

# Differential Cholinergic Innervation Within Functional Subdivisions of the Human Cerebral Cortex: A Choline Acetyltransferase Study

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## ABSTRACT

The distribution of cholinergic fibers in the human brain was investigated with choline acetyltransferase immunocytochemistry in 35 cytoarchitectonic subdivisions of the cerebral cortex. All cortical areas and all cell layers contained cholinergic axons. These fibers displayed numerous varicosities and, on occasion, complex preterminal profiles arranged in the form of dense clusters. The density of cholinergic axons tended to be higher in the more superficial layers of the cerebral cortex. Several distinct patterns of lamination were identified. There were also major differences in the overall density of cholinergic axons from one cytoarchitectonic area to another. The cholinergic innervation of primary sensory, unimodal, and heteromodal association areas was lighter than that of paralimbic and limbic areas. Within unimodal association areas, the density of cholinergic axons and varicosities was significantly lower in the upstream (parasensory) sectors than in the downstream sectors. Within paralimbic regions, the non-isocortical sectors had a higher density of cholinergic innervation than the isocortical sectors. The highest density of cholinergic axons was encountered in core limbic structures such as the hippocampus and amygdala. These observations show that the cholinergic innervation of the human cerebral cortex displays regional variations that closely follow the organization of information processing systems.

**Key words:** cholinergic fibers, human brain, cerebral cortex

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The cerebral cortex receives widespread cholinergic innervation (see Mesulam, '88 for review). The organization of these afferents varies from one cytoarchitectonic region to another (Lehmann et al., '84; Mesulam et al., '86; DeLima and Singer, '86; Lysakowski et al., '89; Lewis, '91). In the monkey, for example, limbic and paralimbic regions of cortex receive a substantially higher density of cholinergic input than sensory association cortex (Mesulam et al., '86). Observations based on acetylcholinesterase histochemistry suggested that similar patterns were likely to exist in the human cerebral cortex (Green and Mesulam, '88; Mesulam and Geula, '88; Geula and Mesulam, '90). The purpose of this report is to pursue this question further with the help of choline acetyltransferase immunohistochemistry, which allows a more specific visualization of cholinergic axons.

## METHODS

The right cerebral hemispheres were obtained from three men who died at 20 (one case) and 55 (two cases) years of

age. Mode of death was sudden (gunshot in one and heart attacks in the other two). Extensive clinical information indicated that there was no prior neurological or dementing disease. The specimens were fixed, cryoprotected, and sectioned coronally at a thickness of 40  $\mu$  as previously described (Mesulam et al., '91). Two of the specimens were cut in whole brain sections.

One set of matching sections was stained with cresyl violet for cytoarchitectonic identification and another with choline acetyltransferase (ChAT) immunocytochemistry by using a well-characterized polyclonal antibody raised against human placental ChAT (German et al., '85). An additional set processed with the immunocytochemical method was counterstained with neutral red for the correlation, within

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Accepted December 16, 1991.

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the same section, of cholinergic fibers with cortical laminae and cytoarchitectonic divisions.

The immunocytochemical procedure, based on double-bridging and avidin-biotin complex formation, followed the methodology that we have described previously (Mesulam et al., '91). Additional intensification was obtained with a method described by Kitt et al. ('88). Two types of control procedures were used. In one set of control sections, an irrelevant IgG was substituted for the ChAT antibody. In additional adsorption controls, the antibody was first incubated in the presence of pure ChAT before being used for immunocytochemistry. Both control sections also underwent the intensification method.

Major cytoarchitectonic subdivisions of cortex were identified on sections stained with cresyl violet. The guidelines for cytoarchitectonic identification were based on Braak ('80), Krieg ('73), and Conel ('67).

All sections were subjected to a general survey and representative fields from 35 cytoarchitectonic areas were photographed at 40 $\times$  magnification under dark-field illumination and at 100 $\times$  magnification under bright-field illumination in order to provide full-depth cortical montages of ChAT-positive cortical fibers. This material helped to establish laminar distributions and relative densities. Since the superficial layers of cortex contained the highest density of cholinergic input, a third set of photomontages was obtained in all of these areas at the higher magnification of 200 $\times$  but spanning only the superficial half of the cerebral cortex. This material provided the basis for establishing a more accurate assessment of comparative densities of cholinergic axons. In the case of unimodal association areas, these photomicrographs were printed to obtain a final magnification of 450 $\times$ . A clear acetate sheet scored at 2.5 mm intervals was placed on these enlargements and the number of ChAT-positive axons and varicosities was counted within ten 5  $\times$  200 mm strips (long axis oriented parallel to the cortical surface) obtained at 50 mm intervals throughout the depth of cortical layer 3. Means of the ten counts were computed for each relevant area and were subjected to a paired t-test analysis.

Laminar distribution and cytoarchitectonic localization were further verified by checking conclusions based on ChAT immunostained sections with sections where ChAT immunostaining was accompanied by counterstaining with neutral red. In selected cytoarchitectonic areas, more than one photomontage was obtained in order to establish the uniformity of laminar and density patterns within individual cytoarchitectonic areas.

## RESULTS

All parts of the human cerebral cortex contained ChAT immunoreactive (cholinergic) axons. These axons displayed multiple varicosities, which probably represent presynaptic specializations. A small minority of the cholinergic axons also displayed extremely complex dense clusters of preterminal profiles (Fig. 1). Observations on counterstained sections showed that cholinergic axons seemed to come in contact with the cell body and dendrites of cortical neurons and also with intracortical vessels. Control sections obtained by substituting ChAT with an irrelevant IgG and also immunocytochemical preparations based on a ChAT-adsorbed antibody did not reveal immunopositive axonal profiles in the cerebral cortex. All three specimens displayed similar patterns of laminar and regional distribution.

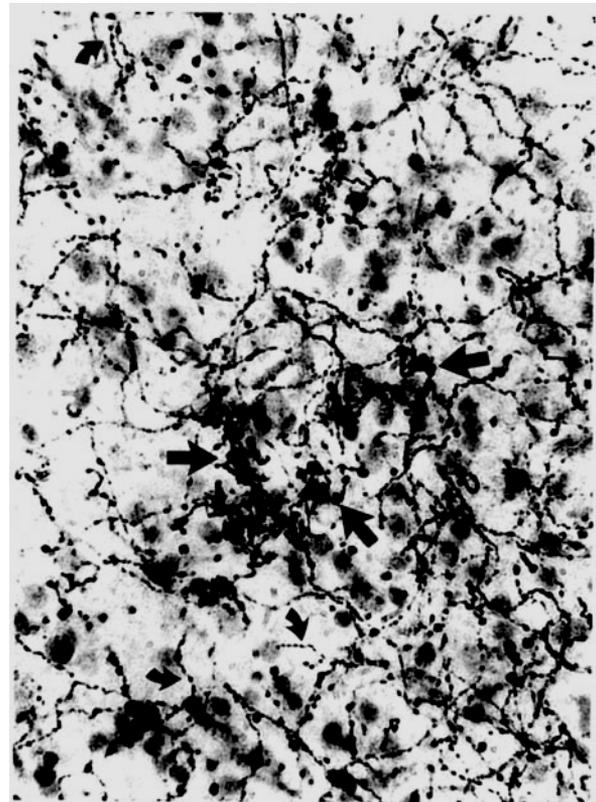


Fig. 1. Choline acetyltransferase-positive (cholinergic) fibers in the human temporopolar cortex. The axons display multiple varicosities (curved arrows). A small minority of these axons gave rise to extremely complex glomerular dense clusters (outlined by straight arrows).  $\times 500$ .

The laminar arrangement of cholinergic axons in the various cytoarchitectonic areas displayed several general patterns. All cortical areas contained a combination of cholinergic axons that were vertical, horizontal and oblique to the cortical surface. Horizontal fibers tended to predominate in layer 1 and occasionally in layer 2. Vertically oriented fibers predominated in the deeper layers, except for entorhinal cortex which had a thick band of horizontally oriented fibers in layer 4. The superficial layers (deeper part of layer 1, layer 2, and the most superficial parts of layer 3) contained either the highest density of cholinergic innervation or as high a density as in any other layer of the same cortical area. In general, cholinergic axons in deeper layers and in the subcortical white matter tended to be thicker than those in more superficial layers. These observations suggest that cholinergic fibers enter the cerebral cortex from the subcortical white matter and that they undergo some intracortical branching in their course towards superficial layers.

In homotypical association cortex (areas 18, 19, 20, 21, 22, 5, 7, 39, 40, and 10), segments of layer 4 displayed a relative decrease in the density of cholinergic axons in comparison to adjacent layers. In primary sensory areas, however, segments of layer 4 occasionally displayed a slightly higher density of these axons than in the immediately adjacent layers.

Primary visual cortex (V1, area 17) and entorhinal cortex had two of the most distinctive laminar distributions (Figs. 2, 3). In area V1, the cholinergic fibers were particularly

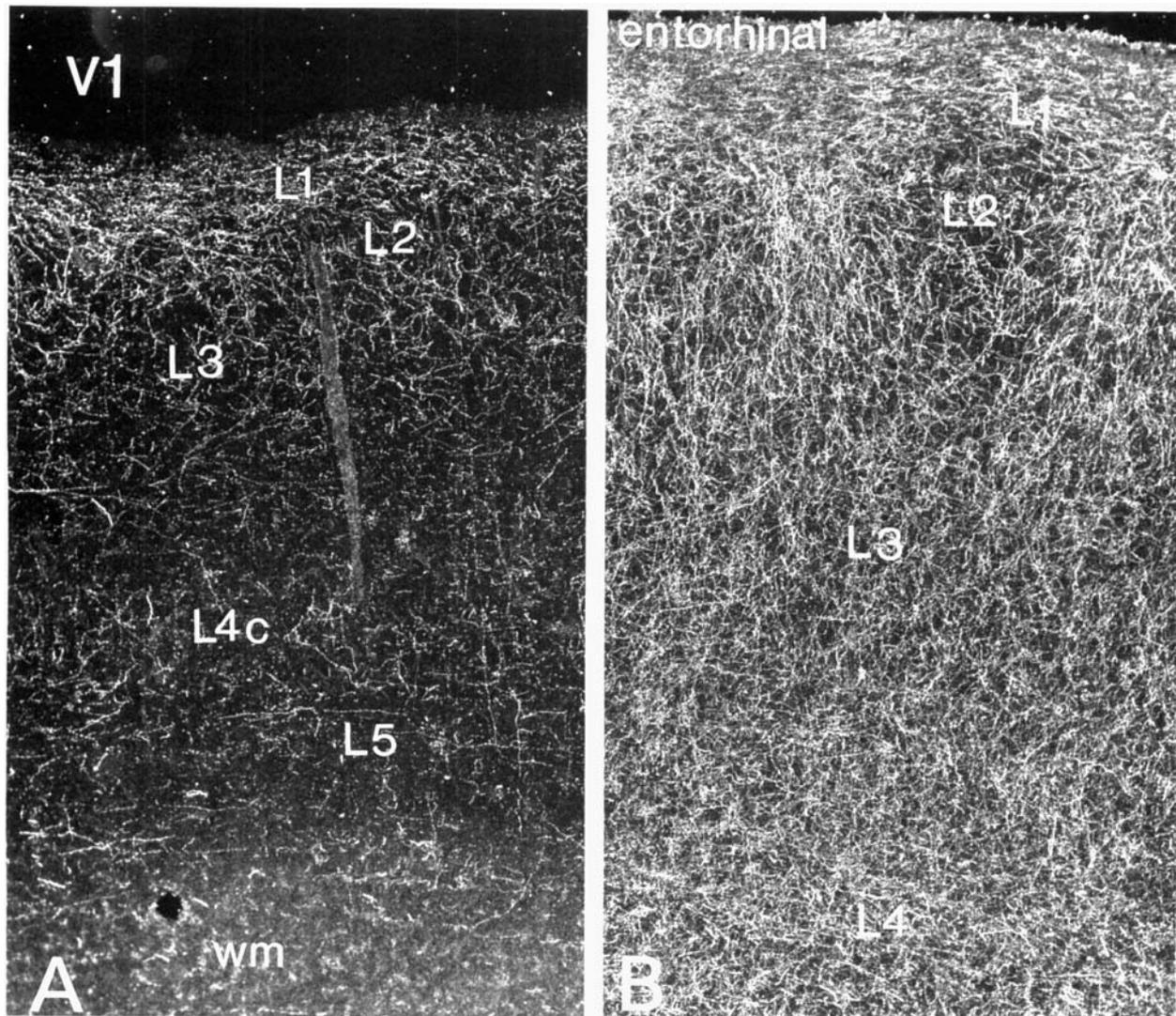


Fig. 2. Darkfield photomicrograph showing the differential density and lamination of cholinergic axons in primary visual cortex (V1) (A) and in entorhinal cortex (B). Note the very substantial difference in overall density. wm, white matter.

intense in layers 1, 2, and the immediately adjacent rim of layer 3. There was a drop in density within the rest of layer 3 and in layers 4a and 4b. Segments of layer 4c tended to display a relatively higher density of cholinergic fibers than layers 4b and 5.

Entorhinal cortex revealed one of the most complex patterns of cholinergic innervation (Figs. 2B, 3B). Layer 1 contained dense fascicles of cholinergic fibers that ran parallel to the surface of the brain. The islands of stellate cells in layer 2 were heavily invested with cholinergic fibers which enveloped the stellate neurons in a matrix of cholinergic axons. Layer 3 contained a dense mesh of vertical, horizontal, and oblique fibers. Some of the vertical axons in layer 3 formed bundles directed towards the layer 2 islands. Layer 4 (lamina dissecans) contained prominent horizontally directed cholinergic fibers whereas layers 5 and 6 contained a mixture of vertical, horizontal, and diagonal fibers.

### Regional variations in the density of cholinergic innervation

The primate cerebral cortex can be divided into five major functional groups: 1) primary sensory and motor areas; 2) modality-specific (unimodal) association areas; 3) heteromodal association areas, principally those in prefrontal and posterior parietal regions; 4) paralimbic formations of the parahippocampal, cingulate, orbitofrontal, temporopolar, and insular areas; and 5) the limbic formations of the amygdaloid complex, hippocampus, and piriform cortex (see Mesulam, '85 for review). Unimodal areas can further be divided into upstream (parasensory) components that are only one synapse away from the corresponding primary sensory area and downstream areas that are synaptically more distant (Pandya and Yeterian, '85). On the basis of neuronal architecture, paralimbic areas can be divided into isocortical (granular) and non-isocortical (dysgranular and

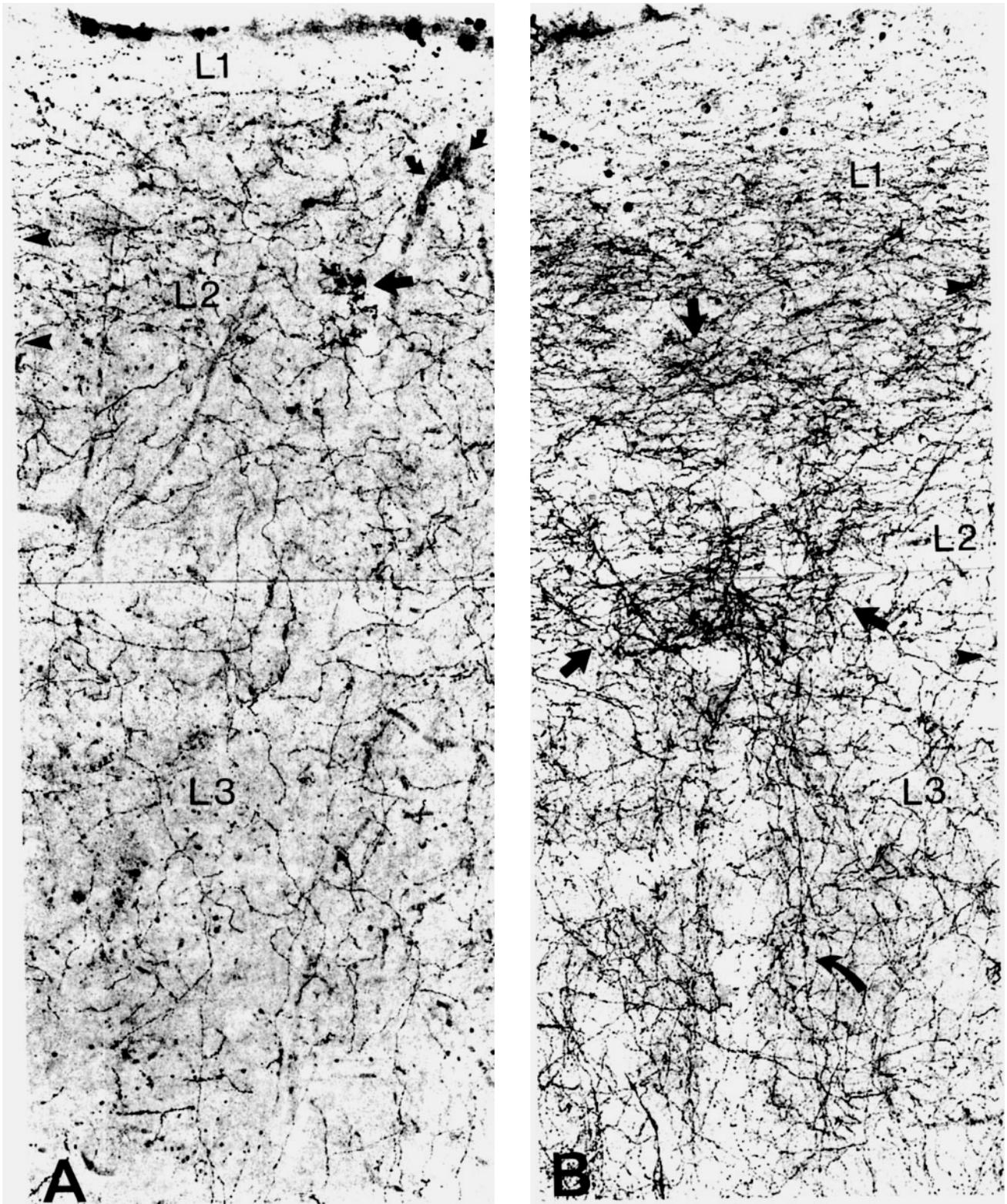


Fig. 3. Brightfield photomicrograph of axonal detail in area 18 (A) and entorhinal cortex (B). Arrowheads point to laminar boundaries. The curved arrows in A point to a cortical capillary that appears to be making contact with multiple varicosities belonging to cholinergic axons. The straight arrow points to one of the complex dense clusters formed by an incoming cholinergic axon within layer 2 of area 18. The

arrows in B outline the boundaries of a layer 2 island of stellate neurons in entorhinal cortex. This island is heavily invested with cholinergic axons. The curved arrow in B points to a vertically directed cholinergic axon coursing through layer 3 towards the island of stellate neurons.  $\times 266$ .

agranular) sectors. The regional density of cholinergic fibers was analyzed with respect to these functional and cytoarchitectonic subdivisions of cortex (Figs. 4–9).

**Unimodal association areas.** In the visual modality, the upstream peristriate association cortex (areas 18 and 19) displayed a density of cholinergic innervation substantially lower than that in the synaptically more downstream visual association cortex (areas 20 and 21) of the middle and inferotemporal areas (Fig. 4A,B). A similar but more subtle gradient was observed in the other two sensory modalities. In the auditory modality, the upstream association cortex of the posterior superior temporal gyrus (area 22) contained a lower density of cholinergic innervation than the synaptically more downstream auditory association cortex in the anterior portions of the superior temporal gyrus (Fig. 4C,D). In the somatosensory modality, the upstream association area of the superior parietal lobule (area 5) contained a lower density of cholinergic innervation than the postero-dorsal granular insula (I<sub>g</sub>), which can be considered a downstream somatosensory association region (Fig. 5A,B). A quantitative evaluation of ChAT-positive axons and varicosities, done in a different set of high-power brightfield photomontages, showed these differences to be statistically significant (Table 1).

**Heteromodal association cortex.** Prefrontal granular cortex (areas 9 and 10) and the inferior parietal lobule (areas 39 and 40) displayed very similar patterns of cholinergic innervation (Fig. 5C,D). The overall density of this innervation was comparable to that in unimodal areas but was substantially lower than the density in the immediately adjacent paralimbic parts of the brain.

**Paralimbic areas.** The entorhinal region contained a very high density of cholinergic innervation (Figs. 2B, 3B). Equally high densities were observed in the cingulate gyrus and the non-isocortical (dysgranular) components of orbitofrontal (OF<sub>dg</sub>), temporopolar (TP<sub>dg</sub>), and insular (Id<sub>g</sub>) cortex (Fig. 6). Within the same paralimbic areas, the more differentiated granular components had a lower density of cholinergic innervation than the immediately adjacent dysgranular components (Fig. 7). This gradient was most pronounced in orbitofrontal cortex and of least magnitude within the insula.

**Limbic areas.** The cholinergic innervation in limbic structures such as the amygdala and hippocampus was higher than in any other parts of the cerebral cortex (Fig. 8A,B). In the amygdala, the basolateral complex contained the highest intensity of cholinergic innervation and displayed an uninterrupted and extremely dense sheet of cholinergic terminals. Other amygdaloid nuclei also contained high densities of cholinergic innervation (Fig. 8B). In the hippocampus, the CA4, CA3, and CA2 subsectors and the molecular layer of the dentate gyrus contained the densest distribution of cholinergic terminal profiles. The CA1 sector contained a slightly less intense collection of cholinergic fibers and the subiculum a still lesser density. Even in the subiculum and CA1 sectors, however, the density of cholinergic innervation was very high. Our observations in the hippocampus agree with those of Ransmayr et al. ('89) but not with those of Lim et al. ('91).

**Primary sensory areas.** The density of cholinergic innervation was lowest in primary visual cortex (area 17) but of moderate density in the primary auditory (area 44) and somatosensory (areas 3b–l) areas (Figs. 2A, 8C, 8D).

**Motor areas.** Motor (area 4) and premotor (area 6) cortex contained cholinergic innervation of moderate density (Fig. 9).

In overall ranking, the density of cholinergic axons was lowest in area 17 (V1), moderate within modality-specific and heteromodal association areas, high in paralimbic areas (especially in their non-isocortical sectors), and very high in core limbic structures such as the amygdala and hippocampus.

## DISCUSSION

The connections of the cerebral cortex can be divided into three broad groups: 1) *local circuit* projections, 2) *discrete* corticocortical and corticothalamic projections, and 3) *diffuse* (widely distributed) regulatory projections. The regulatory pathways of the cerebral cortex arise from the intralaminar thalamic nuclei, the hypothalamus, the basal forebrain, and the brainstem. Except for the projections arising from the intralaminar thalamus, these regulatory pathways employ small amines such as serotonin, dopamine, norepinephrine, histamine, and acetylcholine as their neurotransmitters. A diverse set of observations suggest that the regulatory pathways of the cerebral cortex can modulate the overall state of information processing in a way that can alter the tone and flavor of experience and its impact on the individual (Mesulam, 1990). In keeping with this formulation, the regulatory pathways and their transmitters have been implicated in the organization of global behavioral states such as mood, motivation and arousal.

Cholinergic afferents probably constitute the single most substantial regulatory pathway of the cerebral cortex. The vast majority of these afferents arises from the nucleus basalis of Meynert, a nucleus which is a telencephalic extension of the brainstem reticular formation and also a major component of the limbic forebrain (Ramon-Moliner and Nauta, '66; Mesulam, '85). This duality in the nature of the nucleus basalis is reflected in the two major behavioral affiliations of its corticopetal projections: the modulation of cortical arousal and the maintenance of effective memory processes. Experiments in rats, for example, have shown that the cortical cholinergic projections of the nucleus basalis play a major role in sustaining the arousal-related low voltage fast activity in the cortical EEG (Stewart et al., '84; Buzsaki et al., '88). In monkeys, lesions of this nucleus can cause severe impairments of memory that can be reversed by the systemic administration of cholinergic agonists (Ridley et al., '86; Irle and Markowitsch, '87).

Physiological and anatomical experiments are beginning to shed some light into the cellular mechanisms that may underlie these two behavioral affiliations of cortical cholinergic afferents. The major effect of acetylcholine is to cause a relatively prolonged reduction of potassium conductance so as to make cortical cholinceptive neurons more susceptible to other excitatory inputs (McCormick, '90; Steriade et al., '90). In primary visual cortex, for example, cholinergic stimulation does not alter the orientation specificity of a given neuron but increases the likelihood that the neuron will fire in response to its preferred stimulus (Sato et al., '87). An analogous effect has been described in somatosensory cortex (Metherate et al., '88). Single unit studies in monkeys have also shown that the nucleus basalis neurons that give rise to the corticopetal cortical cholinergic projections are particularly sensitive to stimulus novelty and to the motivational relevance of sensory cues (Wilson and Rolls, '90a,b). These observations suggest that cholinergic afferents are in a position to alter the neural impact of sensory experience (and the resultant level of cortical

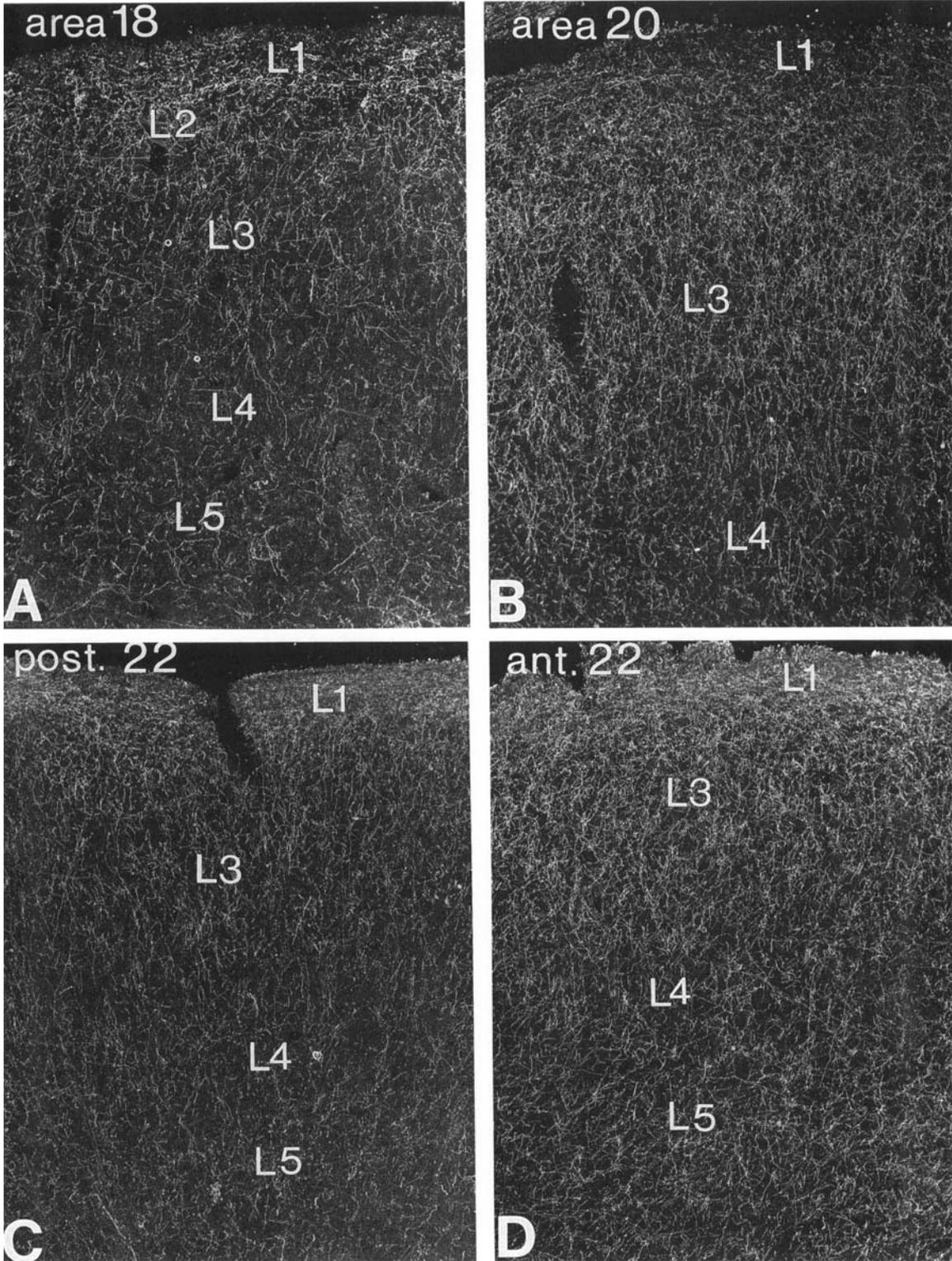


Fig. 4. Darkfield photomicrographs of cholinergic axons in area 18 (A), area 20 (B), posterior parts of area 22 (C), and anterior parts of area 22 (D).  $\times 45$ .

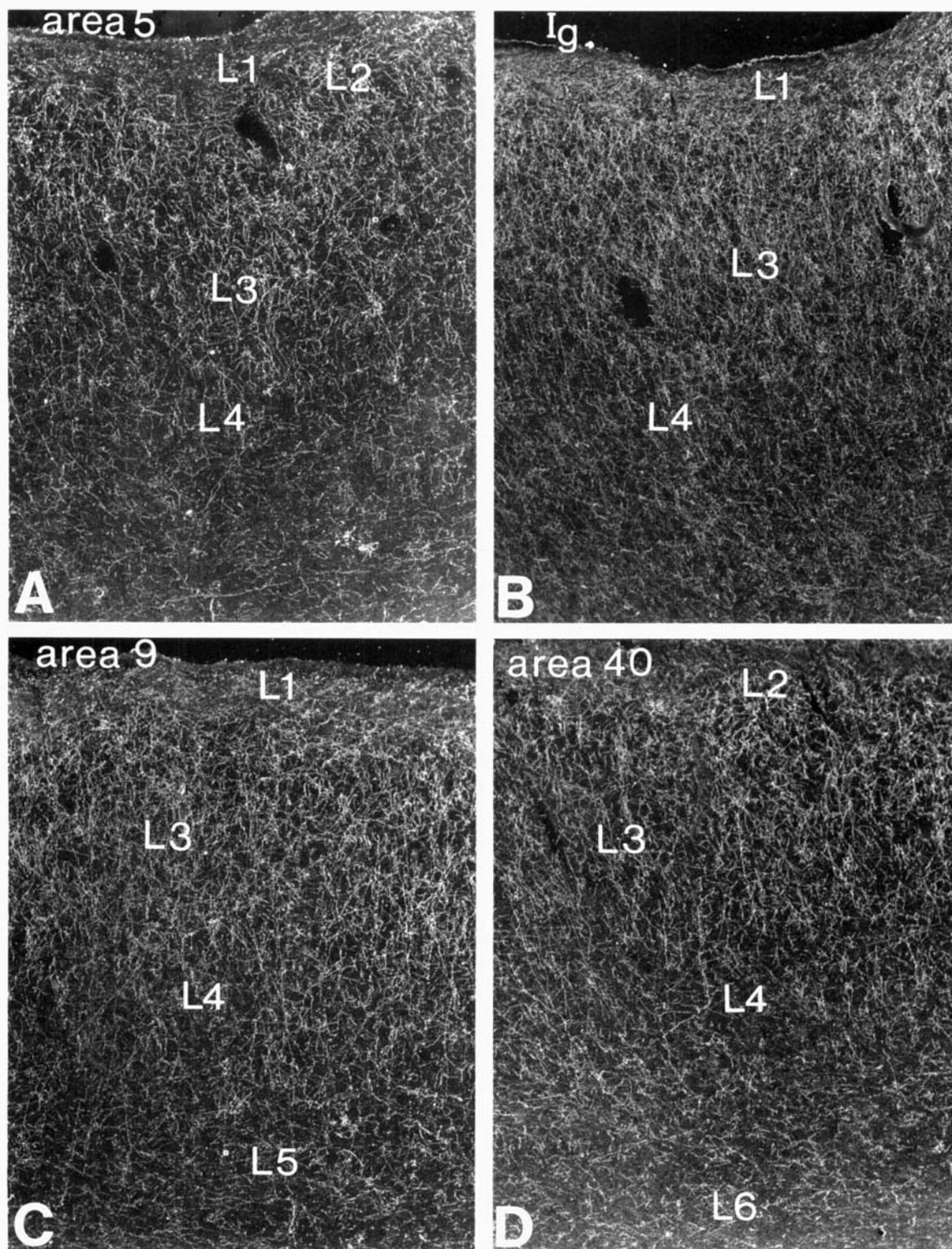


Fig. 5. Darkfield photomicrographs of cholinergic axons in area 5 (A), the granular sector of the insula (Ig) (B), prefrontal area 9 (C), and posterior parietal area 40 (D).  $\times 45$ .

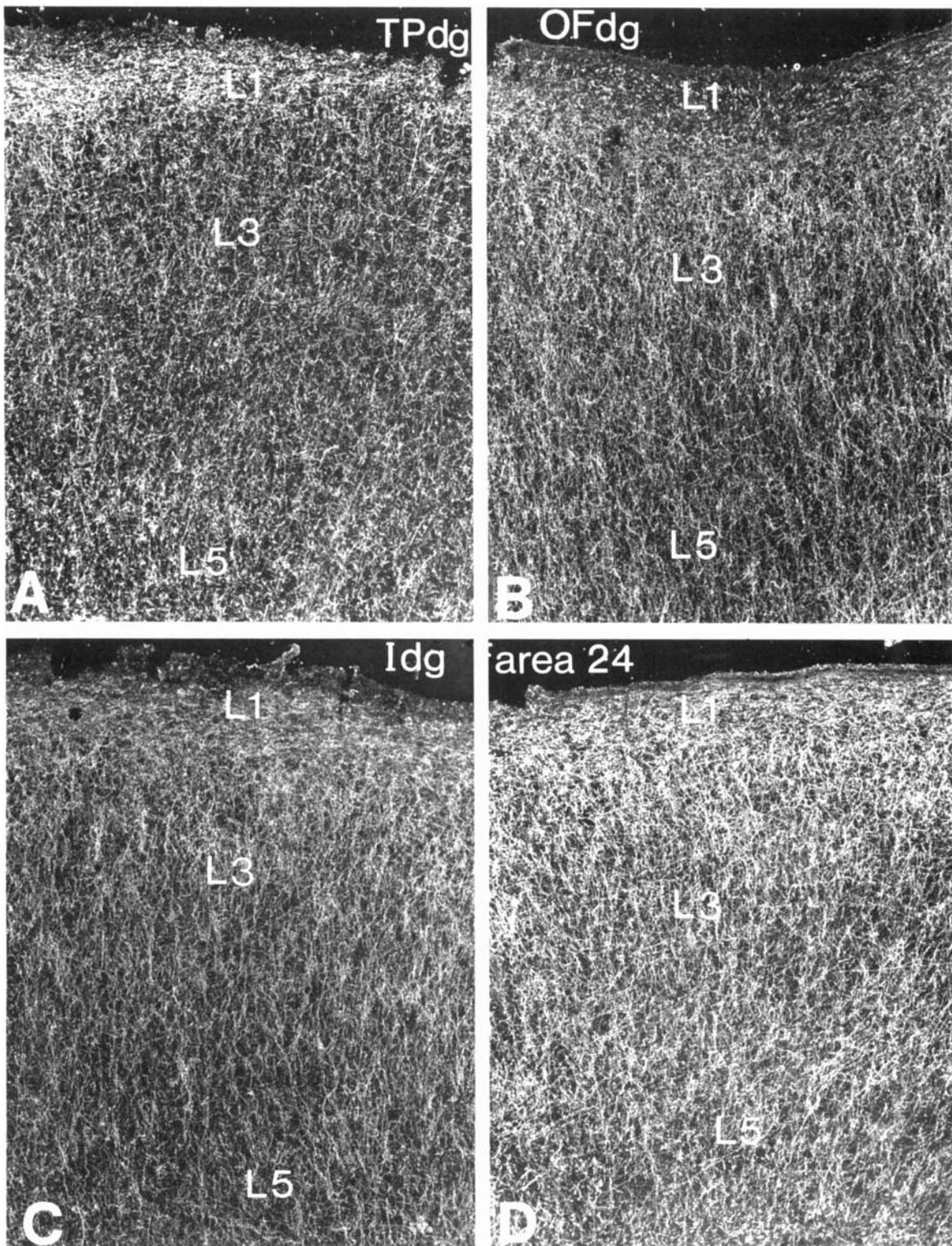


Fig. 6. Darkfield photomicrographs of cholinergic axons in non-isocortical sectors of paralimbic areas. The density of cholinergic axons is high in the dysgranular sector of the temporal pole, TPdg (A), the

dysgranular sector of orbitofrontal cortex, OFdg (B), the dysgranular sector of the insula, Idg (C), and in the ventral parts of area 24 in the cingulate gyrus (D).  $\times 45$ .

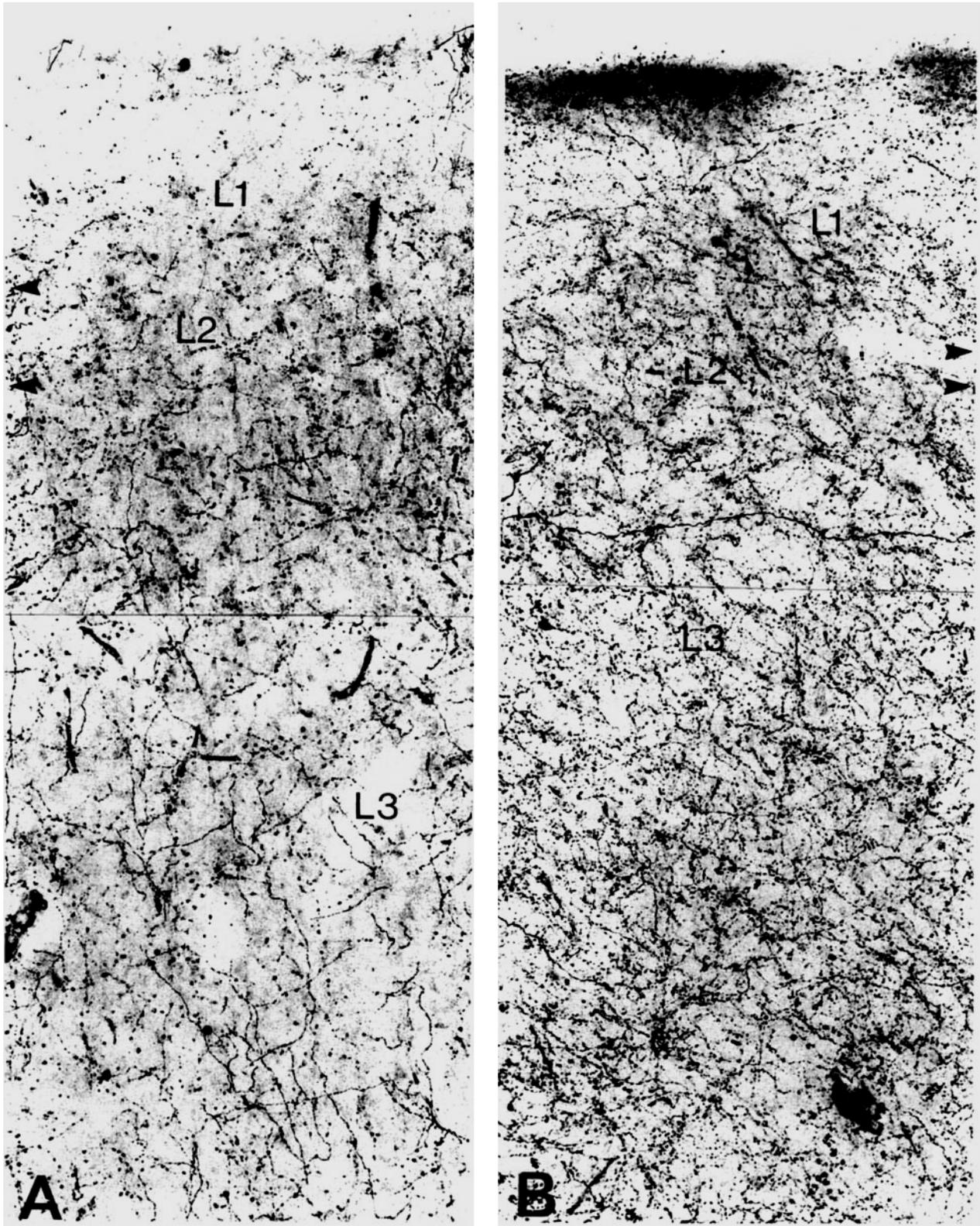


Fig. 7. Brightfield photomicrographs comparing the density of cholinergic axons in **A**, the granular (OFg), and **B**, dysgranular (OFdg) sectors of orbitofrontal cortex. The density is substantially higher in the dysgranular sector, which is synaptically closer to core limbic structures. Arrowheads point to laminar boundaries.  $\times 266$ .

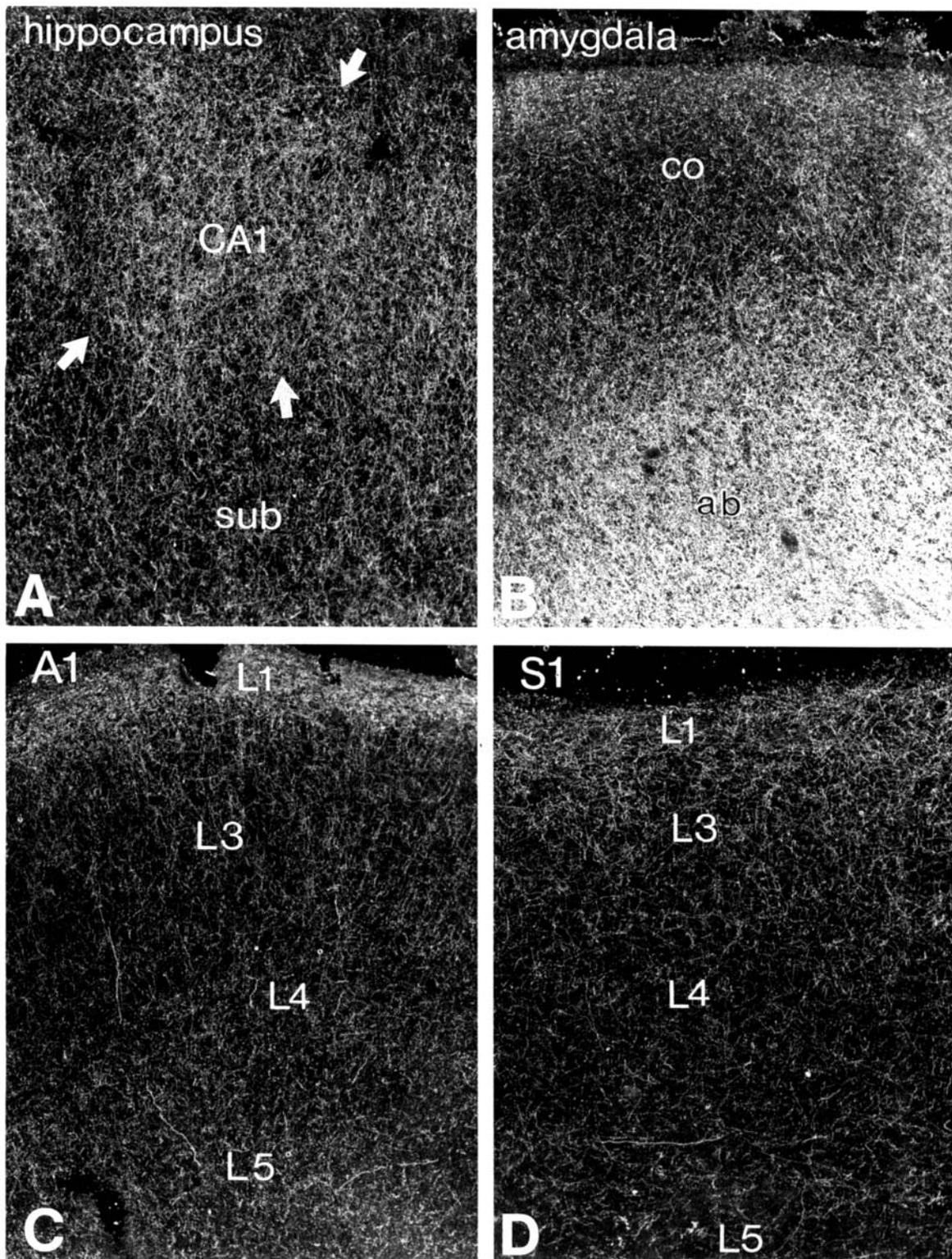


Fig. 8. Darkfield photomicrographs of cholinergic axons and terminals at the junction of the CA1 sector and subiculum (sub) within the hippocampal complex (A), in the cortical (co) and accessory basal (ab) nuclei of the amygdala (B), primary auditory cortex A1 (C), and primary somatosensory cortex S1 (D). In A, the white arrows point to the boundaries between CA1 and the subiculum. The density of cholinergic

terminals is higher in the CA1 sector than in the subiculum. The cortical and accessory basal nuclei of the amygdala shown in B display a very high density of cholinergic input. The basal lateral nucleus of the amygdala, not shown in this photomicrograph, contains an even higher density of cholinergic input.  $\times 45$ .

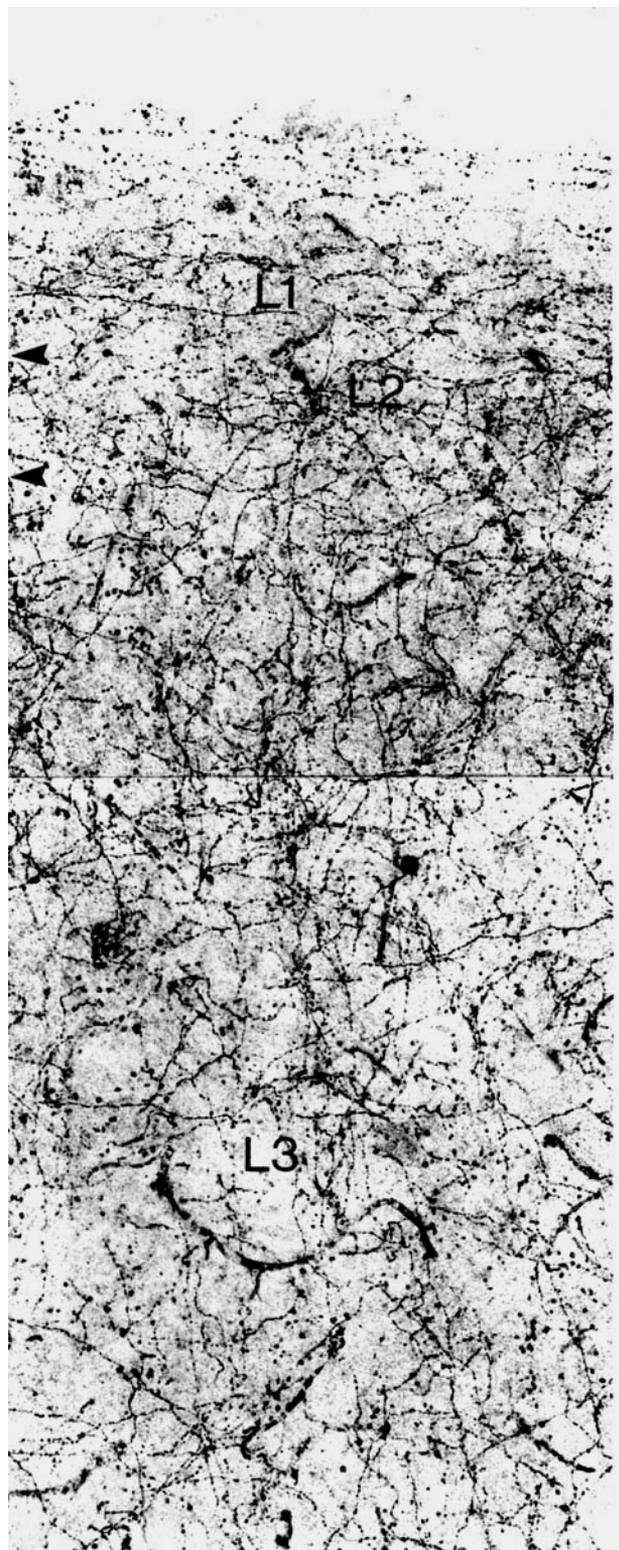


Fig. 9. Premotor cortex (area 6) displays a moderate density of cholinergic innervation. Arrowheads point to laminar boundaries.  $\times 266$ .

arousal) according to its behavioral significance. In keeping with this interpretation, the muscarinic blocking agent scopolamine attenuates the cortical P-300 arousal re-

TABLE 1. Quantitative Evaluation of ChAT-Positive Axons and Varicosities

	Upstream sensory association areas	Downstream sensory association areas
Visual	29.0 $\pm$ 11.1	93.2 $\pm$ 20.8
Auditory	59.8 $\pm$ 10.9	111.2 $\pm$ 27.2
Somatosensory	52.0 $\pm$ 11.2	84.1 $\pm$ 14.7

In photomontages printed at  $450\times$  magnification, horizontal strips 5 mm deep and 200 mm long (long axis parallel to the pia) were outlined at 50 mm vertical intervals throughout layer 3. Counts were obtained in ten strips per area. The numbers represent mean counts and standard deviations of ChAT-positive varicosities and axons per strip. In each modality, paired t tests showed the differences between upstream and downstream unimodal association areas to be highly significant with P less than .0001. The following areas were included in the counting: Upstream Visual = area 18, Downstream visual = area 20, Upstream auditory = posterior area 22, Downstream auditory = anterior area 22, Upstream somatosensory = area 5, Downstream somatosensory = granular insula.

sponse that is normally elicited by novel or surprising stimuli (Hammond et al., '87).

Studies on patients with Alzheimer's disease had provided indirect evidence that the cholinergic innervation of the cerebral cortex in the human also arises principally from the nucleus basalis (Mesulam and Geula, '88). The observations in this report, based on choline acetyltransferase immunocytochemistry, showed that all cytoarchitectonic fields of the human cerebral cortex, including primary sensory-motor, association and limbic areas receive cholinergic innervation. Although cholinergic innervation was ubiquitous in all cortical areas that we examined, major variations of density were also detected. These variations obeyed the cytoarchitectonic and functional subdivisions of the cerebral cortex as well as the organization of multisynaptic information processing streams that convey sensory information into the limbic system.

Many of the corticocortical pathways that link one association area of the primate cerebral cortex to another are organized in parallel. Embedded within this parallel circuitry, however, it is also possible to discern a serial hierarchy, especially in the arrangement of sensory-limbic pathways (Van Hoesen, '72; Mesulam et al., '86). The process starts within the primary sensory areas of the cerebral cortex which provide a portal for the entry of sensory information into cortical circuitry. These primary areas project primarily to upstream (parasensory) unimodal association areas, which, in turn, project to downstream unimodal areas and heteromodal cortex. The heteromodal and downstream unimodal areas collectively provide the major source of sensory information into paralimbic and limbic areas of the brain (Mesulam, '85; Pandya and Yeterian, '85). Experimental evidence leads to the conclusion that these sensory-limbic pathways play pivotal roles in a wide range of behaviors related to emotion, motivation, and memory (Mishkin, '82; Mesulam, '85).

Our observations show that the density of cholinergic innervation was lower within unimodal and heteromodal association areas than in the paralimbic areas of the brain. In the unimodal areas, the downstream sectors had a higher density of cholinergic innervation than the upstream sectors. Within all major paralimbic areas, the non-isocortical subsectors, known to have the more extensive interconnections with limbic structures, also had a higher density of cholinergic innervation. Core limbic areas such as the amygdala and hippocampus had the highest densities of cholinergic innervation. Regional variations in the specific activity of choline acetyltransferase enzyme activity had revealed a nearly identical pattern of distribution in the cerebral cortex of the monkey brain (Mesulam et al., '86).

This pattern of differential distribution had led us to propose that sensory information is likely to come under progressively greater cholinergic influence as it is conveyed along the multisynaptic pathways leading to the limbic system (Mesulam et al., '86). As a consequence of this arrangement, cortical cholinergic innervation may help to channel (or gate) sensory information into and out of the limbic system in a way that is sensitive to the behavioral relevance of the associated experience. The memory disturbance that arises after damage to the nucleus basalis or after the systemic administration of cholinergic antagonists may therefore reflect a disruption of sensory-limbic interactions, which are crucial for effective memory and learning.

The distinctly higher concentration of cholinergic innervation in all limbic and paralimbic regions also suggests that cholinergic neurotransmission is likely to have a particularly strong influence upon the behavioral affiliations of the limbic system. The effect of cholinergic drugs on memory is consistent with this interpretation. Additional observations indicate that cholinergic neurotransmission may also play a particularly important role in the modulation of mood, reward, and aggressive behaviors (Yoshimura and Ueki, '77; Yeomans et al., '85; Gillin et al., '91). The role of acetylcholine in hippocampal long-term potentiation (Tanaka et al., '89) may provide one of the several cellular mechanisms that underly the relationship of cholinergic pathways to memory. There is also some evidence that cholinergic innervation may participate in diverse phenomena related to cortical plasticity (Bear and Singer, '86).

In all cytoarchitectonic areas of the human brain, the superficial layers of cortex contained the highest densities of cholinergic innervation. This arrangement is consistent with muscarinic receptor autoradiography in the rhesus monkey which showed that the M1 pharmacophore, the single most numerous cholinergic receptor subtype in the primate cerebral cortex, displayed its highest concentration within the superficial layers of cortex (Mash et al., '88).

Electron microscopic observations in rodents show that the cholinergic axons of the cerebral cortex are unmyelinated and that they make symmetrical and asymmetrical synaptic contacts through axonal varicosities (Wainer et al., '84; Frotscher and Leranth, '85). The possibility has also been raised that a substantial number of these varicosities may not form conventional junctional synapses and that the acetylcholine they release may act by more extensive diffusion in the extracellular space (Umbriaco et al., '90).

In addition to numerous varicosities, we also observed that a small minority of cholinergic fibers displayed conspicuous dense clusters sporadically distributed throughout the cerebral cortex. The physiological correlates of these complex formations remain unknown but could conceivably reflect events of local plasticity and rearrangement. In the rat brain, cortical cholinergic innervation influences blood flow (Arneric et al., '87) and muscarinic receptors have been detected in arteries of the human cerebral cortex (Tsukahara et al., '85). Our observations provide indirect evidence that arterioles and capillaries in the human cerebral cortex may be innervated by cholinergic axons. If ultrastructural and physiological studies were to support this possibility, then an additional effect of cholinergic innervation upon cortical activity could be mediated through the modulation of regional blood flow.

The vast number of cortical cholinergic axons, especially in paralimbic and limbic areas was truly impressive. The purpose served by such an exuberant influx of cholinergic

axons is incompletely understood. Its size alone indicates that this pathway is likely to constitute the single most massive regulatory afferent system of the cerebral cortex. At least one major set of functions that can be attributed to this pathway is the state-dependent modulation of neural responsiveness and perhaps also the fine-tuning of sensory-limbic interactions. Conceivably, future research may also link the cholinergic innervation of the human brain to a host of additional phenomena ranging from the control of blood flow to the regulation of neural plasticity.

## ACKNOWLEDGMENTS

We thank L. Christie, K. Bouve, and C. Calabrese for expert secretarial and technical support. We thank Dr. C. Kitt for providing us with a detailed protocol of the intensification procedure that we employed. This work was supported in part by a Javits Neuroscience Investigator award (NS20285), an Alzheimer's Disease Research Center grant (AG05134), and the Alzheimer's Disease and Related Disorders Association, NS25785, AG05893, and AG08013.

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