was removed from the skull, cut into 1 cm-thick blocks and postfixed in 4% paraformaldehyde for six hours at 4°C. It was then immersed in phosphate-buffered saline (PBS) until processing. Rats were anesthetized with xylazine and ketamine (10 mg/kg and 30 mg/kg, respectively, i.m.) and then perfused through the heart with 1% paraformaldehyde for 1 minute, then 4% paraformaldehyde for 8 minutes.

The brains were cut either coronally or parasagittally. For the monkeys, the following blocks were taken: a parasagittal block through the central sulcus at the level of the arcuate spur containing portions of both the primary motor cortex (area M1) and the primary somatosensory cortex (area S1); a coronal block close to the occipital pole containing a portion of the primary visual cortex (area V1); a coronal block through the principal sulcus containing prefrontal cortex; a coronal block of anterior cingulate cortex just caudal to the genu of the corpus callosum; a coronal block of posterior cingulate cortex at the midlevel of the intraparietal sulcus; a block through the hippocampal formation anterior to the level of the lateral geniculate nucleus; a parasagittal block of cerebellum and transverse blocks of midbrain. Unless otherwise noted, all incubations were performed at room temperature, shaking. All tissues were cut on a Vibratome at a setting of 50 μ m. The free-floating sections were then incubated with a preblocking solution containing 20% normal horse serum (NHS), 1% gelatin and 1 mg/ml bovine serum albumin (BSA) for 30 minutes, then a solution consisting of PBS with 5% NHS, 1% gelatin and 1 mg/ml BSA, and either the supernatant (1:20 to 1:30) or ascites (1:1000) containing the primary antibody. Pre-blocking and blocking with serum, gelatin and BSA were only necessary for incubations using the supernatant form of the primary antibody. Sections were incubated in this solution overnight at 4°C. They were then rinsed with PBS, placed in a blocking solution of PBS with 2% NHS and 1% gelatin for 30 minutes, and then into a biotinylated anti-mouse IgG secondary antibody solution (Vector Laboratories, Burlingame, CA; 1:200) for 1 hour. In addition, some sections were double-labeled with antibodies against the D3 receptor and either glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO; 1:500) or the calcium-binding protein calbindin (Swant, Bellinzona, Switzerland; 1:1500). For brightfield microscopy, the sections were processed with the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB) as a chromogen, rinsed, mounted onto chrom-alum coated slides, air dried, and immersed in 0.067 % OsO₄ for 8

minutes to intensify the DAB. For fluorescence and confocal microscopy, sections were placed into a solution containing FITC-avidin D (1:200) for two hours. For sections that were incubated in both anti-D3 receptor and anti-GFAP or anti-calbindin antibodies, rhodamine-conjugated anti-rabbit IgG secondary antibody (Boeringer Mannheim, Germany; 1:100) was added to the biotinylated anti-mouse IgG secondary antibody. Prior to visualization they were coverslipped with Vectashield mounting medium. For brightfield and epifluorescence microscopy, sections were analyzed on a Zeiss Axiophot microscope equipped with appropriate filters to visualize FITC and rhodamine. For confocal analysis, sections were scanned on a laser scanning microscope (Zeiss LSM 410) using a Zeiss NeoFluar 63x or 40x objective. For visualization of rhodamine, an ArKr 488/568 laser was used with a 590 nm long pass emission filter, and for visualization of FITC, the same laser was used with a 515-540 nm bandpass emission filter. For electron microscopy, sections were treated as for brightfield material, except that after incubation in secondary antibody they were immersed in 2% glutaraldehyde for 20 minutes. After reaction with 3,3'-diaminobenzidine (DAB), they were rinsed, placed in 1% OsO₄ for one hour, rinsed again, and immersed in 2% uranyl acetate for 30 minutes. They were then dehydrated through a series of graded alcohol solutions, immersed in propylene oxide, placed into Araldite resin and cured in an oven overnight at 60°C. Ultrathin sections from areas of interest were cut on an Ultracut E ultramicrotome (Reichert-Jung, Germany) at approximately 60 nm. Sections were picked up with 200 mesh copper grids, post-stained with uranyl acetate and analyzed on a Zeiss CH10 electron microscope.

RESULTS

The characterization of the anti-D3 monoclonal antibody is presented in Appendix 1.

Light microscopy

In the macaque monkey, immunoreactivity was observed in all brain regions analyzed. Immunoreactive neuronal and glial perikarya were found in all examined structures. The striatum consistently contained high numbers of immunoreactive neurons, possibly representing medium spiny cells, and many, but not all, of the large interneurons were intensely labeled (Fig. 6.1A). The neuropil consisted of a fine feltwork of immunoreactive processes (Fig. 6.1B). No differences were apparent either in numbers of stained cells or in intensity of labeling between ventral and more dorsal portions of the striatum. In the substantia nigra, neuronal somata were moderately stained (Fig. 6.1C), as were those of the neighboring oculomotor or red nuclei. The pyramidal neurons of the olfactory tubercle were labeled (Fig. 6.1D), as were those of the adjacent piriform cortex. In the cerebellum, the most prominent labeling was found in the Purkinje cell layer, where it was virtually restricted to presumptive Bergmann glia somata (Fig. 6.1E). Their radial processes extending through the molecular layer were also stained (Fig. 6.1F). Purkinje cell somata were conspicuously unstained (Fig. 6.1F), but their dendrites in the molecular layer were labeled (see confocal analysis below). Golgi cells of the granule cell layer were intensely immunoreactive (Fig. 6.1F). In addition, the somata of basket and outer stellate cells were lightly labeled, and the granule cells appeared to be lightly stained either in their cytoplasm or their membranes (Fig. 6.1F). All of the deep cerebellar nuclei contained both stained and unstained neurons, and some of both groups were surrounded by an immunoreactive layer that also proved to be glial in origin. No differences in staining pattern or intensity were observed among cerebellar lobules.

In neocortex, many immunoreactive neuronal somata were found in all layers, with the exception of layer IV, which contained few, lightly stained small neurons (Fig.

6.2C). Regional variations in the staining pattern were occasionally subtle but very consistent across animals. In prefrontal cortical area 46, as in most areas examined, labeled pyramidal neurons were very conspicuous, and constituted by far the majority of stained elements (Fig. 6.2C). Their somata were moderately to intensely immunoreactive. At the border of layers I and II, apical dendrites could be observed bifurcating, and the deep half of layer I contained a fine meshwork of lightly immunoreactive processes (Fig. 6.2D). Basal dendrites were also stained, most prominently in layer V. Dendrites were smooth, with no evidence of immunoreactive spines. Immunoreactive glia were found in the cortex and in the subcortical white matter. In the primary motor cortex, immunoreactive neurons were observed in all cellular layers, and clusters of large layer Vb neurons (Betz cells) were intensely labeled (Fig. 6.2A). Apical dendrites were seen throughout cortical layers II-VI and could be seen ascending, sometimes for hundreds of μm , toward the pial surface. Fine immunoreactive processes were found throughout the cortical neuropil (Fig. 6.2B). In the primary somatosensory cortex as well, intensely immunoreactive neurons were found in all cellular cortical layers (Fig. 6.3A), and in Brodmann's area 1 in the postcentral gyrus, large immunoreactive pyramidal neurons appeared in the deepest portion of layer III. Here, too, stained dendrites, presumably from neurons in layers III through VI, could be seen ascending toward the pial surface, and the neuropil contained numerous, very fine immunoreactive processes (Fig. 6.3B). Layer IV contained immunoreactive neuronal somata, but the neuropil in this layer was somewhat more lightly stained than in the other cortical layers. In the primary visual cortex (V1), in contrast, the neuropil was markedly devoid of stained dendrites, except in layers I and II, the superficial portion of layer III, and layer VI. (Fig. 6.3C-E) In addition, pyramidal neuronal somata were lightly labeled, although Meynert cells were more intensely stained (Fig. 6.3E) In the anterior cingulate cortex, pyramidal neurons were well labeled, but the neuropil contained few immunoreactive processes (Fig. 6.4A). In the posterior cingulate cortex, somata were also well labeled, and layer IV contained numerous lightly stained

perikarya (Fig. 6.4B). In contrast to the anterior cingulate cortex, the neuropil of layers II through VI contained many immunoreactive fibers (Fig. 6.4B). In the hippocampal formation, dentate granule cells were lightly labeled, pyramidal cells in Ammon's horn and subiculum were labeled more intensely, and the dendrites of the latter were distinguishable in the molecular layer of the hippocampal gyrus (Fig. 6.4D). In the stratum lucidum, the dendrites of the CA3 pyramidal neurons were labeled; the neuropil, however, was unlabeled (Fig. 6.4C).

In the rat, the patterns of immunoreactivity in general closely resembled those observed in the monkey. In no examined structure was immunoreactivity absent from neuronal and glial somata, but there were subtle variations in somal staining intensity, as well as differences in the labeling of the neuropil. In the caudate/putamen, for instance, neuronal somata of presumptive medium spiny neurons were stained (Fig. 6.5A), as were some somata of the large putative interneurons. In addition, there was relatively intense labeling of fine processes in the neuropil (Fig. 6.5B). In the globus pallidus, however, while neuronal somata were stained, the neuropil was virtually unstained (Fig. 6.5A). As in the monkey, no differences were observed between ventral and more dorsal portions of the striatum. The pyramidal neurons of the olfactory tubercle were labeled, and the granule cells of the islands of Calleja were lightly stained (Fig. 6.5D). The large cells of the islands of Calleja were notably unlabeled (Figs 6.5C and 6.5D).

In the hippocampal formation, dentate granule cells and pyramidal neurons in all the hippocampal subfields were labeled (Fig. 6.5E). Dendrites of these pyramidal neurons were labeled throughout the molecular layer (Fig. 6.5F). The neuropil in the dentate hilus was more lightly stained than elsewhere in the hippocampus and dentate gyrus (Fig. 6.5E).

In neocortex, pyramidal cell somata were well labeled, and layer IV, where present, was characterized by scattered small immunoreactive cells and a lightly stained neuropil. Layer V pyramidal neurons generally were much more intensely stained than

those in other layers. In the sensorimotor area, numerous apical dendrites of strongly immunoreactive layer V neurons were well labeled (Fig. 6.6A). With higher magnification, as seen in the monkey, the neuropil could be seen to contain many fine processes (Fig. 6.6E). In the anterior cingulate cortex, immunoreactive neurons were present in all cellular layers, but immunoreactive apical dendrites were less numerous than in other cortical areas, and layer V pyramidal neurons were no more intensely labeled than those in other layers (Fig. 6.6B). However, immunoreactive dendrites were prominent at the border between layers I and II (Fig. 6.6D). In the retrosplenial cortex, the layer II granule cells were well labeled, but the neurons immediately subjacent were less frequently stained (Fig. 6.6C). In addition, immunoreactive layer III pyramidal neurons in this area were less numerous than in other areas.

Confocal microscopy

On confocal microscopic analysis, D3 receptor-like immunoreactivity was evident in the cytoplasm of neocortical pyramidal neurons. In some pyramidal neurons, especially those in layer II, an intensely immunoreactive soma gave rise to an apical dendrite with immunoreactivity concentrated at the plasma membrane, and a relatively lightly stained cytoplasm. In most pyramidal neurons, however, the apical dendrite was intensely immunoreactive, and second-order dendrites could readily be seen branching off. No labeled spines were observed at the confocal level. Immunoreactive glial cells were stained throughout their cytoplasm. In the cerebellum, Purkinje cell somata were either unlabeled or very lightly stained, but their dendrites were well labeled (Fig. 6.7A-F). Double-labeling with an antibody to calbindin demonstrated that Purkinje cell spines were also labeled (Fig. 6.7D-F). Immunoreactivity for the D3 receptor appeared gradually, with faint labeling at the most proximal portion of the apical dendrite which increased in intensity at greater distances from the soma (Fig. 6.7A-C). Granule cells were also lightly labeled. Double-labeling with an antibody against GFAP revealed that

the labeled profiles in the Purkinje cell layer were, in fact, Bergmann glia, whose radial processes in the molecular layer were also labeled (data not shown).

Electron microscopy

On electron microscopic analysis, D3 receptor profiles were relatively infrequent. They were most abundant in layer I. When present, electron-dense deposits of DAB could clearly be seen filling dendritic profiles which could often be seen making symmetric synaptic contacts with axonal profiles (Fig. 6.8). Occasionally, *en passant* synapses were seen in contact with immunoreactive dendritic shafts. Asymmetric synapses were occasionally observed on immunoreactive profiles. Immunoreactivity was seen in somata and dendritic shafts; virtually no immunoreactive spines were observed. In addition, no labeled axons were observed, even in layer I.

DISCUSSION

The present study describes the distribution of D3 receptor protein immunoreactivity in selected structures in the rodent and primate CNS using a new monoclonal antibody. The specificity of this antibody is verified by immunoblot analysis of the D3 receptor protein translated *in vitro*, of transfected cells expressing the D3 receptor in an inducible manner, and of monkey brain tissue, as well as by radioimmunoassay. The results obtained were comparable to results with a previously characterized D3-specific polyclonal antibody (Liu et al., 1994; Howe et al., 1995).

The dopaminergic innervation of the primate brain differs significantly from that of the rodent. For instance, in the rat, dopaminergic innervation is found largely in frontal cortical areas, whereas in the monkey, it is found throughout the cerebral cortex (Lewis et al., 1987; Berger et al., 1991). Furthermore, the dopaminergic innervation of the primate cerebral cortex itself is characterized by great regional and laminar heterogeneity, with frontal areas, especially the motor and anterior cingulate cortices receiving the densest innervation, and primary sensory areas the least (Lewis et al., 1986;

Lewis et al., 1987; Lewis et al., 1988; Berger et al., 1991). The present study demonstrates that, at the light microscopic level, the D3 receptor protein is widespread, and displays neither the pronounced species and regional variability characteristic of the dopaminergic innervation of the brain, nor the extreme restriction of the D3 receptor indicated by *in situ* hybridization and ligand binding studies (Sokoloff et al., 1990; Bouthenet et al., 1991; Lévesque et al., 1992; Murray et al., 1992; Sokoloff et al., 1992; Landwehrmeyer et al., 1993a; Landwehrmeyer et al., 1993b; Parsons et al., 1993; Herroelen et al., 1994; Murray et al., 1994; Wallace and Booze, 1995). Findings similar to those reported in the present study were reported previously in two other studies utilizing two different anti-peptide antibodies to the D3 receptor (Ariano and Sibley, 1994; Larson and Ariano, 1995). If it is considered, however, that the relevant postsynaptic features in the dopaminergic system are dendrites and spines, not perikarya, these apparent conflicts might be resolved. At the regional level, although the differences in the present study among cortical areas stained for the D3 receptor were relatively subtle, the neuropil in the primary visual cortex was characterized by much less staining of fine processes than in most other cortical areas. Since the bulk of the dopaminergic terminals may synapse on such fine dendrites, the relative paucity of these fibers may reflect the very low density of dopaminergic terminals that characterizes this area (Lewis et al., 1987). This notion is supported by the fact that the only layers in primary visual cortex containing significant D3 receptor-immunoreactive neuropil were layers I and II which receive the bulk of the dopaminergic input to this area (Lewis et al., 1987; Berger et al., 1991). In addition, the small number of immunoreactive profiles observed at the electron microscopic level, in spite of the fact that most cortical neuronal somata at the light level appear to be immunoreactive, suggests that somal immunoreactivity does not ensure immunoreactivity at the synaptic level. Conversely, the observations made of cerebellar Purkinje cells indicate that absence of somal immunoreactivity does not indicate absence of the protein from the dendritic compartment. In addition, the absence

of immunoreactive spines in the cerebral cortex in the same brains which had immunoreactive Purkinje cell spines implies a further restriction within the dendrite, depending on cell type. These differences suggest that if there is, indeed, functional restriction of D3 receptors, it might be occurring not at the transcriptional or translational level, but by means of anatomic compartmentalization, whereby a nearly ubiquitous receptor protein may be fully functional and accessible only in a limited number of areas. Thus perikaryal immunoreactivity may not serve as an index of *functional* receptor density. This notion, while helping to explain discrepancies between the results of immunocytochemical and ligand binding studies, also indicates that further studies of the anatomic distribution of the D3 receptor be done at much higher levels of resolution than previously suspected.

Although negative findings, especially at the electron microscopic level, must be interpreted cautiously, certain negative results in the present study are worth noting. First, the apparent absence, at both the light and electron microscopic level, of labeled axons, even in layer I of the anterior cingulate cortex, where dopaminergic axons are especially abundant (Lewis et al., 1988), would appear to argue against a predominantly presynaptic role for the D3 receptor. Second, the failure to identify clearly any labeled spines in the cerebral cortex is potentially significant, since the D1 receptor has been described as being present preferentially in spines (Smiley et al., 1994), where dopaminergic axons may have a role in modulating excitatory inputs (Goldman-Rakic et al., 1990). The present findings, however, argue for a somewhat different role for the D3 receptor, one where dopaminergic input to shafts may modulate the excitability of a neuron at a point separate from, and less remote from the soma than, the excitatory input to the spines. Since labeled spines were not apparent, it is not possible to state which cerebral cortical neuronal cell type or types contain the D3 receptor. At the light microscopic level, the vast majority of immunostained neurons were pyramidal in shape, and given the ratio of pyramidal to nonpyramidal neurons in cortex (Hendry et al., 1987;

Ren et al., 1992; Hornung and De Tribolet, 1994), it would stand to reason that the majority of D3 receptor protein-containing neurons were pyramidal. This, however, should not be viewed as excluding particular subpopulations of nonpyramidal cells from the population of D3 receptor-containing neurons.

The widespread presence of immunoreactive glial cells is an unexpected finding. Glial cells are known to bear numerous receptors, such as kainate and AMPA/kainate glutamate receptor subtypes, GABAA, muscarinic, H1 and H2, 5-HT1A, β -adrenergic, and even the D2 receptor (Liu et al., 1993; Rosier et al., 1993; Van Der Zee et al., 1993; Whitaker-Azmitia et al., 1993; Bal et al., 1994; Inagaki and Wada, 1994; Patneau et al., 1994). The role these receptors play is not yet known, but it has been hypothesized that since glial cells take up glutamate at the synaptic cleft in a voltage-dependent manner, changes in glial membrane potential could greatly influence the concentration of synaptic glutamate (Barbour, 1988). Thus if the D3 receptor's second messenger system, which has not yet conclusively been elucidated, could affect this potential, it may be able to modulate glutamatergic neurotransmission at the level of the synapse. In this context, because the D3 receptor has a very high affinity for dopamine (Sokoloff et al., 1990; Lévesque et al., 1992), it may be especially well suited to act as a receptor in locations where dopamine levels are particularly low, as in cerebellum (Ikai et al., 1992). Furthermore, the presence of the D3 receptor in glial cells throughout the brain, even in the subcortical white matter and other regions apparently devoid of dopaminergic terminals, may represent an involvement of this receptor in the mediation of nonsynaptic dopaminergic neurotransmission (Bach-y-Rita, 1993).

The present data suggest that the dopamine D3 receptor has a far more widespread distribution than previously thought, and is thus in a position to play a role in the mediation of dopaminergic neurotransmission not only in limbic structures, but throughout much of the brain, including neocortex. In this context, D3 receptor mRNA has been reported as being reduced in certain cortical areas in schizophrenia (Schmauss et

al., 1993). While the full significance of this finding is not yet understood, immunocytochemical approaches may assist in further investigations of the architecture of the dopaminergic system in the normal brain, as well as in affective disorders.

FIGURE LEGENDS

Fig. 6.1 Immunocytochemical localization of the D3 receptor protein in selected subcortical sites in the macaque monkey. In striatum, large, presumably cholinergic neurons are well labeled (arrows in **A**), as is the striatal neuropil (**B**). Neurons of the substantia nigra, pars compacta are immunoreactive, as are their dendrites in the pars reticulata (**C**). In olfactory tubercle (**D**), granule cells are lightly labeled, whereas pyramidal neurons are more intensely labeled. One superficial island is indicated by arrows. In cerebellum (**E**, **F**), Bergmann glial cells are well labeled (arrowhead), but Purkinje cell somata are notably unstained. Purkinje cell dendrites are, however, immunoreactive (see Fig. 6.9). Granule cells are lightly stained, but Golgi cells in the granule cell layer and outer stellate and basket cells in the molecular layer are well labeled (curved arrow). SNr = substantia nigra, pars reticulata. cp = cerebral peduncle. Scale bar = 150 μ m (**A**, **C**, **D**, **E**), 25 μ m (**B**) and 40 μ m (**F**).

Fig. 6.2 Distribution of dopamine D3 receptor protein in the neocortex of the macaque monkey. In primary motor cortex (**A**), labeled cells are found in all cortical layers, and giant layer V cells of Betz are particularly well stained. At higher magnification, the cortex, such as in the superficial portion of layer V, shown here (**B**), is found to contain numerous immunoreactive fibers, presumably dendrites. In prefrontal cortex (**C**), the greater neuronal packing density is evident, and labeled neurons are seen throughout layers II through VI. The junction between layers I and II in the prefrontal cortex,

(indicated by the arrowhead in D), as in most other cortical areas examined, is the site of immunoreactive branching apical dendrites of pyramidal neurons, and layer I contains a meshwork of immunoreactive processes (D). Scale bar = 150 μm (A, C), 40 μm (C) and 25 μm (D).

Fig. 6.3. Immunocytochemical localization of the D3 receptor protein in the macaque neocortex. Primary somatosensory cortex (A) contains large numbers of intensely immunoreactive neurons and glia. The neuropil in this area also contains numerous immunoreactive fine processes, as shown here in layer III (B). Primary visual cortex, in contrast to all other neocortical areas analyzed in the present study, is characterized by very light immunoreactivity, which is only clear at high magnification. Immunoreactive dendrites are most evident at the border between layers I and II (C), layer V (D), and in layer VI, where Meynert cells were more intensely immunoreactive (E). Scale bar = 150 μm (A), 25 μm (B) and 40 μm (C-E).

Fig. 6.4. Immunocytochemical localization of the D3 receptor protein in the macaque monkey cortex. Anterior cingulate cortex (A) contains intensely immunoreactive neurons in all cellular layers. Posterior cingulate cortex (B) is characterized by more labeled dendrites in the neuropil, which are sparser in layer IV. In the hippocampal formation (C), pyramidal neurons in CA4 through CA1 have labeled somata and apical dendrites. The neuropil of the stratum lucidum is unlabeled except for the apical dendrites of CA3 pyramidal neurons (star in C). At higher magnification, the immunoreactive branching apical dendrites of CA1 pyramidal neurons can be resolved (D). Scale bar = 150 μm (A-C) and 40 μm (D).

Fig. 6.5 Immunocytochemical localization of the D3 receptor protein in selected subcortical and cortical sites in the rat. In striatum (A, B), presumptive medium spiny and large neurons are well labeled (A), and the neuropil appears as a dense immunoreactive meshwork (B). The neuropil of the globus pallidus (GP) is relatively unstained. In olfactory tubercle (C, D), comparison of cytoarchitecture using a Nissl stain (C) with immunoreactivity for the D3 receptor protein (D) reveals that the pyramidal cells are well labeled, but the granule cells of the islands of Calleja are only faintly stained. The large neurons of the hilar regions of the islands of Calleja are apparently unstained. Low magnification photomicrograph of a parasagittal section through the dorsal hippocampus (E) reveals that all subsectors of the hippocampal formation contain immunoreactive neurons. The polymorphic layer of the dentate gyrus, however, is relatively lightly labeled. At high magnification (F), the dendrites of CA1 neurons can be seen to be clearly labeled throughout the molecular layer. Scale bar = 150 μm (A, C, D), 25 μm (B), 300 μm (E) and 40 μm (F).

Fig. 6.6 Immunocytochemical localization of the D3 receptor protein in selected cortical areas in the rat. In sensorimotor cortex, layer V contains large, intensely immunoreactive neurons (A). The neuropil, as is shown for layer V (E) is characterized by abundant fine dendritic staining. Anterior cingulate cortex (B) contains immunoreactive cells and dendrites throughout the thickness of the cortex, and layer I here as elsewhere consists of a rich feltwork of immunoreactive fibers (D). Posterior cingulate cortex contains immunoreactive neurons throughout its thickness, but particularly in layer II-IV (C). Layer III is characterized here by a slightly less robust immunoreactivity than in other cortical areas. Scale bar = 150 μm (A, B, C) and 40 μm (D, E).

Fig. 6.7 Confocal analysis of macaque monkey cerebellum immunostained for calbindin (**A, D**) and D3 receptor protein (**B, E**). **C** and **F** represent overlays of the red and green pseudocolored images. Colocalization is indicated in yellow. Purkinje cell somata are relatively lightly labeled, but immunoreactivity increases in the dendritic arbor with increasing distance from the soma (**A-C**). Many Purkinje cell spines are labeled (**D-F**). **(G-I)** confocal analysis of glial cells in the subcortical white matter of the macaque monkey double labeled for GFAP and D3 receptor protein. Most examined glial cells in cortex and in white matter proved also to be D3 protein-immunoreactive. Scale bar = 50 μm (**A-C**), 15 μm (**D-F**) and 30 μm (**G-I**).

Fig. 6.8 Ultrastructural localization of the D3 receptor protein in the cingulate cortex of the macaque monkey. Cross-sections of immunolabeled dendritic shafts (stars) are seen receiving symmetric synapses. Asymmetric synapses are encountered less frequently. Unlabeled synapses are indicated by double arrows. Scale bar = 0.31 μm (**A**) and 0.25 μm (**B**).

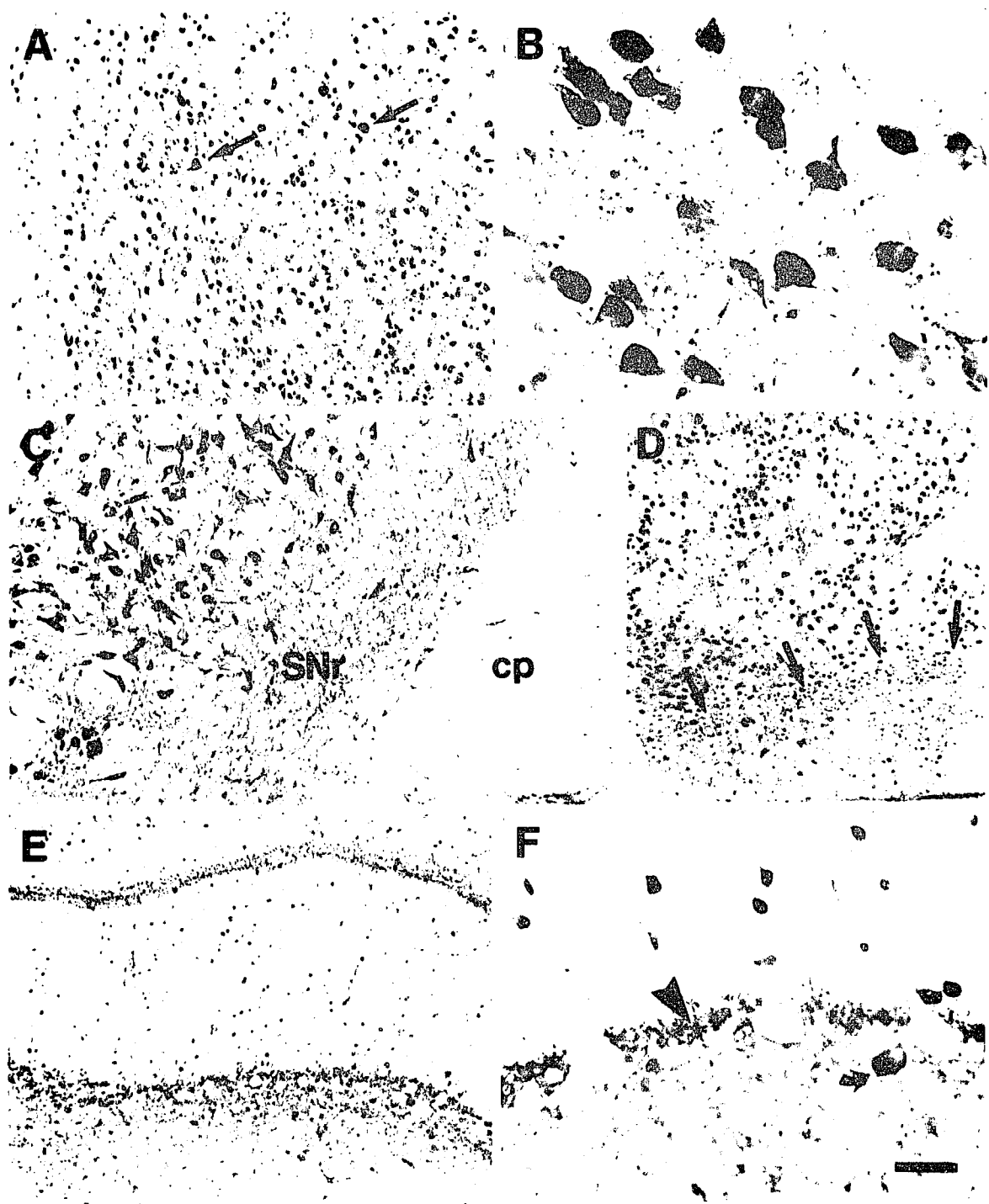


Fig. 6.1

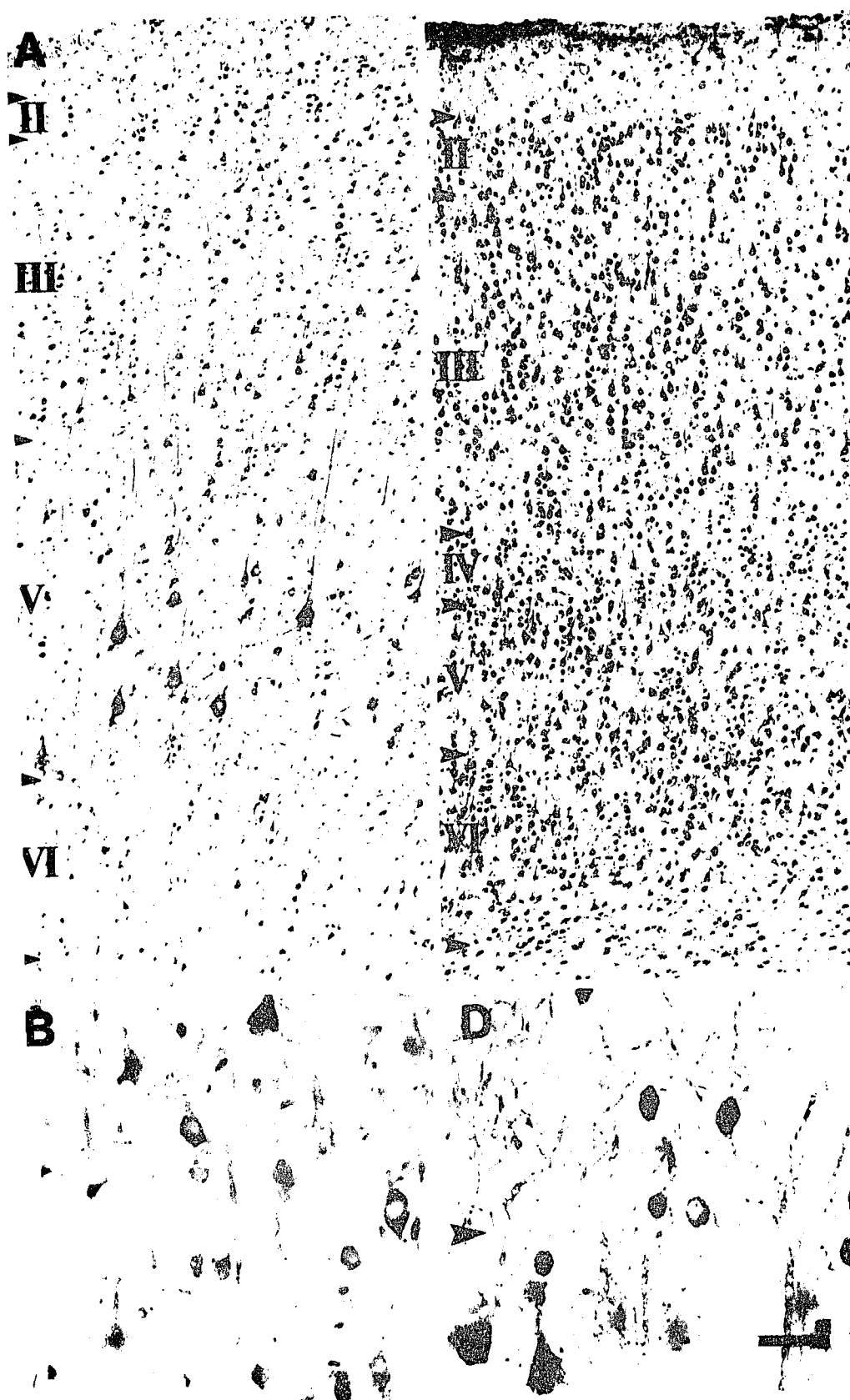


Fig. 6.2

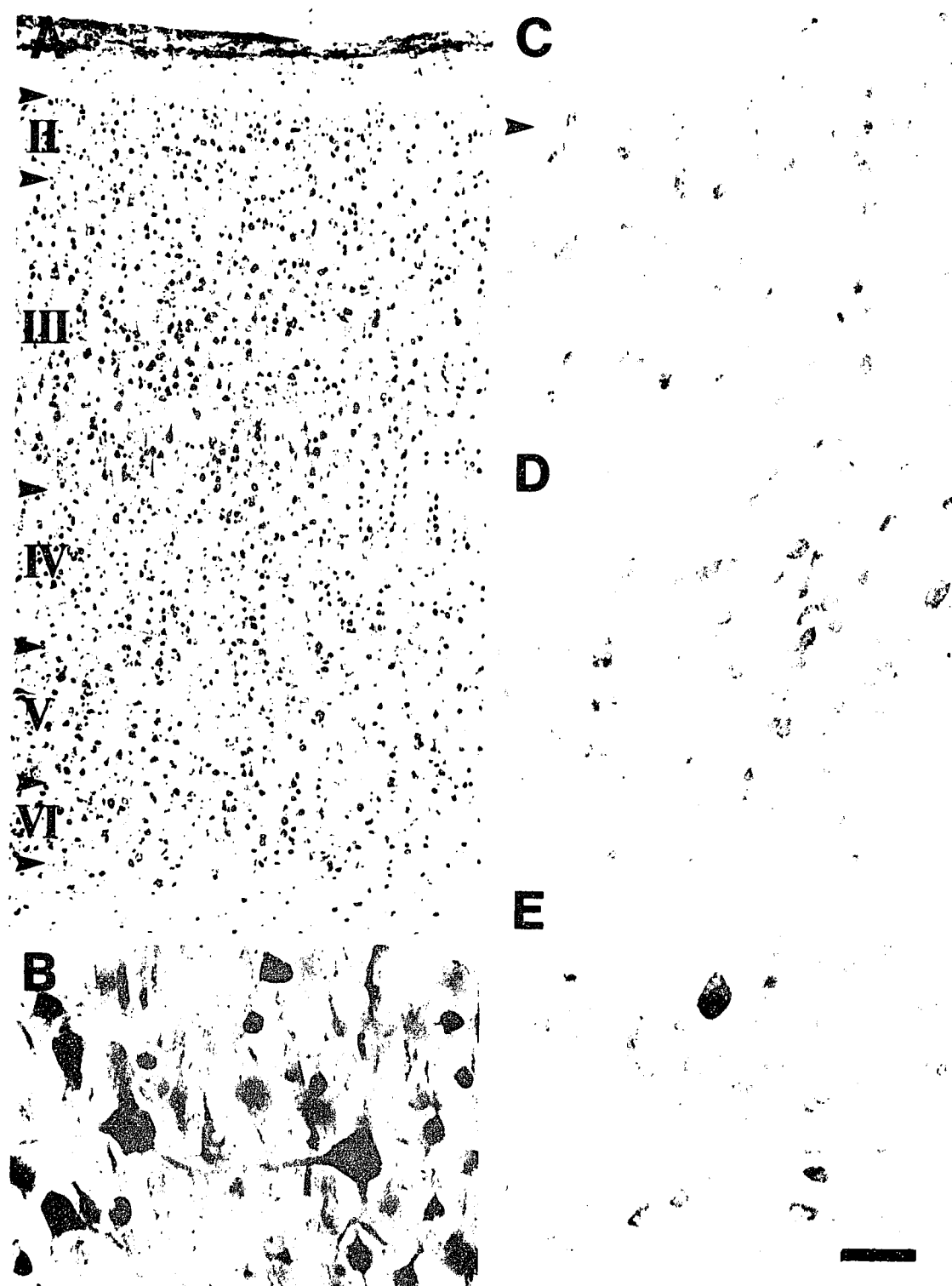


Fig. 6.3

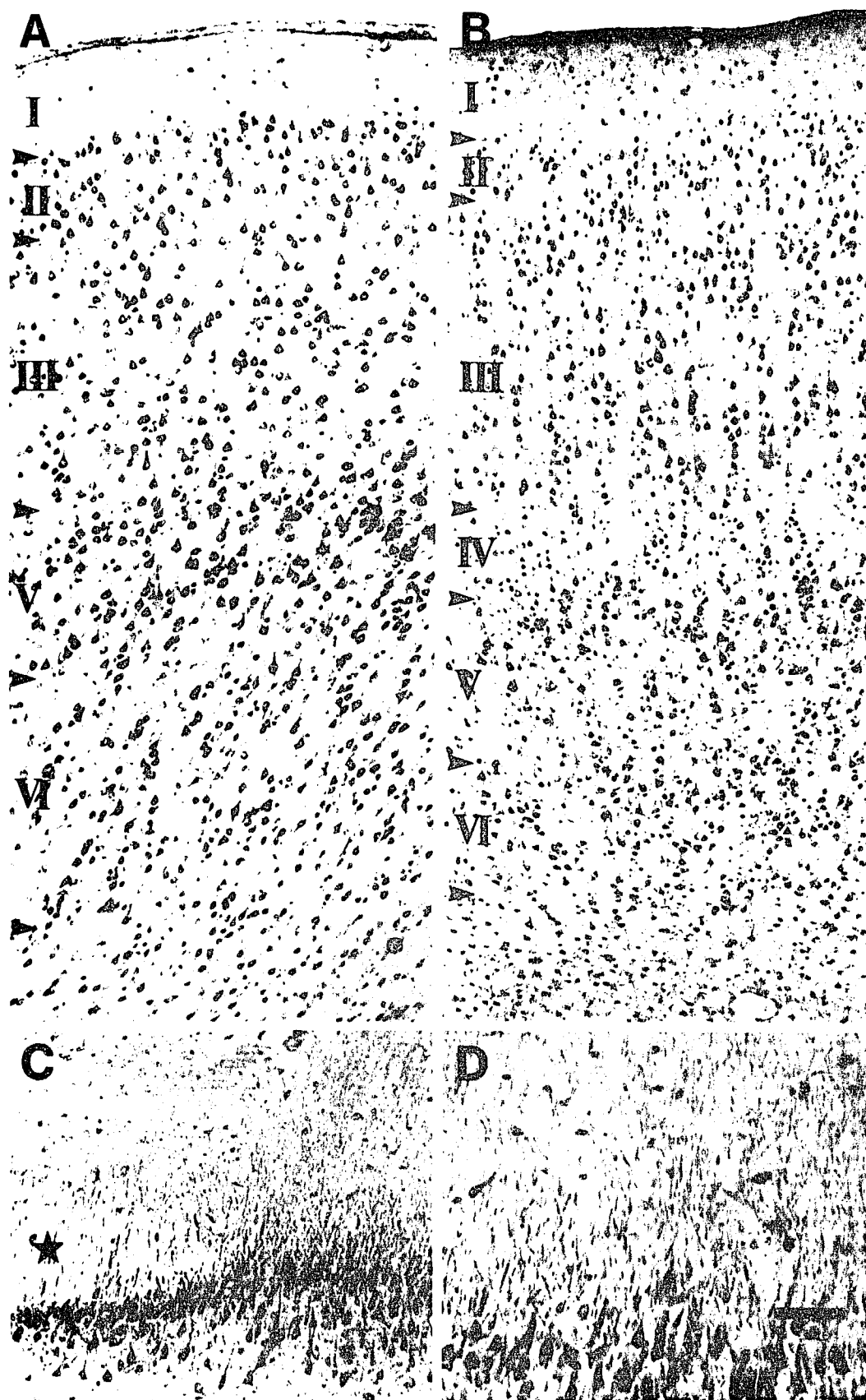


Fig. 6.4

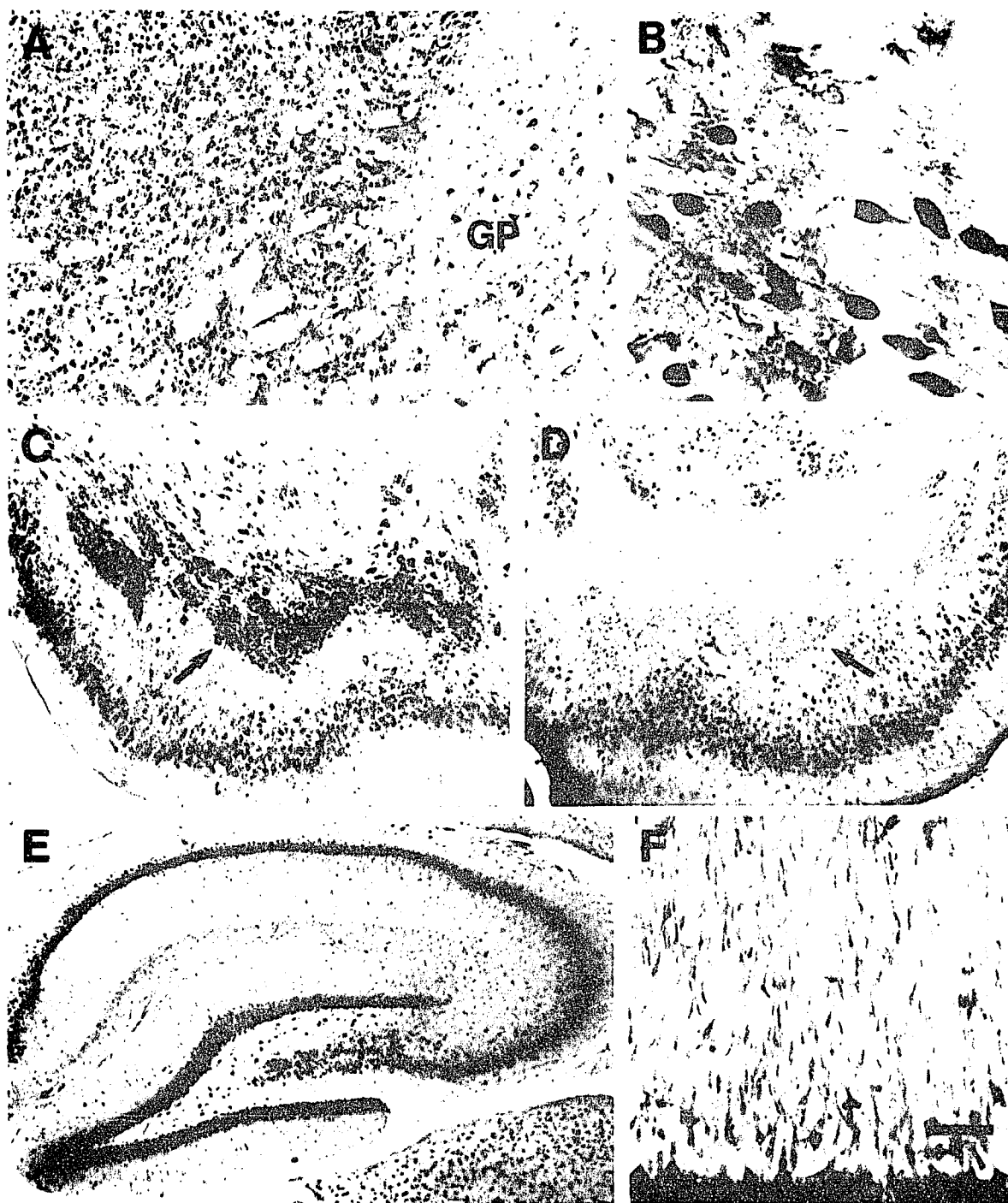


Fig. 6.5

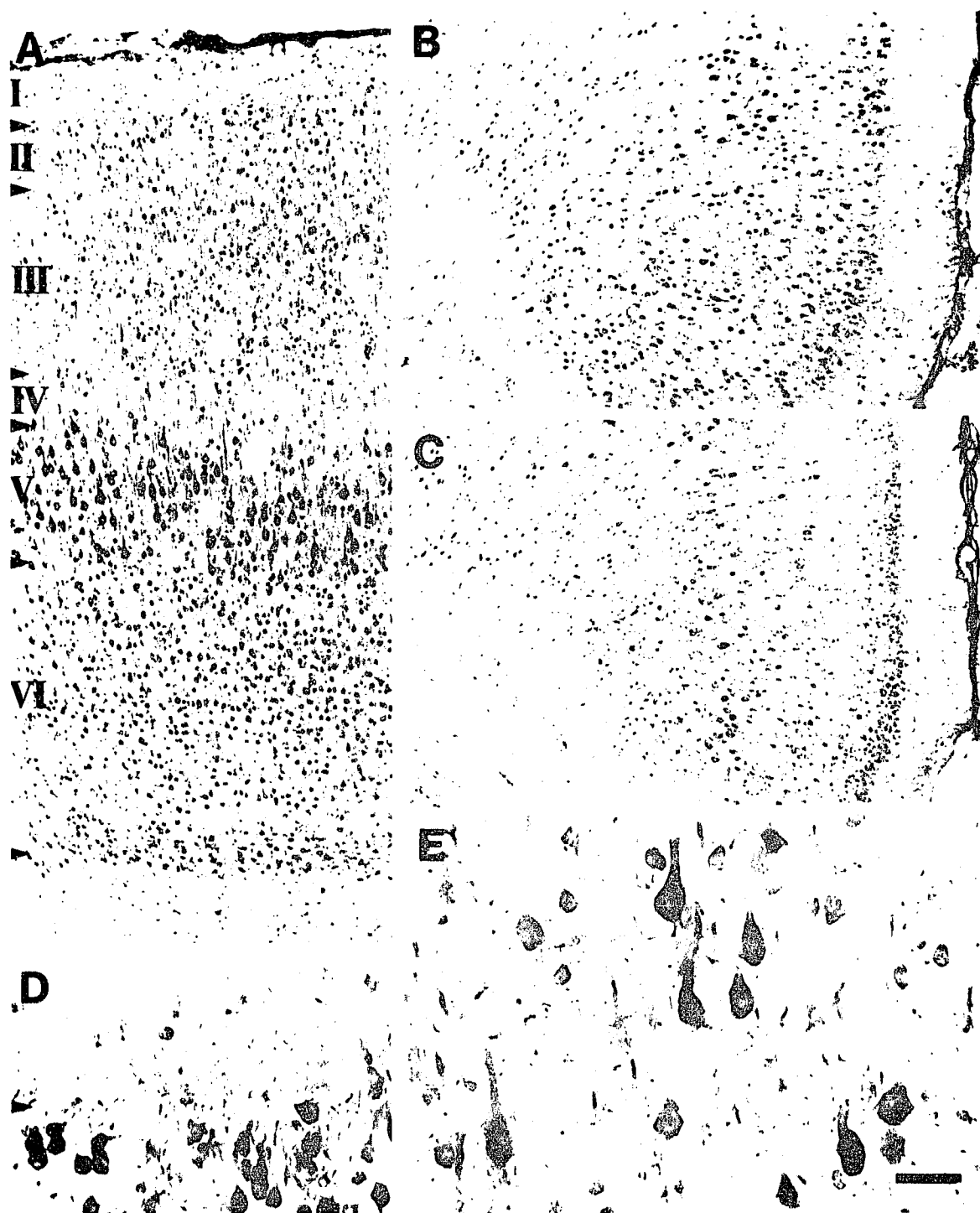


Fig. 6.6

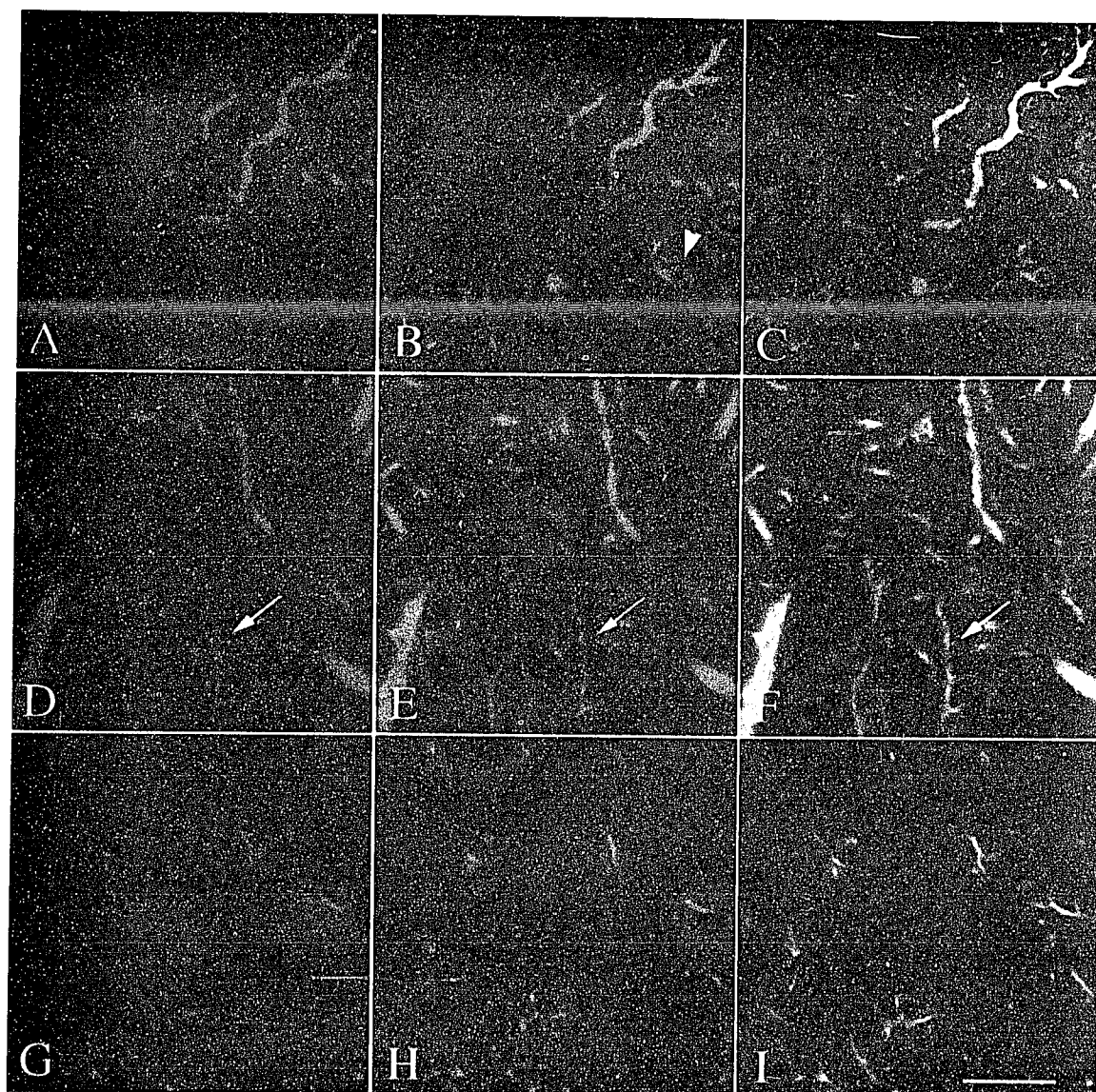


Fig. 6.7

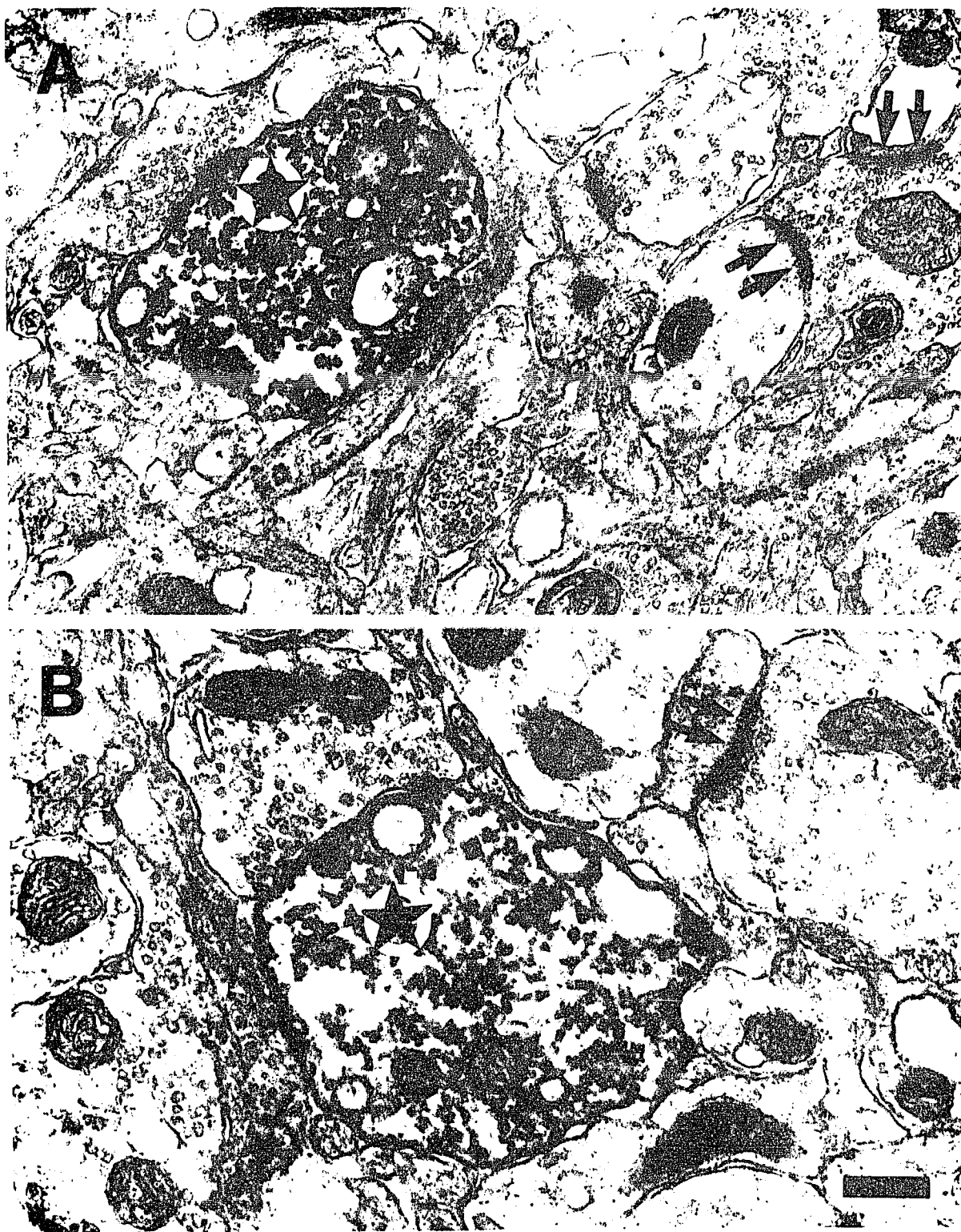


Fig. 6.8

SUMMARY AND CONCLUSIONS

The human cerebral cortex does not readily lend itself to intensive anatomic study. For obvious reasons, the tools available for the study of nonhuman species, such as routine electrophysiologic recordings and tract tracing are virtually inaccessible in the human, except in rare instances such as neurosurgical procedures or naturally occurring focal degeneration. In recent years, improved physiologic techniques, and especially the advent of functional imaging, which permits, for the first time, the routine study of the living normal human brain, have made possible great advances in our knowledge of functional roles of numerous cortical areas in the human and nonhuman primate. At the same time, improved anatomic techniques, notably tract-tracing methods in animals and immunocytochemistry in humans as well, have made it possible to move beyond the Nissl and myelin stains and more intensively to study the anatomy of the cerebral cortex. In a potentially powerful approach, information obtained from correlative connectional and chemoarchitectonic studies in animals, and particularly in nonhuman primates, can be used in conjunction with chemoarchitectonic studies in the human, to extrapolate connectional information on the human brain. The combination of this approach with the physiologic should bring us to a greater understanding of the cerebral cortex.

The studies presented in the preceding chapters represent one small part of this endeavor. The cingulate cortex was chosen for a number of reasons. Anatomically, it is interesting because it possesses a range of cortical types, from the hippocampal rudiment, to agranular cortex, to motor-like areas, to isocortex. It shares this property with other regions of the primate brain that have shown considerable expansion in the human, such as the prefrontal and temporal neocortex. However, unlike these areas, it has retained its characteristic shape and its general topography is readily recognizable in both the monkey and human. More importantly, it has long been recognized as a region which subserves many diverse functions, and it has emerged recently as a participant in numerous sensory, motor and cognitive functions in which it was not previously known to be involved. This

diversity of function makes this complex of areas unique among cortical regions, and an intriguing place to study the possible functional significance of anatomic features.

Comparative chemoarchitecture in the macaque monkey and the human

The human studies presented here were performed on brain material from aged individuals. The notorious difficulty in obtaining normal human material of any age suitable for immunocytochemistry is frequently a barrier to normative studies, and often makes one receptive to suboptimal tissue. In the present studies, we were fortunate enough to obtain very good-quality tissue from nondemented individuals, with short postmortem intervals and fixed with paraformaldehyde. Comparison with human materials from younger individuals described in the literature, as well as with staining with the same antibodies in the macaque monkey convinced us that the quality of staining we obtained was comparable to that found in the normal brain. Quantitative techniques were used cautiously, always in a self-comparative manner, to minimize the bias arising from suboptimal tissue conditions. While these precautions may seem troublesome, and the potential problems arising from a small sample cannot be ignored, there is no replacement for human brain tissue in the study of the human brain, and every effort made to obtain, optimize, and accommodate it is well worth the trouble.

In keeping with the complexity suggested by Nissl studies, the chemoarchitectonic studies in Chapters 2 and 3 of this dissertation present the extreme variability in the biochemically defined structure of the cingulate cortex along its length and breadth. In both the macaque monkey and the human, intrinsic cell populations, both interneuronal and pyramidal cell populations have distributions that depended on the location within the cingulate gyrus, and among nonpyramidal neurons, certain subtypes have more widely variable distributions than others. The double bouquet neurons containing calretinin (Jacobowitz and Winsky, 1991; Rogers, 1992; Lüth et al., 1993; Condé et al., 1994) have a generally consistent distribution throughout the cingulate cortex, although both of these populations are somewhat sparser in the anterior-most

reaches of the cingulate gyrus. In contrast, the larger nonpyramidal neurons containing parvalbumin, that presumably represent chandelier and basket neurons (Stichel et al., 1987; DeFelipe et al., 1989b; Lewis and Lund, 1990; Ribak et al., 1990; Williams et al., 1992; Condé et al., 1994; Del Río and DeFelipe, 1994), are greatly reduced in number in the most anterior and ventral regions of the cingulate cortex.

The apparently atypical nature of the anterior- and ventral-most portions of the cingulate gyrus is a recurrent theme in the human and nonhuman primate, and is also reflected in the differences in the distribution of neurofilament protein-containing pyramidal neurons. This latter difference represents perhaps the most striking change along the cingulate gyrus, and was, in fact, the observation that prompted the subsequent studies presented in this work. The fact that the most obvious change in neurofilament protein-containing neurons occurs in layer III immediately suggested that these neurons might furnish corticocortical connections. But what differences might exist between corticocortically projecting neurons in the anterior cingulate cortex, which contains essentially no neurofilament protein-containing neurons in layer III, and a functionally equivalent set of neurons in the posterior cingulate cortex? First, would they be equally likely to contain neurofilament protein, or would their neurofilament protein content reflect that of the area of origin at large? Second, could their respective content of neurofilament protein be related to other features that might vary among pyramidal cell populations, such as overall dendritic morphology or spine distribution? If so, neurofilament protein could represent an index of still another neuronal property.

Relationship between neurochemical characteristics of projecting neurons and chemoarchitecture

In the absence of physiologic characterization, few corticocortical projections of the cingulate cortex lend themselves to functional interpretation. For instance, the projection from the anterior cingulate cortex to the prefrontal cortex is well described (Baleydier and Mauguière, 1980; Pandya et al., 1981; Bates and Goldman-Rakic, 1993;

Van Hoesen et al., 1993; Arikuni et al., 1995). However, beyond the possibility of referring to it as an "association" type of connection, it is currently all but impossible to describe its relevance to the animal. The cingulate motor areas, however, provide a unique opportunity, since the projections of the cingulate cortex to the primary motor area (Luppino et al., 1990; Morecraft and Van Hoesen, 1992), as well as to the spinal cord (Ghosh et al., 1987; Hutchins et al., 1988; Dum and Strick, 1991; Dum and Strick, 1992; Dum and Strick, 1993; He et al., 1995) are both well described and functionally comprehensible. The present studies suggest that the characteristic pattern of neurofilament protein-containing neurons observed is due, in part, to the presence of cingulate motor areas in the ventral bank of the cingulate gyrus. These two projections differ in their laminar origin, but their neurochemical phenotype is comparable, in that a similar proportion of each contains neurofilament protein. This linkage of neurochemical phenotype with a functional attribute suggests, among other things, that the characteristic chemoarchitecture of an area may be the result, not the cause, of the neurochemical properties of its constituent projections. Of course, the present studies analyzed only those neurons labeled following injections into one area; the same neurons certainly share other collateral projections, and will just as certainly have still other projections that differ. The nature and degree of the divergence of corticocortically projecting neurons is something that may also contribute to their neurochemical characteristics, and adds a further level of complexity to the chemical coding of these circuits. However, the association of neurochemical phenotype with function in the present simple paradigm provides a basis, at least, for further studies into the relevance of neurofilament protein to a given corticocortically projecting neuron.

On the number and distribution of cingulate motor areas in the macaque monkey and human

One finding arising from this study was that the posterior cingulate motor area, CMAC, is located not, as earlier described, in area 23c (Dum and Strick, 1991; Dum and

Strick, 1992; Dum and Strick, 1993) , but in the caudal-most portion of area 24c'. This difference, only a few millimeters in the macaque, has significance for people studying cytoarchitecture, since the presence of a motor area in a granular area such as area 23c would be a unique occurrence in the primate cerebral cortex. The present study finds that, like the other motor fields in the macaque monkey, the cingulate motor areas are located in agranular or dysgranular areas. The difference from earlier work is probably not due to the fact that the present study analyzed corticocortically projecting neurons, whereas the other studies analyzed corticospinal neurons, since these projections are known to overlap almost completely (Dum and Strick, 1991). It is more likely due to the methods used in the present study, where literally adjacent sections were analyzed for retrogradely labeled neurons and cytoarchitecture. Careful scrutiny of the cytoarchitectonic comparison described by Dum and Strick reveals that the area they describe as area 23c is clearly dysgranular, if not frankly agranular (Dum and Strick, 1991). In addition, another study that made careful analyses both of physiological properties and cytoarchitecture also found that the cortical areas where the cingulate motor areas reside are all agranular or dysgranular (Matelli et al., 1991). Finally, since the gigantopyramidal field described by Braak (1976) is always located anteriorly to the border between areas 24 and 23 (Braak, 1976; Vogt, 1995; the present study), these findings support the idea that this unusual field may be part of the cingulate motor area in the human (Vogt et al., 1995).

The number of cingulate motor areas in the macaque monkey is the subject of some debate. Initially, two were described (Hutchins et al., 1988), but subsequent studies by Strick and colleagues identified three, the CMAd and two caudal ones, CMAv in the ventral bank of the cingulate sulcus, and CMAd in its dorsal bank. The present study agrees with the work of other groups which suggests that there are only two. First, no anatomic delineation is apparent between the CMAv and CMAd; the posterior CMA is best described as a single area that spirals through the cingulate sulcus. The confusion probably arises from the fact that the description of three areas is based on the

distribution of neurons in flattened cortical maps, in a system where only "high density bins" are represented. In such a paradigm, the distortions of neuronal distribution at the crests and fundi of sulci and gyri become exaggerated. The fact that all of the boundaries described by Strick and colleagues on flattened representations of the cortex correspond exactly with the crests or fundi of sulci supports this contention.

On a related note, while there is evidence for at least one somatotopically organized motor area in the human cingulate cortex (Matelli et al., 1993; Paus et al., 1993), there is currently no reason to assume there are two. In this context, it is worth noting that despite considerable similarities, the cingulate cortex of the human and the macaque monkey are substantially different. The existence in the human of areas 33 and 31, as well as of spindle neurons and calretinin-immunoreactive pyramidal neurons in area 24, highlights this fact. There has been considerable evolution in the cingulate cortex in the human, and it is equally likely that certain features in the macaque monkey may represent derived characteristics specific to the lineage of Old World monkeys. This concept must be borne in mind when using the macaque monkey as a model for studying the human in general. In the present case, as their resolution improves, functional studies will probably provide the answer to the number and disposition of the cingulate motor areas in the human.

The dopamine D3 receptor

It was hoped for a number of reasons that the dopamine D3 receptor would represent an ideal receptor molecule to study at the cellular level in projection neurons of the cingulate cortex. First, of the neurotransmitter systems known to influence the neocortex, the dopaminergic system has a special significance for the cingulate cortex. The anterior cingulate cortex alone receives the supragenual projection from the substantia nigra (Foote and Morrison, 1987), and in primates, is characterized by one of the densest dopaminergic innervations in the neocortex (Lewis et al., 1987). Furthermore, the dorsal cingulate cortex (areas 24c and 24c') receives an especially dense

dopaminergic input into layer I (Crino et al., 1993). Because of this distribution, dopaminergic receptors could play a role of particular importance in the projections described in Chapter 4. The dopamine D3 receptor is of particular interest for at least two reasons. First, the D3 receptor has been believed to be present in very few regions of the brain, and hardly at all in the neocortex. However, the structures which do contain abundant D3 receptor mRNA and which show putative D3 receptor binding belong to a group of phylogenetically old structures. Among neocortical areas, the anterior cingulate cortex is regarded as one of the oldest, as it is recognizable in all studied mammals (Brodmann, 1909; translation by Garey, 1994). It was therefore thought that if the D3 receptor were to be found in any neocortical area, it might well be the anterior cingulate cortex. The study presented in Chapter 5, was undertaken to characterize a novel monoclonal antibody against the D3 receptor protein, provide the initial anatomic description of its overall distribution in the cerebral cortex, and determine its appropriateness for inclusion into the studies of the projection neurons described in Chapter 4. This study, however, proved that in the case of the D3 receptor at least, meaningful studies of its anatomic distribution would require extensive electron microscopic analysis, and were therefore beyond the scope of this thesis. It is hoped, however, that this study provides the framework, as well as the characterization data, to make such investigations possible in the near future.

Taking all the above together, the primate cingulate cortex emerges as a classic example of the changes the cerebral cortex at large has undergone during mammalian evolution. It has expanded greatly, increasing both the number and size of its areas, and has evolved highly specific cell types, as well as novel functions, possibly by the extension of neighboring fields, as is proposed for the cingulate motor areas, or by the seemingly *de novo* appearance of others, such as those involved in vocalization. By combining its own native properties, such as distinctive thalamic and amygdalar projections, with these new connections, it has given rise to completely novel areas with

potentially unique properties. It remains for functional studies now to elucidate the specific properties that distinguish these unusual cortical areas.

APPENDIX 1

Characterization of a monoclonal IgM antibody against the dopamine D3 receptor

The following is the description of the techniques used to generate and characterize the monoclonal antibodies used in the study presented in Chapter 6. The characterization data are presented on the following pages. The fusion proteins were produced and kindly provided by Dr Allan I. Levey, Department of Neurology, Emory University, Atlanta, Georgia. Antibodies were generated and radioimmunoassays performed by Dr Thomas Moran at the Mount Sinai School of Medicine, New York. The generation of stably D3-expressing cell lines, *in vitro* translation, and immunoblots were performed by Dr Claudia Schmauss at the Mount Sinai School of Medicine, New York.

Generation of monoclonal antibodies. Six- to eight-week old BALB/c mice were immunized with 50 µg of purified fusion protein in Freund's Complete Adjuvant intraperitoneally and boosted similarly 3 weeks later in Freund's Incomplete Adjuvant. Ten days later sera from animals were tested in a radioimmunoassay for binding to fusion protein. The animal with the highest titer was boosted with 24 µg of fusion protein, intravenously, and three days later its spleen was removed and a single cell suspension prepared. One hundred million spleen cells were mixed with ten million SP2/0 myeloma cells in serum free medium and fused using polyethylene glycol 4000 (PEG). Cells were deposited in 5 flat bottom 96 well plates in Iscove's medium, 10% fetal calf serum, gentamycin, sodium pyruvate, L-glutamine, hypoxanthine and thymidine and 24 hours later azaserine added to each well to select for fused cells. Ten to twelve days later, supernatants from wells exhibiting growth were tested for the presence of antibodies which bound to the fusion protein but not to GST alone. Positives were cloned by limiting dilution, and ascites was prepared by injection of 10⁶ hybridoma cells into the peritoneal cavity of pristane primed BALB/c mice.

Radioimmunoassay. A radioimmunoassay for binding to D3 fusion protein was used to screen the hybridoma supernatants. Falcon microtest III flexible assay plates

were coated with 2.5 µg/ml purified GST-D3 fusion protein or GST only. Plates were blocked with PBS-1% BSA and undiluted supernatants added to the wells of both plates. Plates were incubated at room temperature for 90 minutes to allow binding of antibodies. The plates were then washed and 50,000 c.p.m. of ¹²⁵I-iodinated monoclonal rat anti-mouse kappa chain antibody was added to each well to detect bound antibody. Plates were cut up and individual wells counted in a gamma counter. Wells containing antibodies which bound only to the fusion protein were considered positive and cloned by limiting dilution. The same assay was used to test binding of monoclonal antibody to fusion proteins. Plates were coated with 5 µg/ml of fusion proteins 1-7 and undiluted hybridoma supernatant added to each well. Normal mouse immunoglobulin served as negative control. Specific binding is the counts bound minus the background.

In vitro translation of D3-receptor encoded RNA. A T7 RNA polymerase transcript of the human D3 receptor-encoded cDNA was obtained from the linearized plasmid pRc/CMV/hD3. One µg of the *in vitro* synthesized RNA was added to a rabbit reticulocyte lysate mixture (Boeringer Mannheim) and incubated for 1 hour at 30°C. The *in vitro* translation products were separated on 10% SDS polyacrylamide gels (SDS-PAGE).

Generation of stably D3 receptor-expressing GH3 cells, protein extraction, and immunoblot analysis. The stable lines of D3 receptor-expressing GH3 cells with active tetracycline-responsive promoters used in this study are described in Howe et al. (1995). Briefly, the expression of D3 receptors is inhibited when these transfected cells are grown in the presence of tetracycline (1 µg/ml). Removal of tetracycline from the medium leads to a time-dependent increase in the expression of D3 receptor RNA and protein which reaches steady-state levels 24 hours after induction of expression. Cells were collected at various times after induction of D3 receptor expression and proteins were extracted as described (Howe, 1995) Proteins of the monkey temporal cortex were extracted as described in Liu et al. (1994) Proteins (5 µg) were separated on a 10% SDS-PAGE gel

and transferred onto Immobilon PVDF membranes by electroblotting. Blots were blocked in 10% newborn calf serum (Life Technologies), 1x Tris-buffered saline, 0.05% Tween-20 and 0.01% sodium azide for 3 hours. The primary antibodies used were either a rabbit polyclonal anti-peptide antibody (dilution 1:12,500) that was raised against the human D3 receptor (Cambio, Cambridge, U.K.; see Liu et al., 1994; Howe et al., 1995), or the monoclonal IgM anti-D3 receptor antibody (ascites; dilution 1:500). Blots were incubated with primary antibody for 2 hours in the same incubation buffer, washed in 1x Tris-buffered saline, 0.1% Tween-20 for 6 x 10 minutes, and incubated for 1 hour with secondary antibody (peroxidase-conjugated goat anti-rabbit IgG, or goat anti-mouse Ig(G+M); Kirkegaard & Perry Laboratories, Gaithersburg, MD) in the same incubation buffer without sodium azide. Bound antigens were visualized by enhanced chemiluminescence using an ECL kit (Amersham, Arlington Heights, Illinois).

Characterization results

The ability of an IgM anti-D3 monoclonal antibody to recognize the D3 receptor protein was first tested with immunoblots of proteins extracted from stably D3-expressing rat GH3 cells in which the expression of D3 receptors is under the control of a tetracycline-sensitive promotor. A recent study that used a previously characterized polyclonal anti-D3 antiserum (Howe, 1995) showed that induction of D3 receptor expression in these cells correlates with the time-dependent appearance of both D3 mRNA and a major D3 immunoreactive band of approximately 50 kD. As shown in Fig. A.1A, similar results were obtained when immunoblots of proteins extracted from these cells at various times following induction of D3 receptor expression were probed with the monoclonal antibody. The intensity of the approximately 50 kD immunoreactive band increases after induction in a time-dependent fashion and reaches steady-state levels after 24 hours. No immunoreactive signals were obtained on immunoblots of protein extracted from non-transfected GH3 cells.

Furthermore, the monoclonal antibody recognizes on immunoblots an approximately 50 kD protein that resulted from the *in vitro* translation of human D3 mRNA in rabbit reticulocyte lysate. As shown in Fig. A.1B, this result is identical to the result obtained with the polyclonal anti-D3 antiserum, and no immunoreactive signals were obtained when D3 mRNA was omitted from the *in vitro* translation reaction.

Incubation of immunoblots of proteins extracted from macaque monkey temporal neocortex with the monoclonal anti-D3 antibody resulted in the appearance of two major immunoreactive bands of 45 and 58 kD, and a minor band of approximately 50 kD (Fig. A.1C).

FIGURE LEGEND

Fig. A.1. Detection of D3 receptor proteins on immunoblots. (A) Immunoblots of proteins extracted from stably D3 receptor-expressing rat GH3 cells. In the cell line used in the present study, the expression of the human D3 receptor is under the transcriptional control of a tetracycline-sensitive promotor. The blots were probed with the monoclonal antibody (IgM, left), or an affinity-purified rabbit polyclonal antipeptide antibody that was raised against the amino terminus of the D3 receptor (pc, right). The lane marked m contains the molecular size standards. The lanes marked 5, 9, 24 and 48 contain proteins extracted from cells 5, 9, 24 and 48 hours, respectively, after induction of D3 receptor expression by withdrawal of tetracycline from the culture medium. The lane marked nt contains proteins extracted from nontransfected GH3 cells. Five μ g of total cellular protein was loaded onto each lane. (B) Immunoblot of *in vitro* translated D3 receptor protein. An *in vitro* transcribed full-length human D3 receptor-encoded mRNA was incubated with a rabbit reticulocyte lysate translation mixture. A fraction of the translation mixture was electrophoretically separated on a 10% SDS-PAGE gel and transferred onto Immobilon PVDF membrane. The blot was probed with the monoclonal antibody (IgM) and the polyclonal antipeptide antibody. Both antibodies recognize an

approximately 50 kD translation product. The lane marked (-) contains a translation mixture that was incubated in the absence of RNA. (C) Immunoblot of proteins extracted from monkey temporal neocortex. Two major immunoreactive proteins of approximately 58 and 45 kD are recognized by the monoclonal IgM antibody (lanes marked 1 and 2). Approximately 10 μ g of protein was loaded onto the lanes. The lane marked M contains molecular size standards. Bound antigens were visualized by enhanced chemiluminescence using the ECL detection kit (Amersham).

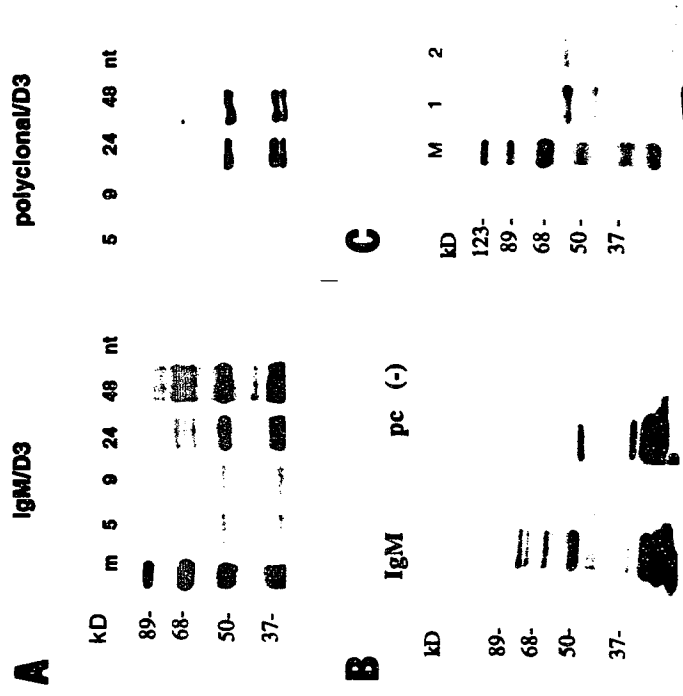


Fig. A1

APPENDIX 2

Manuscripts included in this Dissertation

Chapter 2: Hof, P.R., and E.A. Nimchinsky (1992) Regional distribution of neurofilament and calcium-binding proteins in the cingulate cortex of the macaque monkey. *Cereb. Cortex* 2:456-467.

Chapter 3: Nimchinsky, E.A., B.A. Vogt, J.H. Morrison, and P.R. Hof (in preparation) Chemoarchitecture of the human cingulate cortex. *J. Comp. Neurol.*

Chapter 4: Nimchinsky, E.A., B.A. Vogt, J.H. Morrison, and P.R. Hof (1995b) Spindle neurons of the human anterior cingulate cortex. *J. Comp. Neurol.* 355:27-37.

Chapter 5: Nimchinsky E.A., P.R. Hof, W.G. Young, and J.H. Morrison (submitted). Neurochemical, morphologic and laminar characterization of cortical projection neurons in the cingulate motor areas of the macaque monkey. (*J. Comp. Neurol.*)

Chapter 6: Nimchinsky E.A., P.R. Hof, T. Moran, A.I. Levey, W.G.M. Janssen, C. Schmauss, and J.H. Morrison (in preparation) Regional and cellular distribution of the dopamine D3 receptor in the primate and rodent brain: an immunocytochemical analysis. *J. Neurosci.*

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