

Cholinergic Innervation of the Frontal Cortex: Differences among Humans, Chimpanzees, and Macaque Monkeys

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ABSTRACT

Cholinergic innervation of the frontal cortex is important in higher cognitive functions and may have been altered in humans relative to other species to support human-specific intellectual capacities. To evaluate this hypothesis we conducted quantitative comparative analyses of choline acetyltransferase-immunoreactive axons in cortical areas 9, 32, and 4 among humans, chimpanzees, and macaque monkeys. Area 9 of the dorsolateral prefrontal cortex is involved in inductive reasoning and specific components of working memory processes, while area 32 of the medial prefrontal cortex has been implicated in theory of mind. Area 4 (primary motor cortex) was also evaluated because it is not directly associated with higher cognitive functions. The findings revealed no quantitative species differences in the three cortical areas examined, indicating that human cognitive specializations are not related to a quantitative increase in cortical cholinergic input. However, species-specific morphological specializations were observed. Clusters of cholinergic fibers that may be indicative of cortical plasticity events were present in chimpanzees and humans, but not in macaques. The other significant morphology noted was the common and distinctive oval or ovoid perisomatic staining in macaque cortices. This feature was also sporadically observed in chimpanzee cortex. Our findings suggest a potential alteration of cortical cholinergic afferents within the prefrontal cortex of humans and chimpanzees, to the exclusion of macaque monkeys. *J. Comp. Neurol.* 506:409–424, 2008. © 2007 Wiley-Liss, Inc.

Indexing terms: area 9; area 32; area 4; human evolution; choline acetyltransferase; prefrontal cortex

In contrast to neurotransmitters that act on an immediate and short-term time scale, neuromodulators have effects that are slower, of longer duration, and are more spatially diffuse (Hasselmo, 1995). While many chemicals act as both neurotransmitters and neuromodulators, neuromodulatory actions are defined by their long-term effects on the processing characteristics of cortical networks by influencing synaptic transmission and pyramidal cell adaptation (Hasselmo, 1995; Dreher and Burnod, 2002). The role of neuromodulators in mediating cognitive processes in humans and other animals (Steckler and Sahgal, 1995; Hasselmo, 1995; McGaughy et al., 2000; Sarter and Parikh, 2005) and their involvement in many human neuropsychiatric illnesses are well documented (Akil et al.,

1999; Mega, 2000; Cools et al., 2002; Austin et al., 2002). Because they are integrally involved in cognition, neuro-

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modulatory influence on cortical circuits may have been altered in the evolution of the modern human brain (Previc, 1999).

Acetylcholine (ACh) has been implicated in the chemical regulation of learning, memory deficits associated with aging, as well as in human neurodegenerative diseases (Levin and Simon, 1998; Harder et al., 1998; Hasselmo, 1999; Mega, 2000; Sarter and Parikh, 2005). Studies in rodents and primates have shown that cholinergic axons originate from neurons in the nucleus basalis of Meynert and project to all regions of the cerebral cortex, exhibiting a substantial degree of regional heterogeneity (Mesulam et al., 1983, 1986, 1992; Lehmann et al., 1984; Ichikawa and Hirata, 1986; Lysakowski et al., 1986; Lewis, 1991; Mechawar et al., 2000; Mesulam and Geula, 1991; Mesulam, 2004). Although cortical cholinergic input is ubiquitous, the existence of regional differences in cholinergic innervation of cortical areas and laminar preferences support the concept that cholinergic systems have specific local circuit processing properties. At the cellular level, ACh enhances long-term potentiation by enhancing synaptic modification, having both excitatory and inhibitory effects on pyramidal cells and interneurons (Hasselmo and Bower, 1992; Hasselmo and Barkai, 1995; Patil et al., 1998; Patil and Hasselmo, 1999). Through its actions ACh plays an important role in cortical plasticity (Rasmusson, 2000).

The involvement of ACh in cognition was initially demonstrated in studies using ACh receptor antagonists in humans and rats (Deutsch, 1971; Drachman, 1977). Cholinergic projections to the prefrontal cortex enhance input processing in attentional contexts and facilitate memory encoding (Blokland, 1996; Levin and Simon, 1998; Sarter and Parikh, 2005). For example, the administration of scopolamine, a cholinergic antagonist, eliminates the capacity to form episodic memories and diminishes the ability to analyze information or acquire semantic knowledge in macaque monkeys (Harder et al., 1998). The input provided by ACh to the prefrontal cortex is critical to the learning process and is also important in cognitive flexibility and working memory (Steckler and Sahgal, 1995; Levin and Simon, 1998; Sarter and Parikh, 2005). Furthermore, patients with Alzheimer's disease display reduced ACh activity in prefrontal cortical areas implicated in learning and working memory (Whitehouse, 1992; Mesulam, 1996; Mega, 2000), and disruption of cortical ACh has been implicated in other diseases such as schizophrenia and Parkinson's disease (for review, see Sarter and Parikh, 2005). This evidence indicates that the cholinergic system's projections to the neocortex may be a candidate neurochemical substrate for natural selection to modify in the evolution of specialized human intellectual capacities.

The present study examined whether the human frontal cortex exhibits denser and/or different regional and laminar patterns of cholinergic innervation relative to other primates. A quantitative comparative analysis of choline acetyltransferase-immunoreactive (ChAT-ir) axons was conducted in cortical areas 9, 32, and 4 of humans, chimpanzees, and macaque monkeys. Areas 9 and 32 each make contributions to higher cognitive functions. Area 9 of the dorsolateral prefrontal cortex facilitates higher-order behavioral organization and is activated during inductive reasoning and working memory tasks (Petrides et al., 1993; Petrides, 1995; Aboitiz and Garcia, 1997; Goel et al., 1997; Marklund et al., 2007). Area 32 of the medial pre-

TABLE 1. Samples Used in the Study

	Sex	Age	Brain Weight (g)	PMI	Fixation
<i>Macaca maura</i>	F	5	83.3	N/A	Perfused
<i>Macaca maura</i>	F	7	86.1	N/A	Perfused
<i>Macaca maura</i>	F	7	89.2	N/A	Perfused
<i>Macaca maura</i>	F	8	96.5	N/A	Perfused
<i>Macaca maura</i>	M	8	105.5	N/A	Perfused
<i>Macaca maura</i>	M	10	95.1	N/A	Perfused
<i>Pan troglodytes</i>	F	19	229.2	<14	Immersion
<i>Pan troglodytes</i>	F	27	314.3	<14	Immersion
<i>Pan troglodytes</i>	F	35	348.1	<14	Immersion
<i>Pan troglodytes</i>	M	17	384.0	<14	Immersion
<i>Pan troglodytes</i>	M	19	364.6	<14	Immersion
<i>Pan troglodytes</i>	M	41	377.2	<14	Immersion
<i>Homo sapiens</i>	F	40	1250	17	Immersion
<i>Homo sapiens</i>	F	43	1280	6	Immersion
<i>Homo sapiens</i>	F	53	1350	9	Immersion
<i>Homo sapiens</i>	M	35	1460	11	Immersion
<i>Homo sapiens</i>	M	48	1450	12	Immersion
<i>Homo sapiens</i>	M	54	1450	12	Immersion

N/A, not applicable; postmortem interval (PMI) is reported in hours.

frontal cortex is involved in 'theory of mind' (TOM), the ability to infer the mental states of others, allowing humans to cooperate, deceive, and to predict the actions of others (Adolphs, 2001; Johnson et al., 2002; Gallagher and Frith, 2003). Species differences were not expected in area 4, as cognitive functions are not associated with primary motor cortex.

MATERIALS AND METHODS

Specimens

The nonhuman brain specimens for this research included Moor macaques (*Macaca maura*, four females, two males, age range 5–10 years) and common chimpanzees (*Pan troglodytes*, three females, three males, age range 17–35 years). Human brain specimens were provided by Northwestern University Alzheimer's Disease Center Brain Bank (three women, three men, age range 35–54 years). All human and nonhuman individuals were adult, nongeriatric, and free of gross neuropathologic abnormalities. Human brains were examined for senile plaques and neurofibrillary tangles by the donating brain bank. All individuals received a score of zero for the CERAD senile plaque grade (Mirra et al., 1999) and the Braak and Braak (1991) neurofibrillary tangle stage and showed no evidence of any neurologic or psychiatric disorder at time of death. The nonhuman subjects were housed in social groups and had never been used in studies involving drug treatment. The age, sex, brain weight, and postmortem interval for each specimen can be found in Table 1.

Fixation and sample processing

The macaque monkeys were perfused transcardially with 4% paraformaldehyde as part of unrelated experiments following methods described previously (Hof and Nimchinsky, 1992; Hof et al., 1996). Chimpanzee and human brains were collected postmortem and fixed by immersion in 10% buffered formalin for 7–10 days, then transferred to a 0.1 M phosphate-buffered saline (PBS, pH 7.4) solution containing 0.1% sodium azide and stored at 4°C to prevent further tissue shrinkage and blockade of antigens.

All samples derive from the left hemisphere. For macaque and chimpanzee brains the entire frontal lobe was removed just rostral to the primary motor cortex as a

coronal slab, including areas 9 and 32. For macaque specimens the occipital lobe was removed rostral to the lunate sulcus. This resulted in three blocks for each macaque left hemisphere, the middle block containing the primary motor cortex (area 4). The region of hand representation in the chimpanzee primary motor cortex had been dissected from the left hemisphere of each brain to be processed as small blocks as part of an unrelated project. This region was identified as the area on the lateral surface at the level of the middle genu located within the central sulcus (Yousry et al., 1997). Human samples were dissected from the regions of interest in 4-cm-thick blocks by the donating brain bank. Prior to sectioning, samples were cryoprotected by immersion in a series of sucrose solutions (10%, 20%, and 30%).

Brain specimens were frozen on dry ice and cut into 40- μ m-thick sections using a sliding microtome. As the brain samples were cut sections were placed into individual microcentrifuge tubes containing freezer storage solution (30% each distilled water, ethylene glycol, and glycerol and 10% 0.244 M PBS) and numbered sequentially. Sections were stored at -20°C .

A 1:10 series for all samples was stained for Nissl substance with a solution of 0.5% cresyl violet to reveal cell somata. Nissl-stained sections were used to identify cytoarchitectural boundaries and to obtain neuron densities.

Immunohistochemistry

Floating tissue sections were stained using the avidin-biotin-peroxidase method. Sections were removed from the freezer and rinsed a minimum of 10×5 minutes in PBS. A 1-in-10 series (human samples and chimpanzee primary motor cortex) or a 1:20 series (macaque samples and chimpanzee frontal lobe) for each area was immunohistochemically stained for ChAT using an affinity-purified polyclonal goat anti-ChAT antibody raised against human placental ChAT (AB144P, Chemicon, Temecula, CA) to measure ACh-containing fibers (Ichikawa and Hirata, 1986; Lewis, 1991; Mesulam et al., 1992; Sigle et al., 2003). This polyclonal ChAT antiserum stains a single band of 68 kDa molecular weight on Western immunoblot in human brain tissue (Bruce et al., 1985; Grossman et al., 1995; Gill et al., 2007). Preadsorption experiments have been conducted for this antibody by adding purified ChAT enzyme prior to the primary antibody incubation in macaques (*Macaca nemestrina*) (Rico and Cavada, 1998) and humans (Mufson et al., 1989; Mesulam et al., 1992). For both species preadsorption experiments yielded a complete absence of staining. Mesulam et al. (1992) also tested for antibody specificity in human brain tissue by substituting an irrelevant IgG for the ChAT antibody, with the result being a lack of specific staining. Finally, positive controls for antibody specificity include well-known staining patterns for ChAT-ir cells within defined regions of the telencephalon (i.e., nucleus basalis of Meynert, caudate, and putamen). In the present study the pattern of ChAT-ir staining within these regions of macaques was identical to that previously reported for this species (Mesulam et al., 1983, 1986; Rico and Cavada, 1998). For chimpanzees, stained sections included rostral striatum, exhibiting the expected pattern of ChAT-ir cells (Šimić et al., 1999). Additional immunohistochemical controls included processing sections as described with the omission of either 1) the primary antibody or 2) the secondary antibody. Omission of the primary or secondary

antibody resulted in a complete absence of labeled axons and cells for each species.

Sections were pretreated for antigen retrieval by incubating in 10 mM sodium citrate buffer (pH 3.5) at 37°C for 30 minutes. Sections were then rinsed and endogenous peroxidase was quenched using a solution of 75% methanol, 2.5% hydrogen peroxide (30%), and 22.5% distilled water for 20 minutes at room temperature. Sections were preblocked in a solution of PBS with 2% normal rabbit serum and 0.3% Triton X-100 detergent, then the sections were incubated in primary antibody diluted to 1:500 in PBS for 48 hours at 4°C . After incubation in primary antibody the tissue was incubated in biotinylated secondary antibody (1:200) in a solution of PBS and 2% normal rabbit serum for 1 hour at room temperature. Sections were then incubated in avidin-peroxidase complex (PK-6100, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. A 3,3'-diaminobenzidine-peroxidase substrate with nickel solution enhancement was used as the chromogen (SK-4100, Vector Laboratories). Immunostained sections were counterstained with 0.5% methylgreen to visualize nonimmunoreactive neurons and to aid in identifying layers within the cortex. Robust and full antibody penetration through the tissue sections was observed for each species.

Original photomicrographs were processed using Adobe Photoshop, v. 7.0 (San Jose, CA). Brightness, contrast, and sharpness were adjusted to obtain pictures that most closely resemble the appearance of the tissue as seen through the microscope.

Identifying cortical regions and layers

Cortical regions of interest were identified based on topological location and distinctive regional cytoarchitecture recognizable on Nissl-stained sections. Cytoarchitectural features were relied upon for identification of cortical regions due to individual variation in the gross location of brain regions (Zilles et al., 1996; Amunts et al., 1996; Petrides and Pandya, 1999; Rademacher et al., 2001). Cortical layers were analyzed separately as layers I, II, III, and V/VI. Because there is not a sharp border between the infragranular layers in all cortical areas, layers V and VI were analyzed together. Layer IV was not analyzed, as only area 9 is granular. The borders of cortical areas tend not to be sharp or distinct, thus, sampling was limited to a representative region within the cortical areas of interest.

Area 9 is located in the dorsolateral prefrontal cortex, extending medially to the paracingulate sulcus of humans and the cingulate sulcus of macaque monkeys (Petrides and Pandya, 1999; Paxinos et al., 2000) (Fig. 1). This cortical area is expanded in anthropoids (i.e., monkeys, apes, and humans) and has no defined homolog in other mammals (Preuss and Goldman-Rakic, 1991; Aboitiz and Garcia, 1997). For this study the portion of area 9 sampled was located on the dorsal portion of the superior frontal gyrus in humans and chimpanzees, and in the region referred to as area 9L in macaque monkeys by Paxinos et al. (2000). Area 32 is defined as the portion of the paracingulate cortex anterior to the genu of the corpus callosum (Gallagher and Frith, 2003; Öngür et al., 2003) (see Fig. 1). In chimpanzees the cytoarchitecture of cortex within the anterior paracingulate gyrus was described by Bailey et al. (1950) to closely resemble area FDL in humans (von Economo and Koskinas, 1925), suggesting that

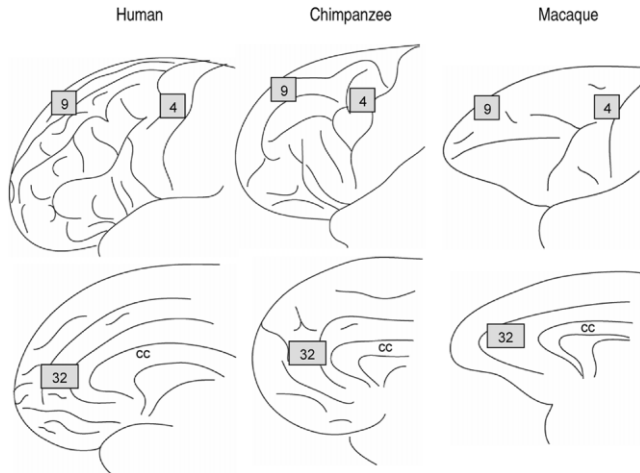


Fig. 1. Lateral (upper) and medial (lower) views of human, chimpanzee, and macaque brains. The positions of the cortical regions sampled in this study are labeled with their respective numerical designations.

they are homologous in structure. Macaques do not possess a strict homolog of the portion of area 32 that is activated in human TOM studies (i.e., anterior cingulate area 32) (Öngür et al., 2003). However, we chose this area to investigate the evolution of the unique human behavioral capacity, TOM. For comparative purposes we were limited to the most similar anatomical cortical territory of the medial prefrontal cortex area 32 in macaques, defined as prelimbic cortex (Öngür et al., 2003). Sampling within area 4 was restricted to the portion associated with hand representation (see Fig. 1). This restriction was implemented to avoid potential differences of cholinergic innervation in the various regions of primary motor cortex. Species differences were not expected in area 4, as it is not associated with cognition and is thought to perform a similar function across primates (e.g., Rizzolatti et al., 1998; Kaas, 2004).

Axon length density

Quantitative analyses were performed using computer-assisted stereology. This system consisted of a Zeiss Axio-plan 2 photomicroscope equipped with an Optronics MicroFire camera, a Ludl XY motorized stage, Heidenhain z-axis encoder, and StereoInvestigator software, v. 6 (MBF Bioscience, Williston, VT). Once the cortical area of interest was identified using both topological location and cytoarchitecture (Preuss and Goldman-Rakic, 1991; Petrides and Pandya, 1999; Lewis and van Essen, 2000; Öngür et al., 2003; Petrides, 2005) in Nissl-stained sections, two to five equidistantly spaced sections per area of interest per individual were used. Care was taken to ensure sampling within the middle of the cortical area in order to avoid transition zones between cortical areas. The variance in section number was dependent on the number of sections available for that cortical area. In some instances the blocks of human tissue obtained from the brain bank yielded only 20 to 30 sections. Once the area of interest was identified the separate cortical layers (I, II, III, and V/VI) were individually traced using the software at low magnification (4× Zeiss Achroplan, N.A. 0.10). On the

occasion when the methyl-green counterstain was too light to identify laminar boundaries, individual layers were traced from adjacent Nissl-stained sections and transferred to the immunostained sections by lining up the tracing with the same area on the immunostained section.

The mean mounted section thickness was measured on every fifth sampling location. Axon length was assessed using the SpaceBalls probe under Koehler illumination at 63× (Zeiss Plan-Achomat, N.A. 1.4) (Calhoun and Mouton, 2000; Mouton et al., 2002; Calhoun et al., 2004; Kreczmanski et al., 2005), a stereological tool that places sampling hemispheres for lineal features in the context of a fractionator sampling scheme (Mouton, 2002). A sampling hemisphere is a virtual hemisphere within the defined sampling box that is oriented with the apex of the hemisphere near the top of the tissue section and the base of the hemisphere toward the bottom of the section (in the z dimension). By moving through the tissue section in the z dimension the diameter of the hemisphere increases and fibers were marked where they intersected the outline of the hemisphere. Hemispheres of 10 μm diameter were used for all samples. Total fiber length within the sampled volume of reference was calculated using the following equation (Calhoun et al., 2004):

$$L = 2 \times (v/a) \times (\Sigma is) \times 1/asf \times 1/ssf \times 1/tsf$$

where v/a is the ratio of sampling frame volume to probe surface area, Σis is the sum of the number of intersections between fibers and sampling hemispheres, asf (area sampling fraction; the fraction of the total area sampled) is the area of the counting frame divided by the total area of the reference space, ssf (section sampling fraction) is the number of sections analyzed divided by the total number of sections through the reference space, and tsf (tissue sampling fraction) is the sampling box height divided by mean mounted section thickness. To obtain axon length density the total fiber length was divided by the planimetric measurement of the reference volume that was sampled, as calculated by the StereoInvestigator software. Analyses of ChAT-ir axon length densities were used to analyze species-specific cortical innervation patterns.

Staining for ChAT was robust and visual examination through the z axis revealed full antibody penetration through the tissue sections for each species. An average of 95.29 ± 22.3 (mean \pm SD) sampling hemispheres was placed in each layer/individual/cortical area. A total of 20,560 sampling hemispheres were used, with 115,083 intersections counted.

Neuron density

Neuron density was assessed using an optical disector combined with a fractionator sampling scheme. Layers II, III, and V/VI were outlined within the area of interest at low magnification (4× Zeiss Achroplan, N.A. 0.10). The optical disector probes were performed under Koehler illumination using a 63× objective (Zeiss Plan-Apochromat, N.A. 1.4). Counting frames were set at $40 \times 40 \mu\text{m}$. Neurons were counted when the nucleolus was in focus within the counting frame. Neurons were identified based on the presence of a large, lightly stained nucleus, a distinct nucleolus, and lightly stained proximal portions of dendritic processes (e.g., Sherwood et al., 2005). The counting

frame height was set at 7 μm to allow a guard zone of at least 2 μm at the top and bottom of the sections. Neuron density was calculated as the sum of neurons counted with the optical disectors divided by the product of the disectors and the volume of the disector (Sherwood et al., 2005). To correct for tissue shrinkage in the z axis the height of the disector was multiplied by the ratio of the nominal section thickness (40 μm) to the actual number weighted mean thickness after mounting and dehydration. No correction was necessary for the x and y dimensions because shrinkage in section surface area is minimal (Dorph-Petersen et al., 2001).

For neuron density counts, an average of 102.4 ± 22.5 (mean \pm SD) sampling sites was placed in each layer/individual/cortical area, with a total of 16,591 sampling sites investigated and 40,201 neurons counted. The mean coefficient of error related to sampling (CE; Schmitz and Hof, 2000) was 0.06 with a standard deviation of 0.02.

Axon length density/neuron density ratio

The ratio of axon length density to neuron density (ALv/Nv) was used for comparative analyses among species rather than axon length to avoid several confounding factors. First, cell density per unit volume can vary with changes in brain size (Haug, 1987; Sherwood et al., 2007). Thus, the ratio of ALv/Nv allows for the evaluation of fiber density in the context of species differences in neuron density. As such, this ratio could be interpreted as innervation per neuron. Next, postmortem interval, method of fixation, and amount of time in fixative are factors that contribute to preprocessing tissue shrinkage. Additional tissue shrinkage may occur with histological and immunohistochemical procedures. Thus, the ALv/Nv ratio acts to standardize data for differential tissue shrinkage among species as well as among individuals.

One of the defining features of layer I, the molecular layer, is an absence of neurons, precluding the use of a layer I ALv/Nv. To circumvent this problem so that among-species comparisons could be made we used the ratio of axon length density in layer I to the neuron density of layer II for each species and area.

Statistical analyses

Factorial analysis of variance (ANOVA) with repeated measures design was used to examine differences among macaques, chimpanzees, and humans. The variables were ChAT-ir ALv/Nv for layers I, II, III, and V/VI. A $4 \times 3 \times 3$ mixed-model ANOVA was performed with cortical area (9, 32, and 4) and layer (I, II, III, and V/VI) as within-subjects measures and species as the between-subjects measure. Tukey's Honestly Significant Difference (HSD) post-hoc tests were used to analyze significant results indicated by the ANOVA analyses. Separate analyses were conducted for ChAT-ir axon length density to examine innervation patterns independent of neuron densities and species effects. For axon length density, a 3×4 (area \times layer) repeated measures ANOVA was used to analyze the pattern differences between areas and layers within each species. Tukey's HSD post-hoc tests were used to evaluate significant results. To assess whether post-mortem interval (PMI) affected the intensity of immunohistochemical staining, nonparametric Spearman's correlation coefficients were calculated for PMI and ChAT-ir ALv/Nv in humans. Data on specific PMI were not available for the chimpanzee sample and PMI was not appli-

cable for the macaques as they were perfused. Spearman's rank order correlation was also used to test the strength of the relationship between age and ChAT-ir ALv/Nv within each species. Our sample was restricted to nongeriatric individuals in order to control for the potentially confounding factor of age-related declines in cortical ChAT-ir axon density. An α level of 0.05 was set for all statistical tests.

An additional methodological concern is the effect of immersion (humans and chimpanzees) versus perfusion (macaques) methods of fixation on the reliability of immunohistochemistry. Perfusion is the most effective method of preservation for immunohistochemical procedures (Evers and Uylings, 1997; Evers et al., 1998; Shiurba et al., 1998; Jiao et al., 1999). If this were a factor in this study it would be expected that axons would be overrepresented in all layers and areas of the macaques. However, staining was robust in all species and such an overrepresentation in macaques was not observed. This is evident in the results, wherein macaques do not exhibit uniformly greater densities than either humans or chimpanzees. Rather, the amount of variation observed in macaques relative to other species is layer- and area-specific and not in one consistent direction.

RESULTS

Qualitative description

ChAT-ir axons were present in all layers of the cortical areas examined in each of the species (Fig. 2). Figures 3, 4, and 5 show ChAT-ir tracings in each area for each species. These figures were produced by obtaining montage images of the cortical areas at $20\times$ (Zeiss Achroplan, N.A. 0.50) and tracing individual axons using Adobe Photoshop software. Although there was significant variation among individuals, this variation was not correlated with PMI among humans (all P values > 0.05 , two-tailed). Spearman's rho correlation coefficients were calculated to test the strength of the relationship between age and ChAT ALv/Nv in each species. Of the 36 possible correlations (3 species \times 3 areas \times 4 layers), only 17% (6/36) were significant, indicating that age did not consistently affect ALv/Nv, especially considering that four of the six significant findings were positive, not negative, correlations. Indeed, the eldest in each species possessed ChAT ALv/Nv values that were comparable to values found for the youngest individual within the same species, with no consistent directional shift.

Previous reports have described ChAT-ir distributions within the cerebral cortices of humans (Mesulam et al., 1992; Mesulam, 1996, 2004), long-tailed macaques (*Macaca fascicularis*) (Lehmann et al., 1984; Lewis, 1991), and rhesus macaques (*M. mulatta*) (Mesulam et al., 1983, 1986). The qualitative findings of the present study are in accordance with these earlier descriptions in humans and macaques. This study represents the first analysis of ChAT-ir axons within the chimpanzee cerebral cortex. For the species analyzed here, we found the distribution of ChAT-ir axons to be regionally heterogeneous with discrete laminar patterns.

The majority of the cholinergic axons observed in all species were thin with multiple closely spaced varicosities (Fig. 6). Less common morphological axon types were thicker and smooth or with irregularly spaced

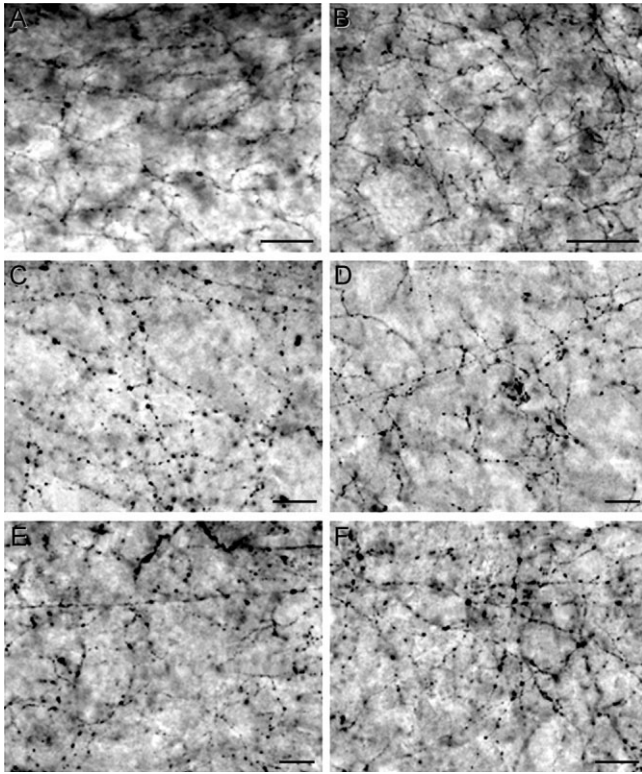


Fig. 2. Brightfield photomicrographs showing ChAT-ir fiber staining in layers I–III and layer III of area 32, respectively, in macaque (A,B), chimpanzee (C,D), and human (E,F). Scale bars = 100 μ m.

oblong swellings (Fig. 7). This less common axon type was more abundant in the infragranular layers. Layer I axons tended to be oriented horizontal to the cortical surface, while axons in layers II–VI were oriented in a variety of directions and formed dense networks of varicose fibers.

Among the cortical areas examined, area 4 displayed the densest complement of ChAT-ir fibers for each of the species. Relative to area 4, areas 9 and 32 were less densely innervated and no obvious qualitative differences were apparent between these two areas within each species (see Figs. 3–5). Distinct species-specific laminar patterns of ChAT-ir axons were observed among cortical regions. In all macaque cortical regions layers I and II were densely innervated, while layers III and V/VI appeared to have a less dense contingent of fibers. This pattern was most pronounced in area 4 of macaques. In contrast to macaques, the innervation in humans and chimpanzees appeared more uniform in areas 9 and 32, with all layers having more or less equally dense innervation. The pattern for human and chimpanzee area 4 was similar to that of macaques, with layers I and II appearing more densely innervated than layers III and V/VI.

The presence of ‘clusters’ of ChAT-ir axons was noted in all areas examined in humans and chimpanzees (Fig. 8), a feature that was observed in humans by Mesulam et al. (1992). Morphologically, clusters consisted of dense tangles of varicose axons that were easily identifiable within the cortical mantle. Comparable features were not observed in macaques. However, all species demonstrated

localized plexuses of ChAT-ir fibers that were denser than the surrounding areas. Another remarkable feature was the common occurrence of dense perisomatic staining present in layers III through V of all cortical areas of the macaques (Fig. 9). This distinctive perisomatic staining surrounded oval or ovoid shaped cells in methyl green counterstain, a morphology that is usually indicative of inhibitory interneurons. None were pyramidal or triangular in shape. This attribute was occasionally observed in chimpanzees, but absent in all human samples.

Within-species analyses

Table 2 lists the mean ChAT-ir axon length density for each species/area/layer. The 4×3 repeated-measures ANOVA used for the analysis of ChAT-ir axon length density within macaques showed a significant interaction between layer and area ($F_{6,30} = 4.68, P < 0.01$; Fig. 10A), and significant main effects of layer ($F_{3,15} = 78.93, P < 0.001$) and area ($F_{2,10} = 6.62, P < 0.02$). The results of the chimpanzee analysis yielded a significant interaction ($F_{6,30} = 5.56, P < 0.01$; Fig. 10B), and significant main effects of layer ($F_{3,15} = 36.91, P < 0.001$) and area ($F_{2,10} = 7.55, P < 0.02$). In humans the interaction was not significant ($F_{6,30} = 0.54, P > 0.05$; Fig. 10C). However, the main effects were each significant: layer ($F_{3,15} = 26.34, P < 0.001$), and area ($F_{2,10} = 6.08, P < 0.02$). For humans, area 4 displayed the highest axon length density in all layers, area 32 was intermediate, and area 9 had the lowest innervation (see Table 2, Fig. 10C). Of the layers, layer II had the highest density, followed by layers I, III, and V/VI.

Tukey HSD post-hoc tests were performed to analyze the significant interactions found in the analyses of data from macaques and chimpanzees. Comparisons were made between layers within each cortical area (Table 3), as well as the differences of layers between cortical regions (Table 4). In all cortical areas of macaques, layers I and II were more densely innervated than either layer III or V/VI. Layer III was more densely innervated than V/VI in area 9 (Table 3). In chimpanzees, layers I and II had a significantly higher axon length density than III or V/VI in area 4 only (Table 3). This matched the patterns found in areas 4 and 32 of the macaques. No laminar differences were detected in area 32 of chimpanzees, and only one difference was found in area 9, with layer II having more axons than V/VI. In the comparisons among macaque cortical areas (Table 4) there were no significant differences between areas 9 and 32. When comparing area 9 to area 4, only layer I was different, being denser in area 4. Finally, in macaques area 4 had a uniformly denser innervation over area 32. The chimpanzee pattern was different from that of the macaques in some of the comparisons (Table 4). Like macaques, chimpanzees displayed no differences between areas 9 and 32. Similar to macaques, chimpanzee area 4 layers I, II, and III were more densely innervated than those of than either area 9 or area 32.

Among-species analyses

The mean ALv/Nv and standard deviation for each cortical area and layer for macaques, chimpanzees, and humans are listed in Table 5 and graphically displayed in Figure 11. In the $4 \times 3 \times 3$ ANOVA with repeated-measures design, the main effect of species was not significant ($F_{2,15} = 0.96, P > 0.05$), although the main effects

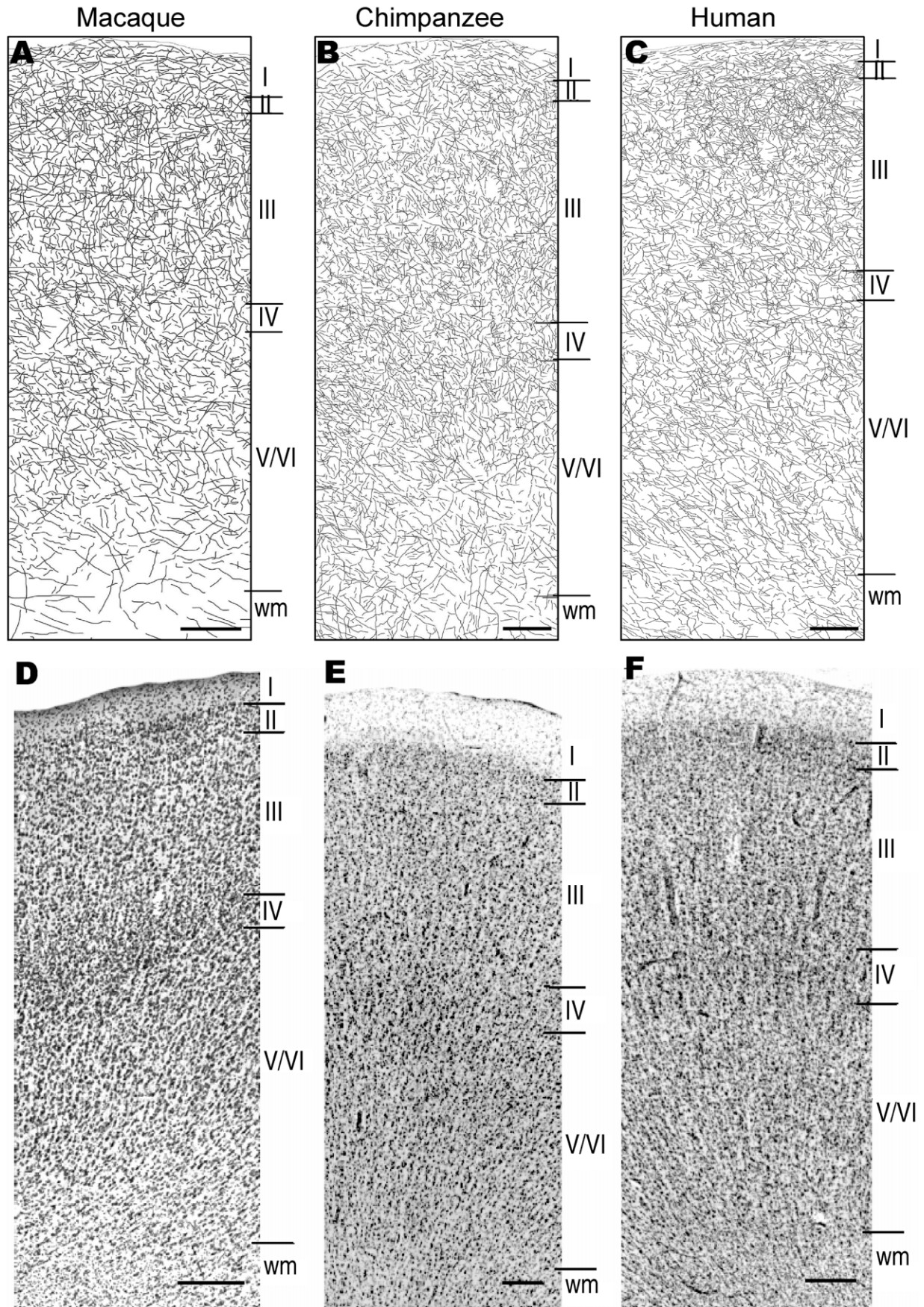


Fig. 3. ChAT-ir axon tracings and Nissl stain in area 9 for macaque (A,D), chimpanzee (B,E), and human (C,F). wm, white matter. Scale bar = 250 μ m.

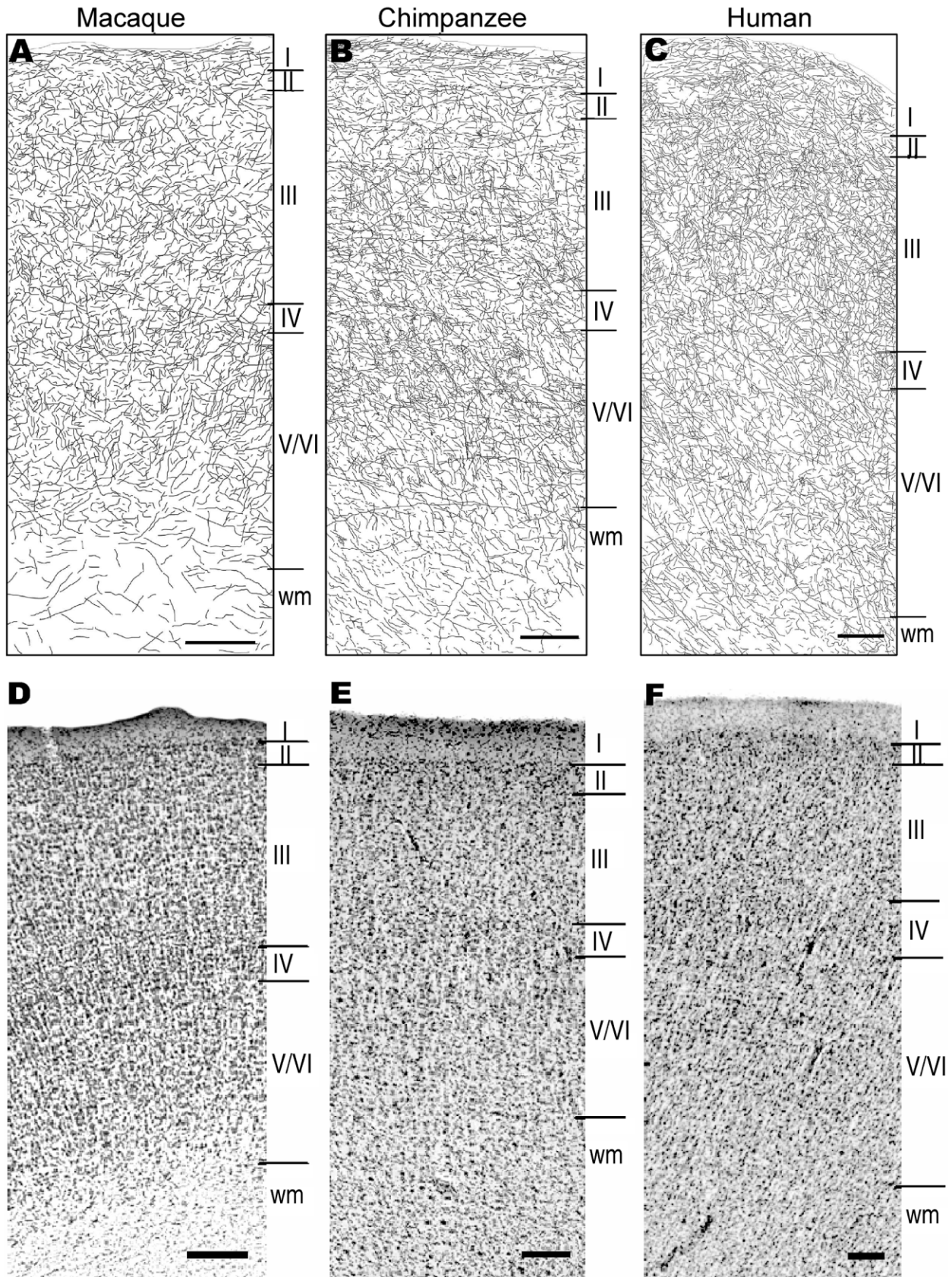


Fig. 4. ChAT-ir axon tracings and Nissl stain in area 32 for macaque (A,D), chimpanzee (B,E), and human (C,F). wm, white matter. Scale bar = 250 μ m.

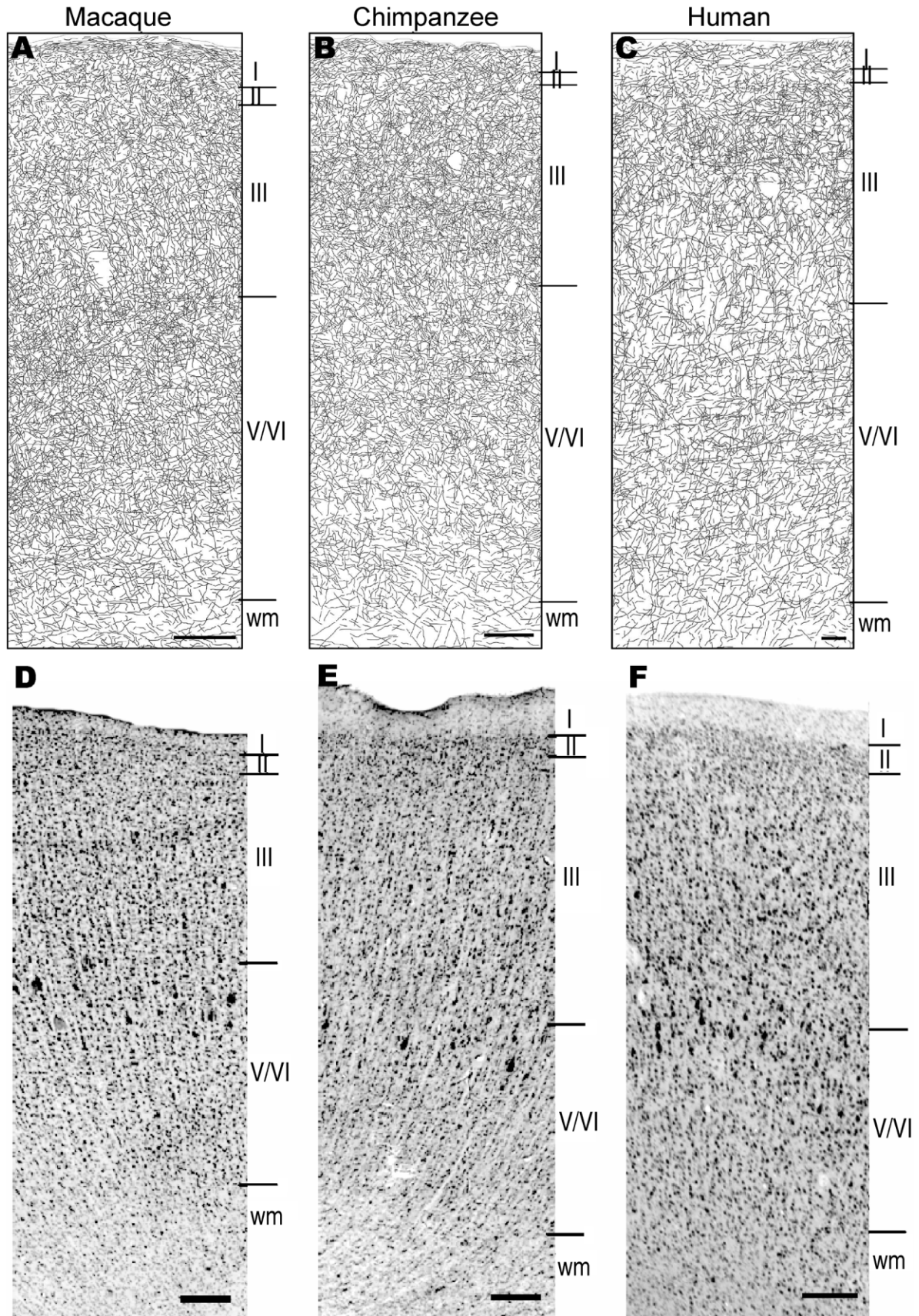


Fig. 5. ChAT-ir axon tracings and Nissl stain in area 4 for macaque (A,D), chimpanzee (B,E), and human (C,F). wm, white matter. Scale bar = 250 μ m.

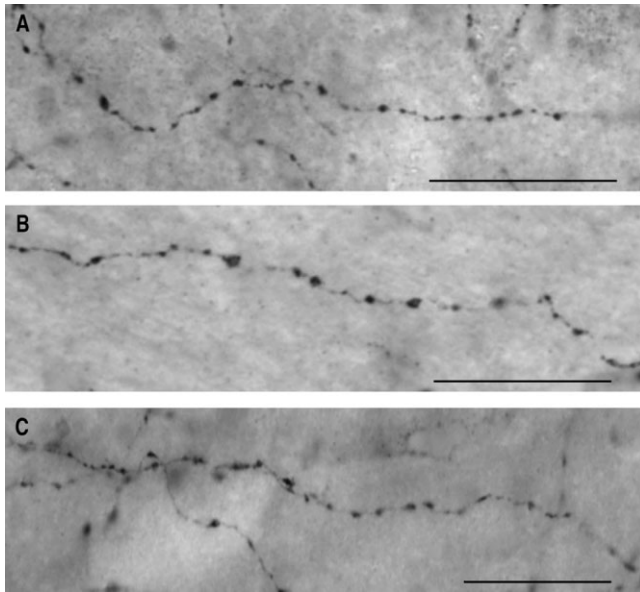


Fig. 6. Examples of thin cholinergic axons with multiple, closely spaced varicosities. Panels show axons in (A) macaque, (B) chimpanzee, and (C) human. Scale bars = 25 μm .

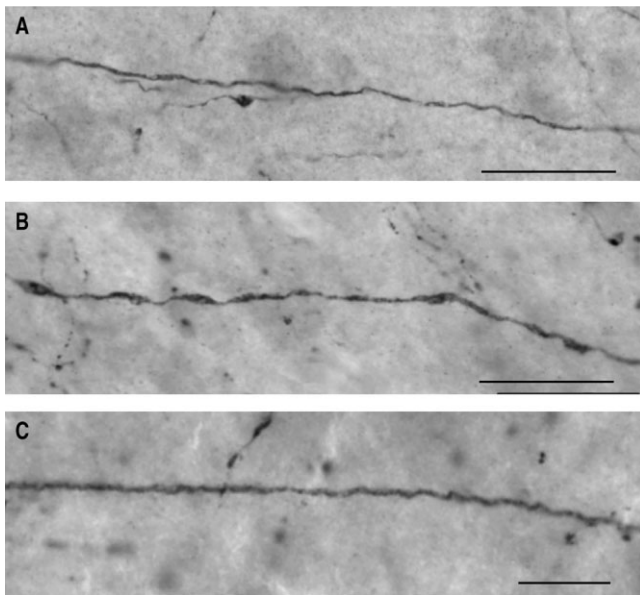


Fig. 7. Cholinergic axons that are thick and smooth or with irregularly spaced oblong swellings. The panels show examples of axons in (A) macaque, (B) chimpanzee, and (C) human. Scale bars = 25 μm .

of layer and area were each significant (layer: $F_{3,45} = 19.58$, $P < 0.001$; area: $F_{2,30} = 28.85$, $P < 0.001$). The three-way interaction among layer, area, and species was not significant ($F_{12,90} = 1.64$, $P > 0.05$). Of the two-way interactions, layer \times species and layer \times area were significant (layer \times species: $F_{6,45} = 9.30$, $P < 0.001$; layer \times area: $F_{6,90} = 11.25$, $P < 0.001$), and area \times species was not ($F_{4,30} = 1.34$, $P > 0.05$). The layer \times area interaction was expected due to the regional and laminar heterogeneity of

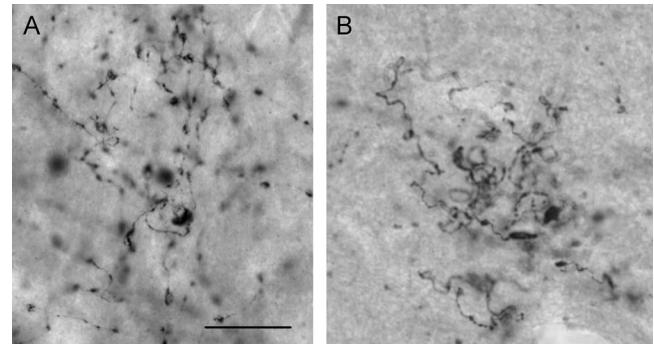


Fig. 8. ChAT-ir axon 'clusters' in human (A) and chimpanzee (B). Scale bar = 25 μm in A.

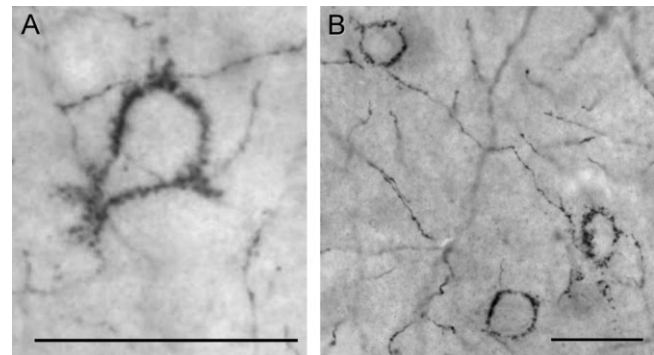


Fig. 9. ChAT-ir perisomatic staining in macaques. Scale bar = 25 μm .

TABLE 2. ChAT-ir Axon Length Densities ($\mu\text{m}/\mu\text{m}^3$) for Each Species, Area, and Layer

Species	Layer	Area 9	Area 32	Area 4	N
<i>Macaca maura</i>	I	0.159 \pm 0.06	0.130 \pm 0.04	0.230 \pm 0.04	6
	II	0.173 \pm 0.06	0.138 \pm 0.03	0.207 \pm 0.04	
	III	0.120 \pm 0.03	0.084 \pm 0.02	0.126 \pm 0.02	
	V/VI	0.082 \pm 0.03	0.067 \pm 0.01	0.113 \pm 0.02	
<i>Pan troglodytes</i>	I	0.064 \pm 0.02	0.061 \pm 0.01	0.111 \pm 0.03	6
	II	0.067 \pm 0.01	0.063 \pm 0.01	0.101 \pm 0.03	
	III	0.051 \pm 0.01	0.050 \pm 0.01	0.070 \pm 0.02	
	V/VI	0.047 \pm 0.01	0.047 \pm 0.01	0.064 \pm 0.01	
<i>Homo sapiens</i>	I	0.067 \pm 0.01	0.084 \pm 0.01	0.101 \pm 0.03	6
	II	0.086 \pm 0.02	0.111 \pm 0.04	0.113 \pm 0.04	
	III	0.056 \pm 0.02	0.074 \pm 0.02	0.084 \pm 0.03	
	V/VI	0.047 \pm 0.01	0.066 \pm 0.01	0.070 \pm 0.02	

Data are expressed as mean \pm standard deviation.

ACh innervation. Overall, the layers of area 4 were more densely innervated than corresponding layers in either area 9 or 32 (Table 5). Post-hoc Tukey HSD tests of the species \times layer interaction revealed no differences between layers within either macaques or chimpanzees (all P 's > 0.05). However, in humans, layer I was significantly different from layer III ($P = 0.001$) and V/VI ($P = 0.007$). Additionally, the pattern of innervation for areas 9 and 32 was different for humans and chimpanzees, in contrast to the macaque monkeys. For macaques, layer V/VI was less densely innervated than the other layers in areas 9 and 32. In contrast, humans and chimpanzees each maintained a higher density of ChAT-ir innervation to these lower layers. All three species had similar innervation patterns for area 4.

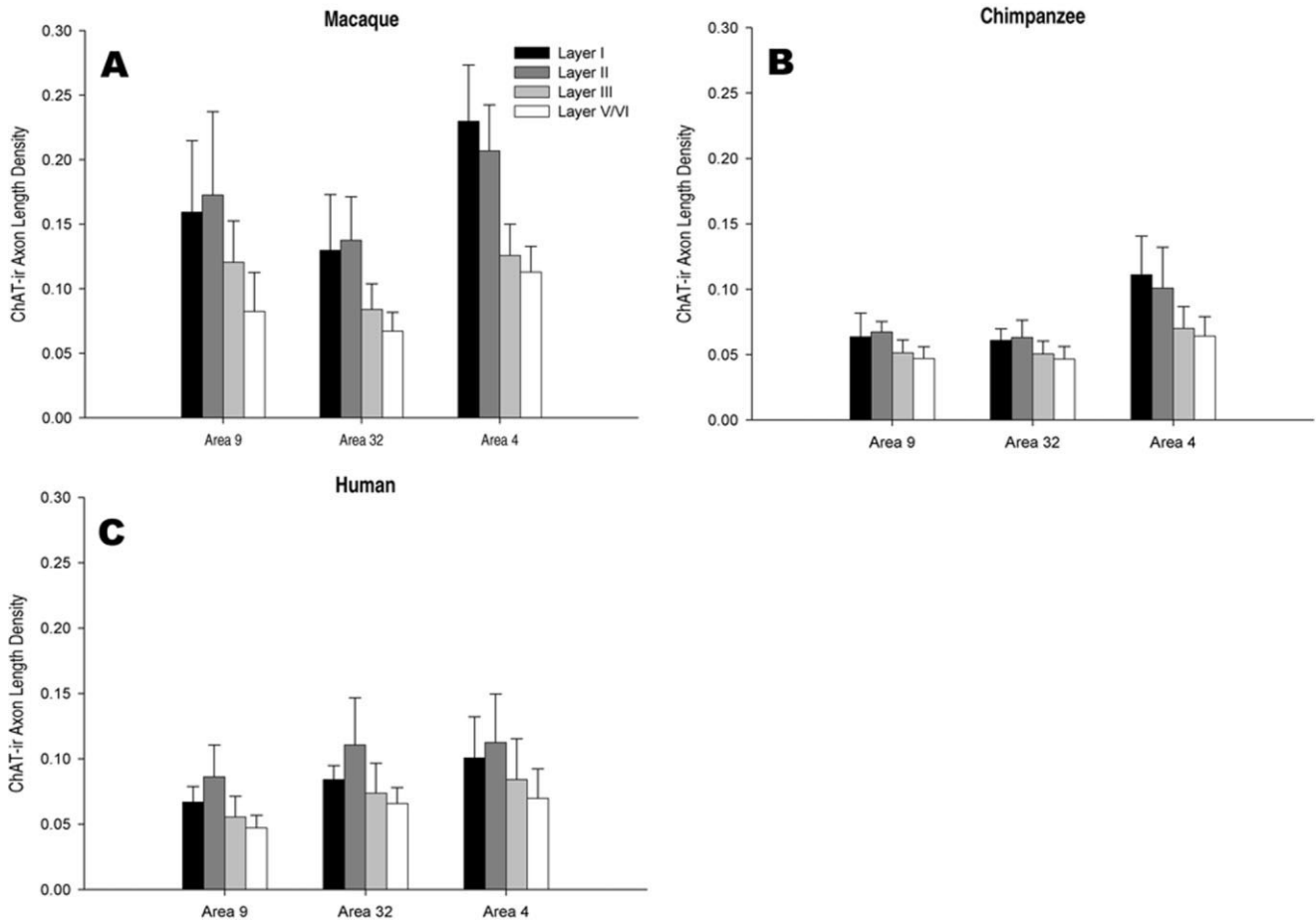


Fig. 10. ChAT-ir axon length densities in each layer, area, and species. Error bars represent standard deviations.

TABLE 3. Probabilities for Tukey HSD Post-hoc Tests of ChAT-ir Axon Length Density

Species	Layers	Area 9	Area 32	Area 4
<i>Macaca maura</i>	I-II	0.98	1.00	0.57
	I-III	0.03* (I)	0.01* (I)	0.00* (I)
	I-V/VI	0.00* (I)	0.00* (I)	0.00* (I)
	II-III	0.00* (II)	0.00* (II)	0.00* (II)
	II-V/VI	0.00* (II)	0.00* (II)	0.00* (II)
	III-V/VI	0.04* (III)	0.88	0.98
<i>Pan troglodytes</i>	I-II	1.00	1.00	0.73
	I-III	0.48	0.70	0.00* (I)
	I-V/VI	0.11	0.25	0.00* (I)
	II-III	0.15	0.41	0.00* (II)
	II-V/VI	0.02* (II)	0.10	0.00* (II)
	III-V/VI	1.00	1.00	0.99

Comparisons between layers within each cortical area for macaques and chimpanzees. *Results statistically significant at the 0.05 level. The layer with the higher axon length density is indicated in parentheses to the right of each significant result.

TABLE 4. Probabilities for Tukey HSD Post-hoc Tests of ChAT-ir Axon Length Density

Species	Layer	Area 9–Area 32	Area 9–Area 4	Area 32–Area 4
<i>Macaca maura</i>	I	0.21	0.00* (4)	0.00* (4)
	II	0.07	0.09	0.00* (4)
	III	0.05	1.00	0.02* (4)
	V/VI	0.94	0.18	0.01* (4)
<i>Pan troglodytes</i>	I	1.00	0.00* (4)	0.00* (4)
	II	1.00	0.00* (4)	0.00* (4)
	III	1.00	0.04* (4)	0.03* (4)
	V/VI	1.00	0.09	0.07

Differences of layers are reported between cortical regions for each species. *Results statistically significant at the 0.05 level. The area with the higher axon length density is indicated in parentheses to the right of each significant result.

DISCUSSION

The cholinergic innervation of the cerebral cortex is ubiquitous but regionally heterogeneous. It has been posited to be the most substantial cortical regulatory pathway in terms of its widespread innervation (Mesulam et al., 1992). Due to the lack of regional uniformity of cortical innervation and the distinct laminar patterning, it is believed that ACh is able to exert differential control over

local circuits rather than acting in a global manner across the cortex (Lysakowski et al., 1986; Lewis, 1991; Mesulam et al., 1992; Mechawar et al., 2000). Lesions or drugs that deplete ACh cortical innervation in primates and rodents impair learning and memory in the acquisition and performance on discrimination tasks that challenge attentional processes (Irlle and Markowitsch, 1987; Fine et al., 1997; Harder et al., 1998; Levin and Simon, 1998; McGaughy et al., 2000; Tsukada et al., 2004; Sarter and Parikh, 2005), and learning and memory deficits are ameliorated with ACh agonists (Levin and Simon, 1998; Wu et

al., 2000). The effects of ACh are important for synaptic modulation and its actions are cell- and receptor-type specific, having both excitatory and inhibitory effects (see Rasmusson, 2000, for a review). Finally, recent evidence indicates an accelerated rate of protein evolution in primates relative to rodents for three separate cholinergic receptor subtypes (Dorus et al., 2004). Collectively, the evidence of regional heterogeneity, combined with studies demonstrating the critical role that cortical ACh input plays in cognition and its depletion in human neurodegen-

erative and neuropsychiatric disorders, were the basis of our hypothesis that the pattern of ACh cortical innervation may have undergone selective evolution within the human lineage. Therefore, we performed a quantitative comparative analysis of ChAT-ir fibers among human, chimpanzee, and macaque brains. Because cortical cholinergic systems influence higher cognitive functions, we expected humans to have significantly different innervation density and laminar profiles relative to nonhuman primates. However, our results demonstrate that humans do not have denser or more extensive cortical ACh input than either chimpanzees or macaques.

The within-species analysis of axon length density demonstrated variation in ChAT-ir axon innervation patterns independent of neuron densities. This measure cannot be used for among-species comparisons because it does not account for differential tissue shrinkage. However, it does allow for an analysis of variation among regions and layers within a species, and the results demonstrated different patterns of cortical cholinergic innervation among the three species examined here, independent of neuron densities. These analyses revealed that area 4 was most densely innervated for all species. For humans, area 32 was second to area 4 in axon length density. For macaques, area 32 received the least cholinergic afferents of

TABLE 5. Mean and Standard Deviation for ChAT ALv/Nv in Each Layer and Cortical Area for Macaques, Chimpanzees, and Humans

Species	Layer	Area 9	Area 32	Area 4	N
<i>Macaca maura</i>	I	1230.40 ± 357.46	1185.63 ± 352.97	1659.37 ± 198.17	6
	II	1328.90 ± 407.69	1266.77 ± 287.51	1502.62 ± 215.76	
	III	1356.37 ± 442.77	1091.29 ± 229.11	1742.49 ± 352.90	
<i>Pan troglodytes</i>	V/VI	885.05 ± 182.39	873.96 ± 191.84	1962.76 ± 323.37	6
	I	909.54 ± 271.06	929.92 ± 231.43	1542.29 ± 232.98	
	II	964.30 ± 161.38	987.11 ± 347.37	395.92 ± 250.91	
<i>Homo sapiens</i>	III	1057.79 ± 224.15	1210.12 ± 386.37	1781.54 ± 225.21	6
	V/VI	1076.05 ± 181.48	1127.47 ± 404.58	1865.25 ± 521.11	
	I	36.71 ± 160.75	829.06 ± 88.61	940.72 ± 308.72	
	II	961.17 ± 375.04	1079.31 ± 277.54	1080.08 ± 407.63	
	III	1134.50 ± 334.42	1357.06 ± 436.03	1723.61 ± 697.59	
	V/VI	1061.52 ± 232.82	1217.47 ± 281.19	1748.02 ± 709.02	

Data are expressed as a mean ± standard deviation.

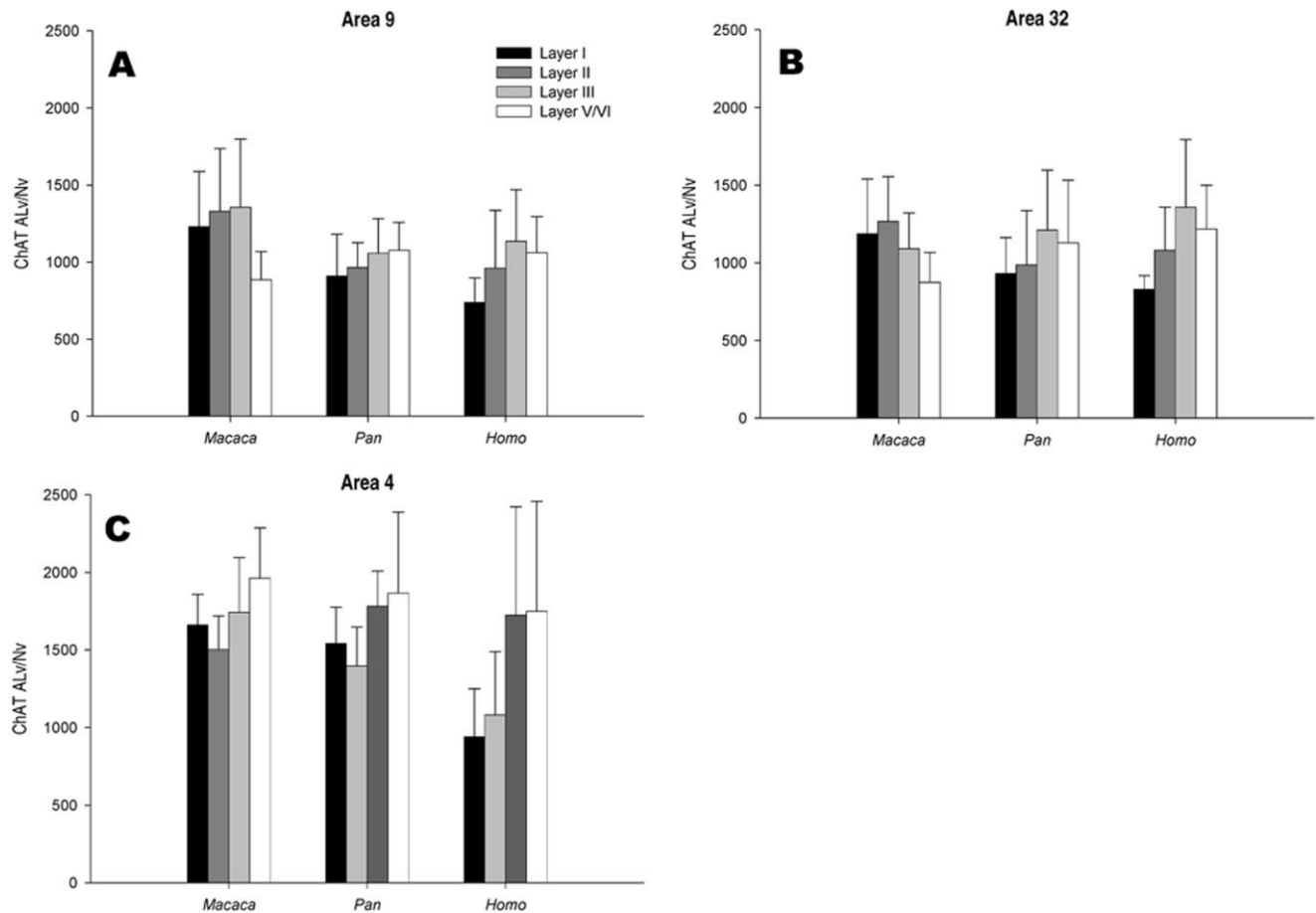


Fig. 11. ChAT-ir ALv/Nv in each layer, area, and species.

the three cortical areas and area 9 and 32 were comparably innervated in chimpanzees.

Comparisons of ChAT ALv/Nv in each layer and area revealed subtle differences among humans, chimpanzees, and macaque monkeys. Post-hoc analyses of the species \times layer interaction indicated that humans had significantly less cholinergic input to layer I when compared to either layer III or layer V/VI. This same pattern was observed in chimpanzees, although it did not reach statistical significance. In contrast, the pattern in macaques for ChAT ALv/Nv was such that the input to layers V/VI was lower compared to layer I for areas 9 and 32, but not for area 4 (Table 4). The pattern of ChAT innervation in the primary motor cortex was similar for all species (Fig. 11). The fact that the innervation patterns differ between the macaques and hominines (i.e., chimpanzees and humans) in areas 9 and 32 is intriguing. This could reflect an alteration in cortical circuitry, putting a stronger emphasis on ACh input in layers III and V/VI of the cortical areas involved in cognition in the evolution of the lineage that includes humans and chimpanzees. Layer III is important because of its putative role as the terminal input layer in controlling corticocortical connections (Fuster, 1997), and layers V and VI receive input from the supragranular layers and mainly provide output to the brainstem, spinal cord, and other subcortical areas. It is possible that these variations may represent a subtle phyletic shift in cortical cholinergic transmission.

Perisomatic staining of what appeared to be inhibitory interneurons was commonly observed in layers III–V in all areas of the macaque cortex. This feature was rarely noted in chimpanzee motor cortex. Similar features were not present in any of the human cortical areas. This distinctive feature was not reported in previous literature in humans or in long-tailed and rhesus macaques (Lehmann et al., 1984; Mesulam et al., 1986, 1992; Lewis, 1991; Mesulam, 2004). It is possible that this feature is unique to Moor macaques. Van der Gucht et al. (2006) recently demonstrated significant variation in the structure of the preunate gyrus among different macaque species, including the Moor macaque, illustrating that substantial neuroanatomical variation is possible among closely related congeners. Further studies will be necessary to determine if it is found in other species and what the functional implications of this perisomatic staining may be.

The presence of ‘clusters’ of ChAT-ir fibers was observed in humans and chimpanzees, but not in macaques. Clusters were described in human cortex by Mesulam (1992) as being complex glomerular dense clusters of varicose axons. We observed these clusters sporadically distributed throughout the cortical mantle in both humans and chimpanzees. What this axon morphology represents is unknown, although it was suggested that their occurrence represents local events of plasticity or circuitry rearrangement (Mesulam et al., 1992). If clusters are representative of cortical plasticity events, their presence in human and chimpanzee, but not macaque, cortices is intriguing. This possibility is made more interesting by the recent discovery of serotonergic and dopaminergic clusters of axons in both human and chimpanzee neocortex and their absence in macaque monkeys (Raghanti et al., 2007, and submitted). This may indicate that human and chimpanzee brains are characterized by a greater capacity for plasticity mediated by neuromodulators possibly contributing to cognitive and behavioral flexibility. In addition, a signifi-

cant amount of individual variation in ChAT-ir fibers was present within our sample. Interindividual variation in cortical cholinergic innervation in the hippocampus, caudate nucleus, and frontoparietal cortex was positively correlated with performance on learning tasks in mice (Durkin et al., 1975), and it has been suggested that variations in cholinergic innervation in human and nonhuman primates would reflect individual differences in learning abilities (Mesulam et al., 1986).

To date, the only comparative study involving cortical ACh input involved measuring the amount of potassium-induced ChAT activity in mouse versus human neocortical slices (Sigle et al., 2003). Relative to mice, only a very low concentration of potassium was required to induce ChAT activity in humans (Sigle et al., 2003). Direct comparisons of ACh cortical input between different species have not been reported. However, some comparisons can be made from the existing literature. Studies of the distribution of ChAT-ir axons in the rat neocortex reported their presence in all cortical areas and layers (Ichikawa and Hirata, 1986; Lysakowski et al., 1986; Mechawar et al., 2000), with the frontal cortex receiving the densest complement of fibers and layer I having the highest laminar density (Mechawar et al., 2000). In contrast, Old World primates exhibit a rostral-caudal gradient of innervation, with innervation in rostral areas of the frontal cortex being less dense than the caudal premotor and motor areas (Mesulam et al., 1986, 1992; Lewis, 1991). A study of ChAT-ir axons in cat sensory and motor cortices demonstrated regional and laminar differences in innervation patterns, with primary visual cortex being less densely innervated than other sensory cortical areas (Avendaño et al., 1996). Another notable difference between primates and other mammals is the occurrence of ChAT-positive neurons in both cat and rat neocortex, albeit with species-specific densities and distributions (Ichikawa and Hirata, 1986; Avendaño et al., 1996; Mechawar et al., 2000; Bhagwandin et al., 2006). ChAT-ir cells have been detected in primate cerebral cortex only during fetal development (Hendry et al., 1987).

Differences among primates have been reported for the localization of galanin relative to cholinergic neurons in the basal forebrain (Kordower and Mufson, 1990; Benzing et al., 1993). Galanin is an inhibitory modulator of acetylcholine in rats (Laplante et al., 2004; Elvander and Ögren, 2005) and galanin-ir fibers are hypertrophied in Alzheimer’s disease, with increased expression of galanin receptors within the nucleus basalis corresponding to late-stage Alzheimer’s disease (Mufson et al., 2000). Galanin hyperfunction is associated with the cholinergic hypofunction and likely contributes to the associated learning and memory deficits characteristic of Alzheimer’s patients (Chan-Palay, 1988). Among primates, humans and apes (gibbons, chimpanzees, and gorillas) displayed a distinctively different localization of galanin-ir relative to monkeys (brown capuchins, rhesus macaques, and baboons) (Benzing et al., 1993). Taken together with the findings reported here, it appears that both cholinergic innervation and modulation were altered in the evolution of apes and humans.

The present research represents the first comparative study of cholinergic innervation of the cerebral cortex between humans and nonhuman primates. Data demonstrating the differences (or similarities) between humans and other species, particularly other primate species, are

critical to our understanding of the evolution of the human brain and its unique cognitive abilities. Our quantitative analyses of ChAT-ir axons revealed no species differences in either primary motor cortex or in prefrontal cortical areas implicated in cognitive functions, indicating that human cognitive specializations are not related to a quantitative increase in cortical ACh input. However, species-specific variation in the morphology of axons was observed. Clusters of ChAT-ir fibers were present in chimpanzees and humans, but not in macaques. It has been suggested that such clusters may represent local areas that have been preferentially involved in cortical plasticity (Mesulam et al., 1992), but their true physiological function is not yet known. Further studies are necessary to determine the importance of this feature and whether it represents a significant specialization of processing capabilities in the cerebral cortex of great apes and humans. The other significant morphology noted was the common and distinctive ovoid perisomatic staining in macaque cortices. This feature was sporadically observed in chimpanzee cortex and never in humans. Earlier studies of long-tailed and rhesus macaques did not mention this morphology (Mesulam et al., 1986; Lewis, 1991). This may represent a unique specialization in Moor macaques, the species used in the present study. Together, the alterations in cortical innervation and morphology shared by humans and chimpanzees may contribute to an increase in cognitive and behavioral flexibility and an increased capacity for learning and memory.

Finally, it should be noted that the inclusion of chimpanzees in this study, a species rarely studied, allowed for additional insight into human-specific specializations that would otherwise not have been possible. We currently have very little knowledge regarding the neural underpinnings of uniquely human cognitive abilities. To gain an understanding of human evolution we must use comparative data from a diverse range of species (Preuss, 2006). Comparisons that include chimpanzees are especially relevant in determining neuroanatomical features that supported human brain evolution due to their close phylogenetic relationship with humans.

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