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Differences in Cortical Serotonergic Innervation among Humans, Chimpanzees, and Macaque Monkeys: A Comparative Study

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In this study, we assess the possibility that the evolution of human intellectual capacities was supported by changes in the supply of serotonin to the frontal cortex. To this end, quantitative comparative analyses were performed among humans, chimpanzees, and macaques. Immunohistochemical methods were used to visualize serotonin transporter–immunoreactive (SERT-ir) axons within the cerebral cortex. Areas 9 and 32 were chosen for evaluation due to their roles in working memory and theory of mind, respectively. Primary motor cortex was also evaluated because it is not associated with higher cognitive functions. The findings revealed that humans do not display a quantitative increase in serotonin innervation. However, the results indicated region- and layer-specific differences among species in serotonergic innervation pattern. Compared with macaques, humans and chimpanzees together displayed a greater density of SERT-ir axons relative to neuron density in layers V/VI. This change was detected in cortical areas 9 and 32, but not in primary motor cortex. Further, morphological specializations, coils of axons, were observed in humans and chimpanzees that were absent in macaques. These features may represent a greater capacity for cortical plasticity exclusive to hominoids. Taken together, these results indicate a significant reorganization of cortical serotonergic transmission in humans and chimpanzees.

Keywords: area 4, area 9, area 32, human evolution, prefrontal cortex, serotonin transporter

Introduction

The role of serotonin (5HT) in cognitive functions, particularly those mediated by the prefrontal cortex, is well established in humans and other mammals (e.g., Meneses and Hong 1995; Steckler and Sahgal 1995; Murphy et al. 2002; Williams et al. 2002; Roth et al. 2004). The pattern of serotonergic cortical innervation observed in primates appears to be more complex and regionally heterogeneous than that of other mammals investigated (e.g., Morrison and Foote 1986; Mulligan and Törk 1988; Audet et al. 1989; Wilson and Molliver 1991; Austin et al. 2002). However, it remains to be determined whether serotonergic systems in the cerebral cortex display significant differences among primate species. This knowledge is particularly relevant in light of 5HT's function in processes such as learning and memory and in uniquely human neurodegenerative disorders, such as Alzheimer's disease and schizophrenia (Naughton et al. 2000; Vergé and Calas 2000; Roth et al. 2004). Do humans have a unique pattern or density of cortical serotonergic innervation compared with other primates? Comparative data assessing differences between species is necessary in order to gain a comprehensive understanding of serotonergic systems and how they function, particularly in humans.

The localization of 5HT receptors in the prefrontal cortex indicates that 5HT contributes to memory and cognition (Buhot 1997; Marek and Aghajanian 1998; Azmitia 1999). In addition, several lines of evidence illustrate the role of 5HT in cognitive functions. For example, 5HT levels were positively correlated with accuracy of performance on an attention task in rats (Puumala and Sirviö 1998). Moreover, drugs that increase 5HT, such as 5HT uptake blockers, improve attention, visual and verbal memory, working memory, and processing speed in intact, healthy rodents as well as in macaques and schizophrenic subjects (Meneses and Hong 1995; Williams et al. 2002; Buchanan et al. 2003). Dysfunctions of serotonergic systems contribute to the cognitive disturbances associated with depression and suicide, obsessive-compulsive disorder, anxiety disorders, and impulse-control disorders (Noguchi et al. 2001; Austin et al. 2002; Bradshaw 2003). In addition, cortical depletion of 5HT has been noted in human neuropathological and neurodegenerative diseases such as Parkinson's disease and schizophrenia (Naughton et al. 2000; Vergé and Calas 2000). For example, Thomas et al. (2006) demonstrated a 47% decrease in serotonin transporter (SERT) density in the prefrontal cortex of Alzheimer's disease patients regardless of depressive symptoms. Austin et al. (2002) found a specific reduction in SERT density only in layer VI in depressed subjects who committed suicide.

The aim of the present study was to obtain quantitative comparative data on cortical serotonergic innervation in humans, chimpanzees, and macaques using SERT immunohistochemistry. We examined areas 9 and 32 of the prefrontal cortex and the primary motor cortex (area 4). Area 9 is expanded in anthropoids (i.e., New World monkeys, Old World monkeys, and apes) with no obvious homologue in other mammals (Preuss and Goldman-Rakic 1991; Aboitiz and Garcia 1997). This area is involved in high-order behavioral organization and is essential to working memory (Petrides et al. 1993; Petrides 1995, 2000; Aboitiz and Garcia 1997; Goel et al. 1997; Marklund et al. 2007). Working memory processes play a critical role in the human language faculty (Coolidge and Wynn 2005), and it has been proposed that the neural circuitry required for complex working memory may have been an essential precondition for the origin of language (Aboitiz and Garcia 1997). Functional magnetic resonance imaging studies in humans have shown that area 32 is involved in the cognitive capacity of "theory of mind" (TOM) (Stuss et al. 2001; Gallagher and Frith 2003). Although debate exists over whether TOM is a uniquely human attribute,

most researchers agree that humans possess a greater capacity for reflecting on, and interpreting, their own mental states as well as those of others (Povinelli and Bering 2002; Roth and Dicke 2005). Therefore, prefrontal cortical areas 9 and 32 represent neural substrates of 2 important human cognitive specializations. If serotonergic cortical innervation underlies human intellectual abilities, differences between humans and other primates would be expected in these areas. For comparison, primary motor cortex (area 4) was also examined. 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 (Rizzolatti et al. 1998; Kaas 2004).

Materials and Methods

Specimens

The nonhuman brain specimens for this research included Moor macaques (*Macaca maura*, 4 females, 2 males, age range 5–10 years), and common chimpanzees (*Pan troglodytes*, 3 females, 3 males, age range 17–35 years). Human brain specimens were provided by Northwestern University Alzheimer's Disease Center Brain Bank (3 females, 3 males, age range 35–54 years). All human and nonhuman individuals were adult, nongeriatric, and free of gross neuropathological abnormalities. The human cases showed no evidence of dementia before death. The nonhuman subjects were housed in social groups. The age, sex, brain weight, and postmortem interval (PMI) for each specimen can be found in Table 1.

Fixation

The macaque monkeys were perfused transcardially with 4% paraformaldehyde as part of unrelated experiments following methods previously described (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 phosphate-buffered saline solution (0.1 M, hereafter referred to as PBS) containing 0.1% sodium azide, and stored at 4 °C to prevent further tissue shrinkage or blockade of antigens.

Sample Processing

All samples derive from the left hemisphere of the brain. For macaque and chimpanzee brains, the entire frontal lobe was removed just rostral to the primary motor cortex as a coronal slab and included areas 9 and 32. For macaque specimens, the occipital lobe was removed rostral to the lunate sulcus. This resulted in 3 blocks for each macaque left hemisphere, the middle block containing the primary motor cortex (area 4). The region of hand representation in 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%).

Table 1
Samples used in this study

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

Note: N/A = not applicable, PMI is reported in hours.

Brain specimens were frozen on dry ice and cut into 40-μm-thick sections using a freezing 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-in-10 series for all samples was stained for Nissl substance to reveal cell somata with a solution of 0.5% cresyl violet. Nissl-stained sections were used to identify cytoarchitectural boundaries and to obtain neuron densities. A 1-in-10 series (human samples and chimpanzee primary motor cortex) or a 1-in-20 series (macaque samples and chimpanzee frontal lobe) for each area was immunohistochemically stained for SERT to measure 5HT-containing fibers (Akil et al. 1999; Austin et al. 2002; Verney et al. 2002).

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 min in PBS. Sections were pretreated for antigen retrieval by incubating in 10 mM sodium citrate buffer (pH 8.5) at 90 °C for 30 min. 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 min at room temperature. Sections were preblocked in a solution of PBS with 4% normal goat serum, 0.3% Triton X-100 detergent, and 3% dried milk. Following this, sections were incubated in primary antibody diluted to 1:12 000 in PBS for 48 h at 4 °C. The antibody used was a rabbit anti-SERT polyclonal antibody directed at the C-terminus of rat SERT peptide 596–622 (a gift from Dr Randy D. Blakely, Vanderbilt University Medical Center, Nashville, Tennessee). After incubation in primary antibody, the tissue was incubated in biotinylated secondary antibody (1:200) in a solution of PBS and 4% normal goat serum for 1 h at room temperature. Sections were then incubated in avidin-peroxidase complex (PK-6100, Vector Laboratories, Burlingame, CA) for 1 h 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 methyl-green (0.5%) to visualize nonimmunoreactive neurons and to aid in identifying layers within the cortex. Negative controls omitted the primary antibody and omitted the secondary antibody, resulting in a complete absence of labeled axons.

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 (e.g., Amunts et al. 1996; Zilles et al. 1996; Petrides and Pandya 1999; Rademacher et al. 2001). Cortical layers were analyzed separately as layer I, layer II, layer III, and layer 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 also tend not to be sharp or distinct; thus, sampling was limited to a representative region within the cortical area of interest.

Axon Length Density

Two to five equidistantly spaced sections per area of interest per individual were used. The variance in section number was dependent upon the number of sections available for that cortical area. The blocks of human tissue obtained from the brain bank were particularly thin and yielded only 20–30 sections in some instances. Once the area of interest was identified, the separate cortical layers (I, II, III, and V/VI) were

individually traced 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 on adjacent Nissl-stained sections and transferred to the immunostained sections.

Quantitative analyses were performed using computer-assisted stereology. This system consisted of a Zeiss Axioplan 2 photomicroscope, equipped with an Optronics MicroFire camera, a Ludl XY motorized stage, Heidenhain z axis encoder, and StereoInvestigator software version 6 (MBF Biosciences, Williston, VT). Mounted section thickness was measured at every 5th sampling location. Axon length was assessed using the SpaceBalls probe under Koehler illumination at 63× (Zeiss Plan-Achromat, 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). 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):

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

where v/a is the ratio of sampling frame volume to probe surface area, Σ 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 axon length densities were used to analyze species-specific cortical innervation patterns.

Neuron Density

Neuron density was assessed using an optical disector combined with a fractionator sampling scheme. Cortical 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 × 40 μm. 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 sectioned 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).

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 the absolute total axon length to avoid several confounding factors. First, cell density per unit volume varies with 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, PMI, 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. These confounding factors are unavoidable and beyond the control of the researchers. However, the ALv/Nv ratio acts to standardize data for differential tissue shrinkage among species as well as between individuals.

One of the defining features of layer I, the molecular layer, is an absence of neurons, precluding the calculation of a layer I ALv/Nv ratio. To circumvent this problem so that between-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 analyses of variance (ANOVAs) with repeated measures design were used to determine differences among macaques, chimpanzees, and humans. The variables were SERT-immunoreactive (SERT-ir) ALv/Nv for layers I, II, III, and V/VI. A 4 × 3 × 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 ANOVAs. Separate analyses within each species were conducted for SERT-ir axon length density to examine innervation patterns independent of neuron densities and species effects. For axon length density, a 3 × 4 (area × layer) repeated-measures ANOVA was used to analyze the pattern of differences between areas and layers within each species. Tukey's HSD post hoc tests were used to evaluate significant results.

To assess whether PMI affected the intensity of immunohistochemical staining, nonparametric Spearman's correlation coefficients were calculated for PMI and SERT-ir ALv/Nv in humans. Data on specific PMI were not available for the chimpanzee sample, and PMI was not applicable 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 SERT-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 decline in SERT-ir density. α was set at 0.05 for all statistical tests.

Methodological Concerns

A final methodological concern would be the effect of immersion (humans and chimpanzees) versus perfusion (macaques) methods of fixation on the reliability of immunohistochemistry results. 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 was 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 did 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

SERT-ir axons were present in all cortical areas and layers examined. Figures 1, 2, and 3 show SERT-ir axon tracings in each area for each species. These figures were produced by obtaining montage images of the cortical areas at 20× (Zeiss Achroplan, N.A. 0.50) and tracing individual axons using Adobe Photoshop software. Examples of SERT immunostained sections for each species are found in Figure 4. Considerable individual variation was observed; however, this variation was not correlated with PMI in humans (all P values >0.05, 2-tailed). Spearman's rho correlation coefficients were calculated to test the strength of the relationship between age and SERT ALv/Nv within each species. Of the 36 possible correlations (3 species × 3 areas × 4 layers), only 11% (4/36) were statistically significant. Two were positively correlated and 2 were negatively correlated, indicating that age was not a confounding factor in this analysis.

Previous reports on the distribution of the serotonergic input to the primate frontal cortex have been published for marmosets (*Callithrix jacchus*) (Hornung et al. 1990), long-tailed

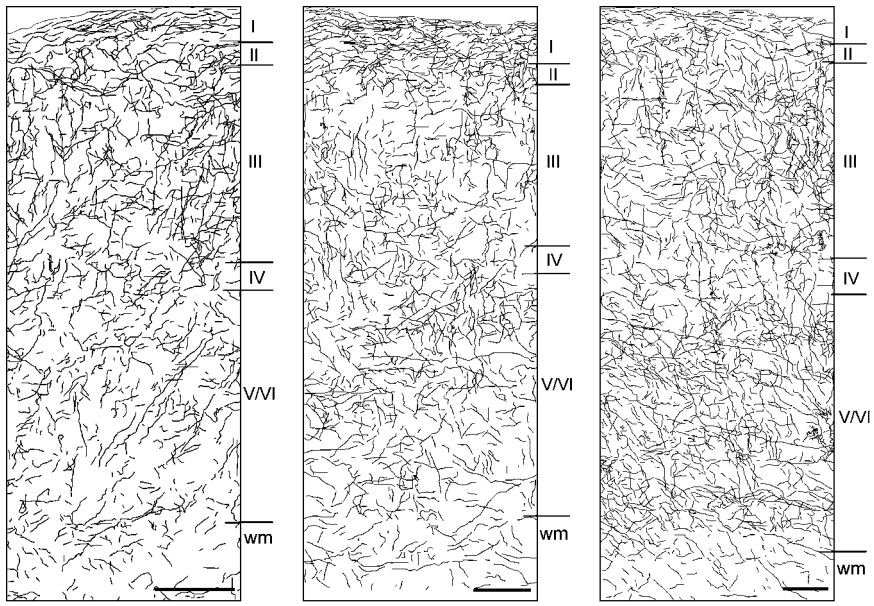

Figure 1. SERT-ir axon tracings in area 9 of macaque, chimpanzee, and human, respectively. Scale bar = 250 μm.

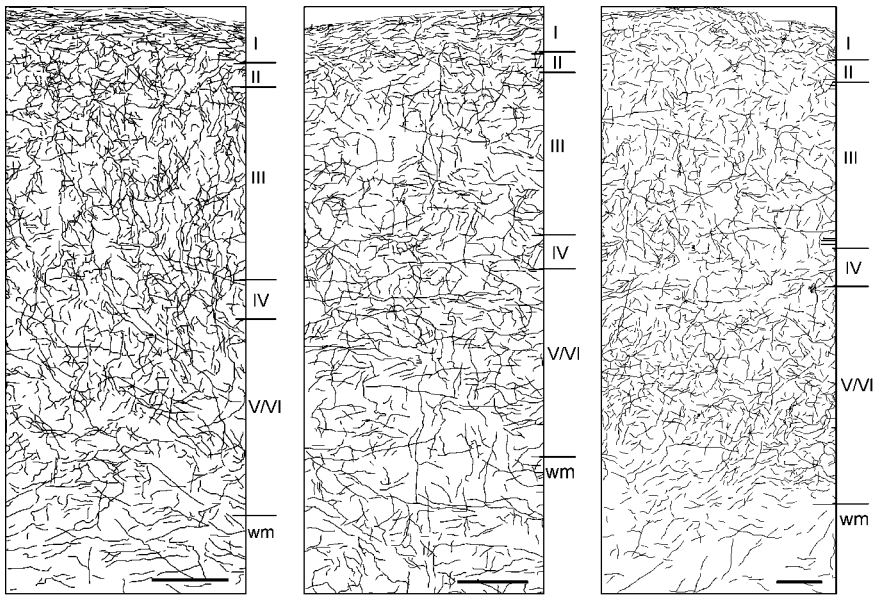

Figure 2. SERT-ir axon tracings in area 32 of macaque, chimpanzee, and human, respectively. Scale bar = 250 μm.

macaques (*Macaca fascicularis*) (Azmitia and Gannon 1986; Berger et al. 1988; Wilson and Molliver 1991), rhesus macaques (*Macaca mulatta*) (Wilson and Molliver 1991; Smiley and Goldman-Rakic 1996), and humans (Trottier et al. 1996; Varnäs et al. 2004). Consistent with these previous reports, we observed 3 distinct morphological types of axons in each species (see Fig. 4); type 1 axons were thin with small ovoid (fusiform and granular) varicosities; type 2 axons were thin with large spherical varicosities (also referred to as "beaded axons"); and type 3 axons were thick with few or no varicosities (Hornung et al. 1990; Wilson and Molliver 1991; Trottier et al. 1996). Type 1 was the main fiber type encountered and was found throughout each cortical area and species. Type 2 fibers were mostly found in the supragranular layers of all species but were also noted occasionally in infragranular layers of chimpanzees and humans. The third, and least common, fiber type was noted throughout the cortex of each species. Type 3 axons are thought to be the stem fibers for type 2 axons (Hornung et al. 1990).

In general, the density of fibers was greater in the supragranular layers relative to infragranular layers in all species and cortical areas. Fibers showed no preferred orientation in all layers except in layer I where the orientation of fibers was predominantly horizontal to the pial surface. This pattern was common to all cortical areas examined here and is consistent with other immunohistochemical studies of serotonergic innervation within primate frontal cortex (Hornung et al. 1990; Wilson and Molliver 1991; Trottier et al. 1996). It should be

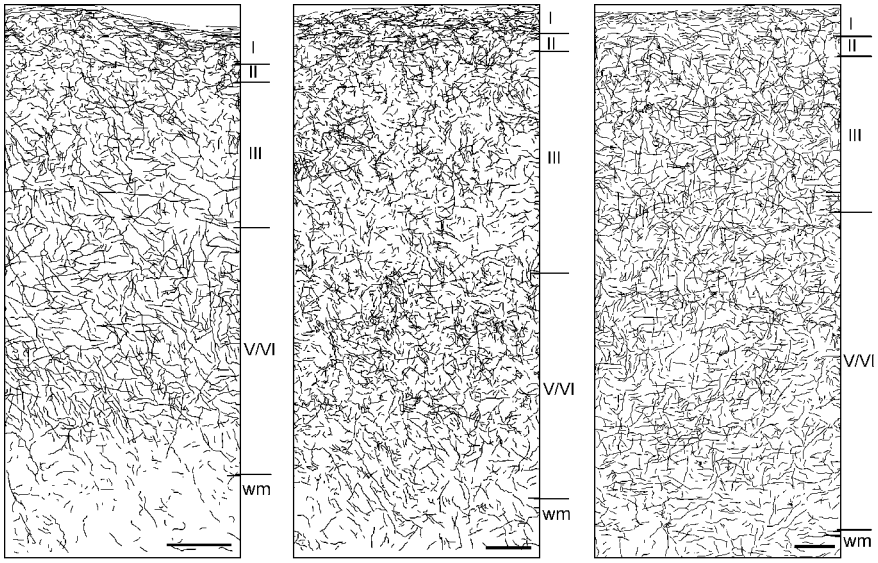

Figure 3. SERT-ir axon tracings in area 4 of macaque, chimpanzee, and human, respectively. Scale bar = 250 μm.

noted, however, that the quantitative analysis conducted in the present study did not distinguish among axon types.

Pericellular arrays (baskets) surrounding nonpyramidal interneurons formed by type 2 axons in the supragranular layers have been reported in cats (Mulligan and Törk 1987; DeFelipe et al. 1991), marmosets (Hornung et al. 1990), and macaques (Foote and Morrison 1984; Wilson et al. 1989; Smiley and Goldman-Rakic 1996). In the present study, pericellular arrays were present in the supragranular layers of all areas of macaques, although this morphological type was more common in areas 9 and 32 than in primary motor cortex (Fig. 5). A similar pattern of pericellular arrays was also found in chimpanzees, with the addition of "clusters" of type 2 axons that were occasionally observed throughout the cortex (Fig. 6). In humans, pericellular arrays were not encountered. Rather, clusters of type 2 axons were observed that were morphologically comparable with those found in chimpanzees (Fig. 7). Clusters of beaded SERT-ir axons were found throughout the human cortex, similar to the chimpanzee condition. In summary, the pericellular arrays found in the macaque and chimpanzee supragranular layers were morphologically very similar to one another and to pericellular arrays described in marmosets and cats (Mulligan and Törk 1987; Hornung et al. 1990). Humans and chimpanzees have what appear to be clusters or "coils" of type 2 axons that were found in all layers and have not previously been described.

Within-Species Analyses

For neuron density counts, an average of 102.4 ± 22.5 (mean ± standard deviation [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 SD of 0.02.

Staining for SERT was robust and full antibody penetration through the tissue sections was observed for each species. An average of 89.1 ± 16.7 (mean ± SD) sampling hemispheres was placed in each layer/individual/cortical area. A total of 19 243 sampling hemispheres were used, with 62 651 intersections counted.

Table 2 lists the mean SERT axon length density for each species/layer/area. The 4 × 3 repeated-measures ANOVA used for the analysis of SERT-ir axon length density within macaques showed a significant interaction between layer and area ($F_{6,30}$ = 3.08, P = 0.02; Fig. 8*a*) and a significant main effect of layer ($F_{3,15}$ = 71.22, P < 0.001). The main effect of area did not reach statistical significance ($F_{2,10}$ = 1.94, P = 0.20). The results of the chimpanzee analysis yielded a significant interaction ($F_{6,30}$ = 5.48, P = 0.001; Fig. 8*b*) and significant main effects of layer ($F_{3,15}$ = 54.92, P < 0.001) and area ($F_{2,10}$ = 7.40, P = 0.01). In humans, the interaction was significant ($F_{6,30}$ = 3.36, P = 0.01; Fig. 8*c*), as was the main effect of layer ($F_{3,15}$ = 51.10, P < 0.001) and area ($F_{2,10}$ = 10.50, P = 0.003). Post hoc Tukey HSD tests were used to evaluate the significant interactions for each species. Comparisons were made between layers within each cortical area (Table 3). Differences of layers between cortical regions are also reported (Table 4). The results demonstrate significant differences in the patterns of serotonergic axon length density among humans, chimpanzees, and macaques, independent of neuron densities. In macaques, layer I was more densely innervated than layers III and V/VI in all cortical areas (see Table 3). Layer II had a higher density than either layer III or V/VI in areas 32 and 4 and was higher than only layers V/VI in area 9. Like macaques, chimpanzee layer I was more densely innervated than III and V/VI in all areas (see Table 3). Additionally, layer I had a greater axon length density than layer II in area 32. Layer II exhibited higher density than layer III in areas 9 and 32 and layer V/VI in areas 32 and 4. Analysis of human data revealed a similar pattern in that layer I had a higher axon length density relative to layers III and V/VI in all cortical regions (see Table 3). In addition, layer I was more densely innervated than layer II in area 4. Finally, human layer II had a higher density than layers III and V/VI only in area 32.

The only among-area differences detected in macaques was a higher axon length density in layers I and II of area 4 relative to area 9 (see Table 4). The chimpanzee analysis demonstrated a higher density in layers II and III of area 4 compared with area 9 (see Table 4). Layer I of area 32 was more densely innervated than that of either area 9 or 32. Further, layer II of area 32 was

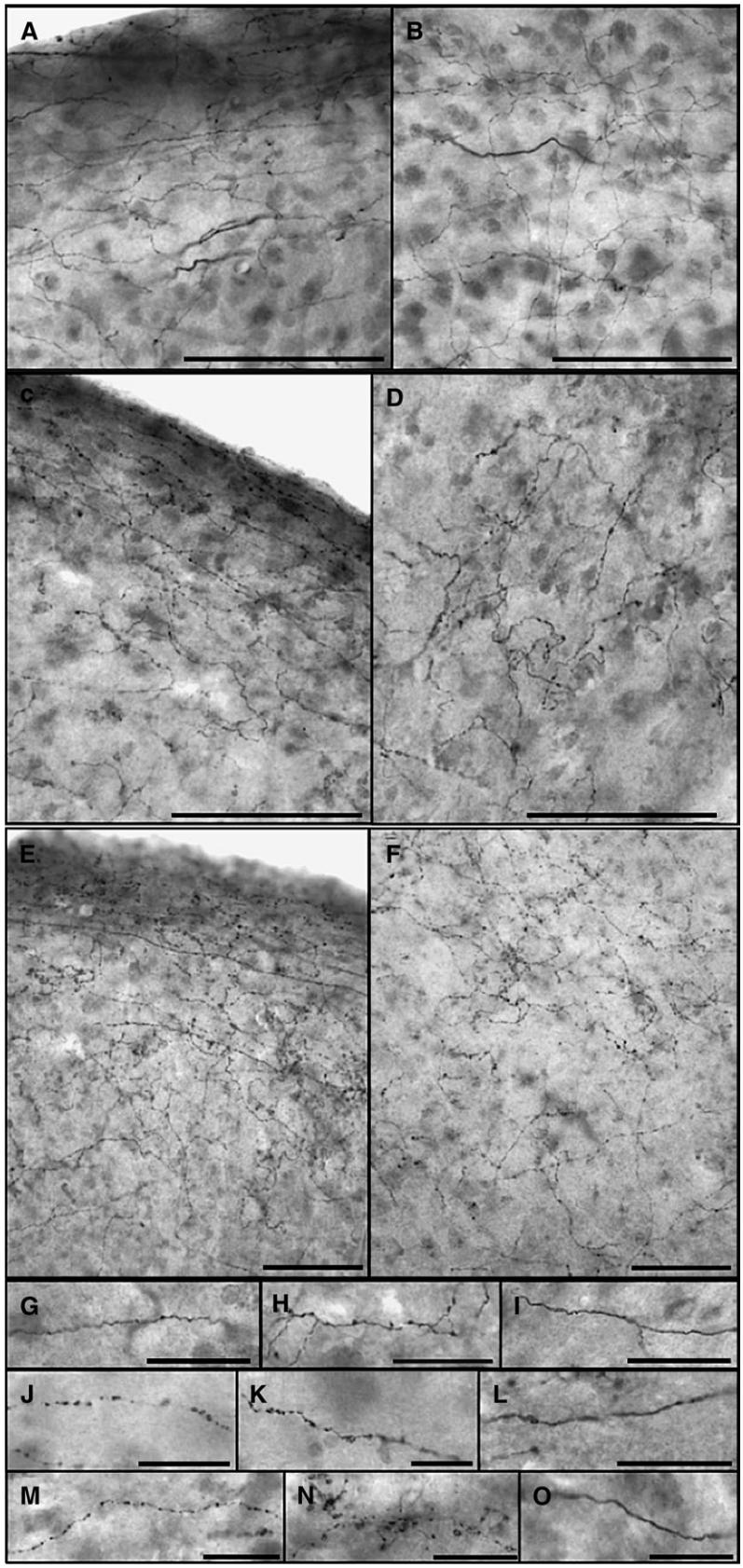

Figure 4. Brightfield photomicrographs in layers I–III and layer III, respectively, in macaque (*A*, *B*), chimpanzee (*C*, *D*), and human (*E*, *F*); scale bars = 100 μm. The lower panels show type 1, type 2, and type 3 axons, respectively, in macaque (*G*, *H*, *I*), chimpanzee (*J*, *K*, *L*), and human (*M*, *N*, *O*); scale bars = 25 μm. Background cells are stained with methyl green.

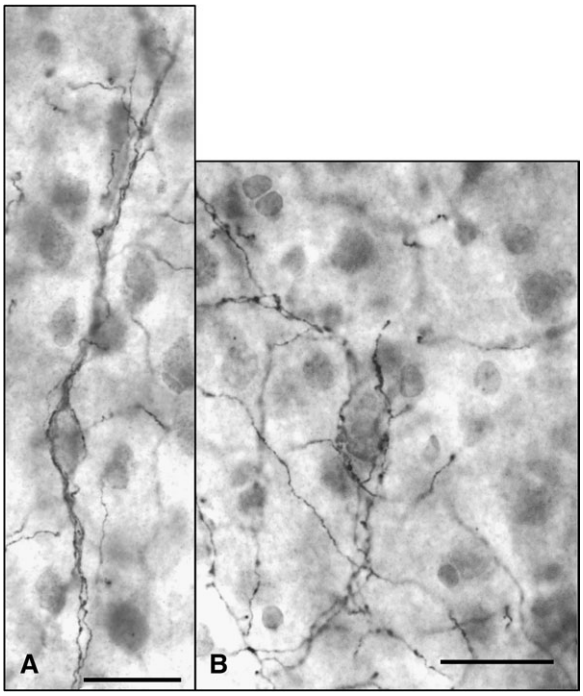

Figure 5. SERT-ir pericellular arrays in macaque. Scale bar = 25 μm. Background cells are labeled with a methyl-green counterstain.

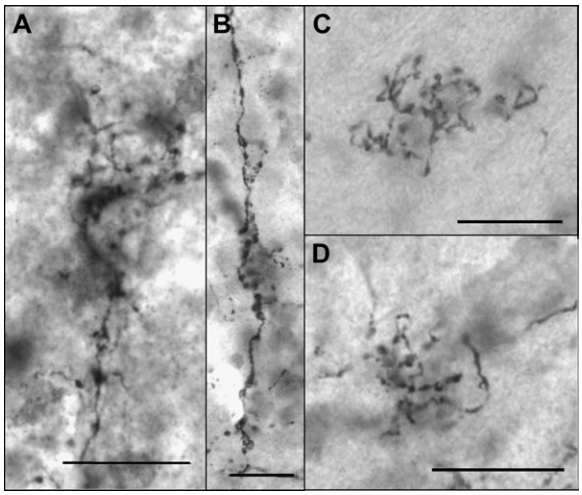

Figure 6. SERT-ir pericellular arrays (*A*, *B*) and clusters (*C*, *D*) in chimpanzee. Scale bar = 25 μm.

also significantly denser than layer II of area 9. The human analysis revealed that area 32 was altogether more densely innervated than area 9 (see Table 4), and area 4 exhibited a uniformly more dense innervation than area 9. Lastly, layer II of area 32 had a greater axon length density than that of area 4.

Among-Species Analyses

The mean SERT-ir ALv/Nv and SD for each layer and cortical area for macaques, chimpanzees, and humans are shown in Table 5. In the 4 × 3 × 3 mixed model ANOVA, the 3-way interaction of layer × area × species was significant ($F_{12,90}$ = 2.83, P = 0.002), as were the main effects of layer ($F_{3,45}$ = 11.54, P < 0.001), area ($F_{2,30}$ = 12.86, P < 0.001), and species ($F_{2,15}$ = 12.30, P < 0.001). The interactions of layer × species and layer × area were each significant ($F_{6,45}$ = 23.48, P < 0.001; $F_{6,90}$ = 7.96, P < 0.001, respectively) and the interaction between area × species was not ($F_{4,30}$ = 0.20, P = 0.94). The 3-way interaction between layer × area × species is represented in Figure 9. Post hoc Tukey HSD tests of this interaction revealed no among-species differences in direct comparisons of layer and area (all P values >0.05). However, in the 3-way interaction, the laminar pattern of serotonergic innervation appeared to be different for the macaques than for the humans and chimpanzees (see Fig. 9). For this reason, a separate 4 × 3 × 2 mixed model ANOVA was conducted collapsing humans and chimpanzees into a group referred to as Homininae (used to refer to the clade that includes only humans, common chimpanzees, bonobos, and their fossil ancestors) that was then compared with the macaques. In this analysis, the significant interactions included layer × species ($F_{3,48}$ = 31.55, P < 0.001) and layer × area ($F_{6,96}$ = 4.97, P < 0.001). The area × species interaction was neither significant ($F_{2,32}$ = 0.01, P = 0.99) nor the 3-way interaction between layer, area, and species ($F_{6,96}$ = 1.93, P = 0.08). The main effects were as follows: layer ($F_{3,48}$ = 7.23, P < 0.001); area ($F_{2,32}$ = 11.98, P < 0.001); and species ($F_{1,16}$ = 0.56, P = 0.46). The layer × area interaction was expected, as regional and laminar heterogeneity of 5HT innervation is well documented in primates (Morrison and Foote 1986; e.g., Wilson and Molliver 1991; Trottier et al. 1996). Of special note was the layer × species interaction (Fig. 10). When broken down by area (Fig. 11), the main difference between the hominines and macaques exists in

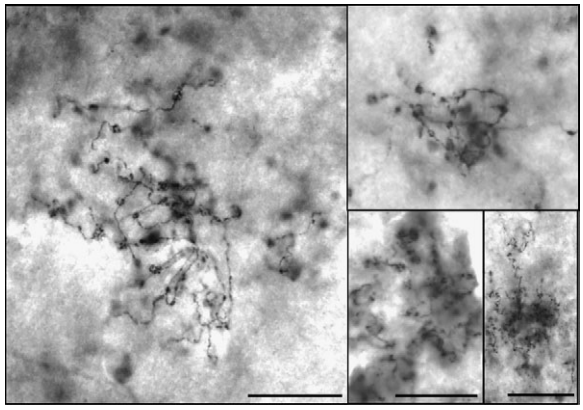

Figure 7. SERT-ir clusters in human. Scale bar = 25 μm.

Table 2
SERT-ir axon length densities (μm/μm^3) for each genus, area, and layer

Species	Layers	Area 9	Area 32	Area 4	*N*
Macaca maura	I	0.098 ± 0.03	0.123 ± 0.04	0.142 ± 0.06	6
	II	0.083 ± 0.02	0.114 ± 0.03	0.135 ± 0.06	
	III	0.055 ± 0.02	0.065 ± 0.01	0.061 ± 0.03	
	V/VI	0.030 ± 0.01	0.040 ± 0.01	0.047 ± 0.03	
Pan troglodytes	I	0.065 ± 0.02	0.100 ± 0.02	0.081 ± 0.02	6
	II	0.051 ± 0.01	0.071 ± 0.01	0.069 ± 0.01	
	III	0.029 ± 0.004	0.044 ± 0.01	0.052 ± 0.01	
	V/VI	0.045 ± 0.01	0.046 ± 0.01	0.051 ± 0.02	
Homo sapiens	I	0.045 ± 0.01	0.076 ± 0.01	0.070 ± 0.02	6
	II	0.036 ± 0.01	0.067 ± 0.01	0.053 ± 0.01	
	III	0.026 ± 0.003	0.042 ± 0.01	0.041 ± 0.01	
	V/VI	0.030 ± 0.01	0.046 ± 0.004	0.044 ± 0.01	

Note: The numbers reported are the mean ± SD.

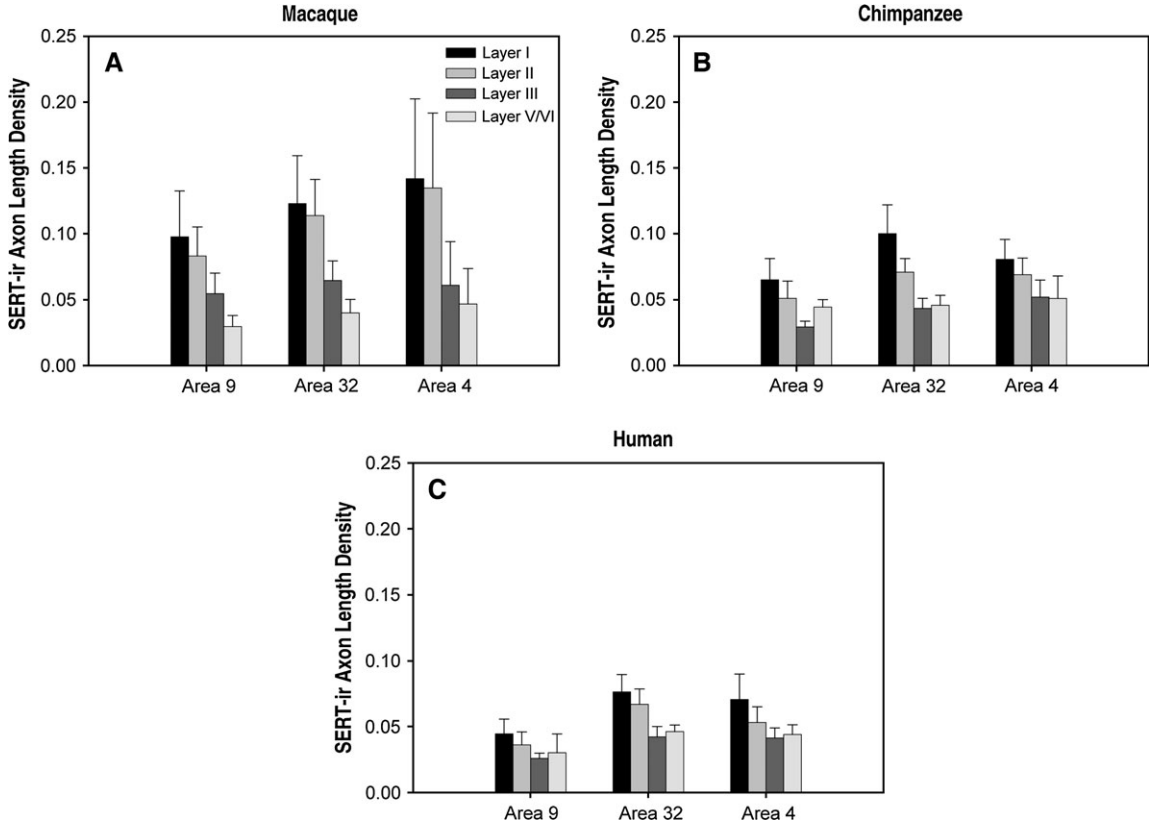

Figure 8. Mean SERT-ir axon length density for (*A*) macaques, (*B*) chimpanzees, and (*C*) humans, error bars represent SDs.

Table 3
Probabilities for Tukey HSD post hoc tests of SERT-ir axon length density

Species	Layers	Area 9	Area 32	Area 4
Macaca maura	I–II	0.87	1.00	1.00
	I–III	0.00* (I)	0.00* (I)	0.00* (I)
	I–V/VI	0.00* (I)	0.00* (I)	0.00* (I)
	II–III	0.09	0.00* (II)	0.00* (II)
	II–V/VI	0.00* (II)	0.00* (II)	0.00* (II)
	III–V/VI	0.21	0.23	0.88
Pan troglodytes	I–II	0.20	0.00* (I)	0.47
	I–III	0.00* (I)	0.00* (I)	0.00* (I)
	I–V/VI	0.01* (I)	0.00* (I)	0.00* (I)
	II–III	0.01* (II)	0.00* (II)	0.06
	II–V/VI	0.97	0.00* (II)	0.04* (II)
	III–V/VI	0.14	1.00	1.00
Homo sapiens	I–II	0.52	0.33	0.00* (I)
	I–III	0.00* (I)	0.00* (I)	0.00* (I)
	I–V/VI	0.02* (I)	0.00* (I)	0.00* (I)
	II–III	0.23	0.00* (II)	0.11
	II–V/VI	0.89	0.00* (II)	0.39
	III–V/VI	0.99	0.99	1.00

Note: Comparisons made between layers within each cortical area for each species.
*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 SERT-ir axon length density

Species	Layer	Area 9–Area 32	Area 9–Area 4	Area 32–Area 4
Macaca maura	I	0.19	0.00* (4)	0.58
	II	0.05	0.00* (4)	0.43
	III	0.99	1.00	1.00
	V/VI	0.99	0.72	1.00
Pan troglodytes	I	0.00* (32)	0.12	0.02* (32)
	II	0.01* (32)	0.04* (4)	1.00
	III	0.21	0.00* (4)	0.84
	V/VI	1.00	0.97	0.99
Homo sapiens	I	0.00* (32)	0.00* (4)	0.91
	II	0.00* (32)	0.00* (4)	0.04* (32)
	III	0.01* (32)	0.01* (4)	1.00
	V/VI	0.01* (32)	0.03* (4)	1.00

Note: 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.

layers V/VI of areas 9 and 32, wherein hominines have a higher ALv/Nv ratio than do macaques, with no overlap between the 2 groups.

Discussion

The present study represents the first quantitative comparative analysis of cortical serotonergic innervation between humans and other primate species and is the first report of 5HT in chimpanzee neocortex.

There exists a duality of serotonergic innervation in the mammalian cerebral cortex, with type 1 axons arising from cells within the dorsal raphe and type 2 (and, presumably, type 3) axons originating from cells within the median raphe (e.g., Kosofsky and Molliver 1987; Mulligan and Törk 1988; Hornung et al. 1990; Wilson and Molliver 1991; Trottier et al. 1996; Miner et al. 2000). This duality of innervation and the variations observed in the local patterns of cortical serotonergic afferents suggest that the separate classes of axons affect different types of cortical neurons, thereby selectively affecting specific elements of cortical networks (e.g., Hornung et al. 1990; Wilson

Table 5
Mean and SD for SERT ALv/Nv in each layer and cortical area for macaques, chimpanzees, and humans

Species	Layers	Area 9	Area 32	Area 4	*N*
Macaca maura	I	750.61 ± 227.38	1121.04 ± 296.97	1043.33 ± 502.53	6
	II	647.28 ± 167.23	1040.16 ± 205.46	1002.40 ± 489.98	
	III	602.14 ± 127.04	837.90 ± 167.70	855.17 ± 509.33	
	V/VI	336.15 ± 105.14	594.74 ± 139.41	817.79 ± 478.44	
Pan troglodytes	I	936.21 ± 267.55	1524.49 ± 452.80	1131.19 ± 128.07	6
	II	728.73 ± 186.55	1083.04 ± 252.65	978.01 ± 158.96	
	III	605.23 ± 92.86	1024.30 ± 203.90	1341.66 ± 274.25	
	V/VI	1019.19 ± 82.98	1186.35 ± 394.57	1446.48 ± 399.00	
Homo sapiens	I	486.28 ± 96.53	748.92 ± 79.59	681.94 ± 288.09	6
	II	394.40 ± 84.16	659.00 ± 97.36	510.34 ± 176.70	
	III	529.56 ± 66.52	767.31 ± 76.46	832.99 ± 168.85	
	V/VI	666.25 ± 256.19	935.84 ± 111.84	1055.70 ± 267.94	

and Molliver 1991). The additional evidence of the role of 5HT in higher cognitive functions and their susceptibility in neuropathologies that afflict humans suggest that serotonergic cortical systems may have been modified in the evolution of human cognitive specializations (e.g., Steckler and Sahgal 1995; Azmitia 1999; Naughton et al. 2000; Vergé and Calas 2000; Williams et al. 2002; Roth et al. 2004).

The goal of the present comparative study was to assess whether the human cerebral cortex receives a relatively denser contingent of serotonergic input relative to other primates, potentially underlying unique human cognitive specializations. To examine this hypothesis, we conducted a rigorous quantitative analysis of SERT-ir fibers in cortical areas 9, 32, and 4 among humans, chimpanzees, and macaques. Although our results did not reveal overall denser or more extensive cortical 5HT input in humans relative to the other species, we found important phylogenetic variation that may have functional implications for cognitive processing.

The within-species comparisons of axon length density revealed variations in innervation patterns among the 3 species analyzed, independent of neuron densities. This analysis found similar laminar patterns within cortical areas among the 3 species. In addition, the pattern of innervation across all areas among species was similar, with area 4 having the densest innervation, followed by area 32. Area 9 received the lowest number of serotonergic afferents in all layers (see Fig. 8). For humans, there was a relatively stronger serotonergic input to area 32. Chimpanzees were similar to humans in this emphasis, but to a lesser extent.

Although our initial statistical analysis among humans, chimpanzees, and macaques did not detect species differences in SERT-ir ALv/Nv, it did reveal a difference in innervation pattern between the macaques and the hominines (i.e., chimpanzees and humans) in the infragranular layers. By employing a subsequent analysis wherein chimpanzees and humans were grouped together and compared with macaques, we found that, collectively, humans and chimpanzees have a greater SERT ALv/Nv in layers V/VI of areas 9 and 32. Hornung et al. (1990) found that the only consistent difference between earlier macaque studies and their analysis of the New World monkey marmoset cerebral cortex was a weaker 5HT innervation of the infragranular layers in marmosets. Although a single species should not be assumed to be representative of a broader phylogenetic clade, it is tempting to speculate that changes in cortical 5HT innervation might have occurred since New World monkeys and catarrhines (i.e., Old World monkeys and apes) split from a common ancestor approximately 40 million years ago. Such generalizations, however, should be taken as preliminary until more species are examined. Nonetheless, if the few species studied to date do indeed represent their taxonomic groups, this trend is suggestive of a phylogenetic shift within the order Primates, with increasing amounts of serotonergic input to infragranular layers of prefrontal cortical areas that may have occurred at the origin of catarrhines, and then again at some point since the branch of Old World monkeys and apes. Taking a broader phylogenetic view, it is interesting that DeFelipe et al. (1991) noted that infragranular layers were sparsely innervated relative to layers I–IV in cats. Although carnivorans are only very distantly related to primates, these findings raise the possibility that primates differ from other mammals in displaying greater serotonergic innervation of infragranular layers. However, it should be noted that these differences neither were the result of a direct comparative study nor are the cortical areas compared necessarily homologous. To explore more fully evolutionary changes in cortical serotonergic systems, future studies should incorporate standard methodologies to the examination of a greater diversity of primates, including strepsirrhines (i.e., lemurs, lorises, and galagos), as well as other mammalian species.

Collectively, layers V and VI receive input from supragranular layers and provide output to other cortical regions, subcortical structures, brain stem, and spinal cord (Haines 1997). The increase in 5HT afferents exclusively to the infragranular layers of areas 9 and 32 in chimpanzees and humans indicates a specialization of specific cortical output functions. This is particularly interesting in light of the findings by Austin et al. (2002) in human depressed subjects committing suicide wherein SERT-ir fibers were decreased significantly in layer VI of prefrontal cortical area 46. Their analysis did not include layer V; however, their findings further indicate that localized and layer-specific alteration in cortical serotonergic transmission in humans may contribute to the working memory deficits that are characteristic of major depression (Pelosi et al. 2000). Together with our findings, the denser innervation found in layers V/VI and the exclusive sensitivity of layer VI to neuropathology in humans indicate that the increase of serotonergic afferents to the infragranular layers is functionally significant.

The increase in serotonergic input demonstrated in humans and chimpanzees may contribute to cognitive specializations shared by apes, but absent in other primates. For example, the increased innervation in area 9 may support human language and the increased capacity for learning symbolic language systems in the laboratory in the genus *Pan* (e.g., Gardner RA and Gardner BT 1969; Savage-Rumbaugh et al. 1980; Rivas 2005). Comparable language acquisition skills have not been documented in macaques or other monkey species. In addition, the increased afferents in area 32 may function in support of TOM attributes. Humans and chimpanzees share several behavioral capacities related to TOM, such as the ability for deception, gaze following, reconciliation, cooperative hunting, and tool use (Heyes 1998; Suddendorf and Whiten 2001; Povinelli and Bering 2002; Keller 2004). Whereas gaze following has recently been documented in macaques (Flombaum and Santos 2005), their TOM capacities do not appear to be as developed as in chimpanzees. Chimpanzees consistently exhibit mirror self-recognition, a behavior that is not observed in macaques (Povinelli and Preuss 1995). Additionally, macaques fail, but chimpanzees excel at, a test designed to assess the ability to

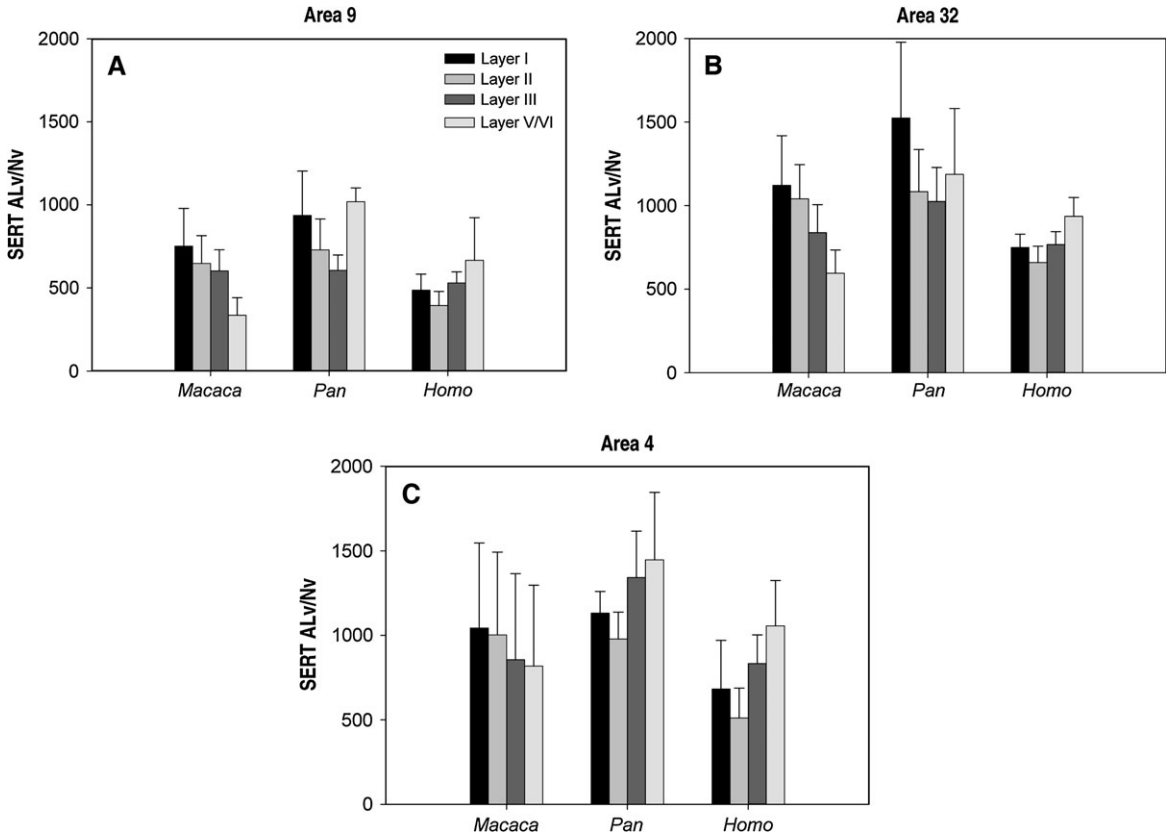

Figure 9. SERT-ir ALv/Nv in each layer of (*A*) area 9, (*B*) area 32, and (*C*) area 4. Error bars represent SDs.

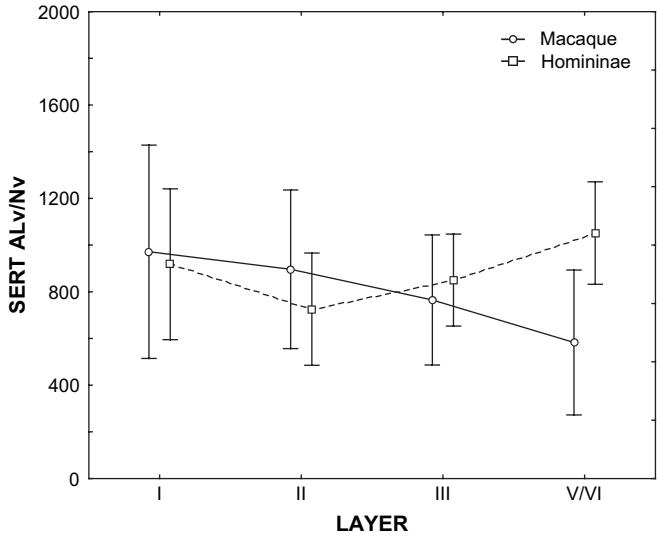

Figure 10. SERT-ir ALv/Nv 2-way interaction between the macaques and the Hominini. Error bars represent SDs.

infer goals and desires of others in a cooperative role-reversal task (Povinelli and Preuss 1995).

We identified pericellular arrays in the supragranular layers of both macaques and chimpanzees, but not in humans. Pericellular arrays, or baskets, have been described in cats (Mulligan and Törk 1987, 1988), marmosets (Hornung et al. 1990), and rhesus and long-tailed macaques (Foote and Morrison 1984; Wilson et al. 1989; Smiley and Goldman-Rakic 1996). Earlier reports indicated that the pericellular arrays were less elaborate in macaques compared with those found in cat and marmoset (Hornung et al. 1990). However, the pericellular arrays observed in this study appear comparable with those found in the literature on cats (Mulligan and Törk 1987) and marmosets (Hornung et al. 1990). In marmosets, pericellular baskets were most common in the frontal, anterior parietal, cingulate, and superior temporal cortices and in the hippocampus (Hornung et al. 1990). In cats, pericellular baskets expressed γ-aminobutyric acid (DeFelipe et al. 1991) and were mostly found in the suprasylvian, auditory, and entorhinal cortices as well as in the hippocampus (Mulligan and Törk 1987). Available evidence indicates that these baskets in the cerebral cortex surround calbindin-containing inhibitory interneurons (Hornung et al. 1990; DeFelipe et al. 1991; Hornung and Celio 1992). The methyl-green counterstain used in the present study did not allow for unambiguous identification of cell types that were contained within the serotonergic baskets. However, the cells appeared oval or ovoid in shape when the morphology of the cell was apparent, indicative of inhibitory interneurons (DeFelipe 2002).

An additional morphological feature of SERT-ir type 2 fibers was found in chimpanzees and humans, but not in macaques. These features were morphologically similar to coils described for tyrosine hydroxylase–immunoreactive axons in humans (Gaspar et al. 1989; Benavides-Piccione and DeFelipe 2003) and clusters described for choline acetyltransferase–containing fibers, also in humans (Mesulam et al. 1992). Coils and clusters have not been described in other primate or nonprimate species for any of these neuromodulators (5HT, dopamine, and acetylcholine). Mesulam et al. (1992) speculated that the occurrence

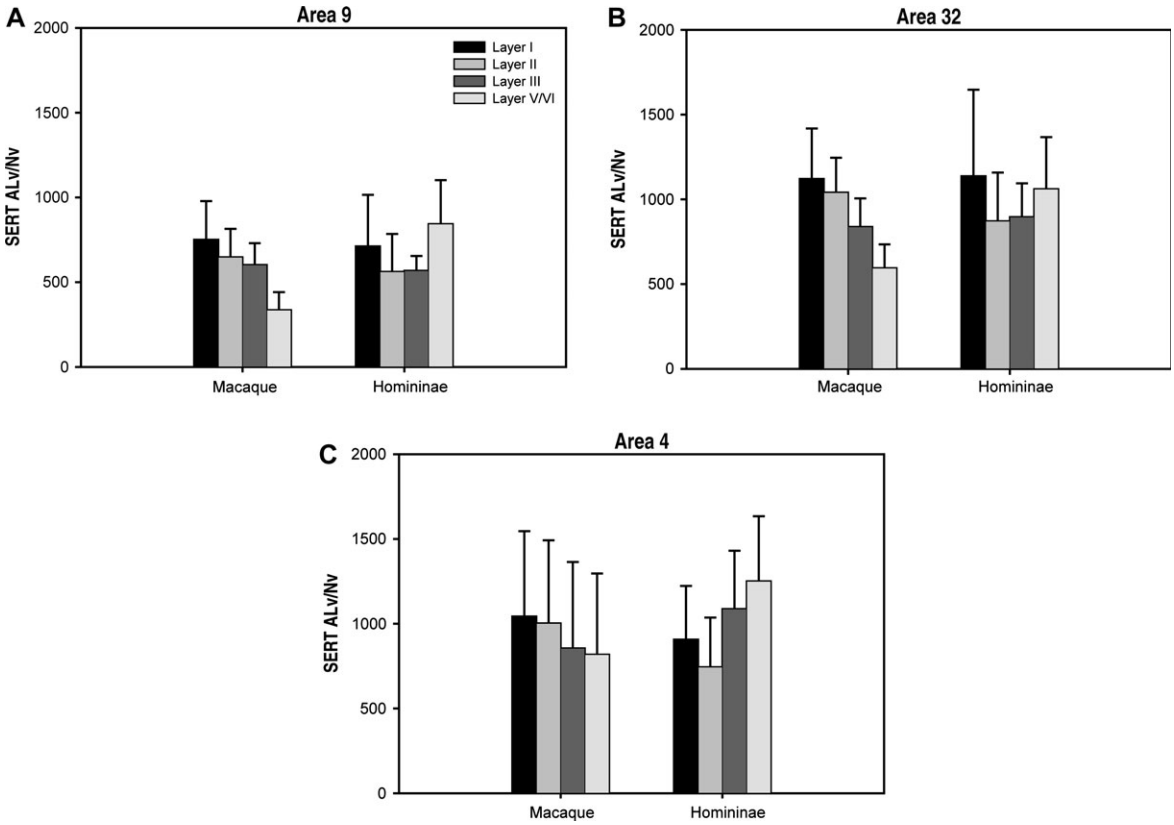

Figure 11. SERT-ir ALv/Nv between macaques and the Homininae in (*A*) area 9, (*B*) area 32, and (*C*) area 4. Error bars represent SDs.

of this type of morphology may represent local events of plasticity or circuitry rearrangement. This possibility is intriguing and their distribution in humans and chimpanzees to the exclusion of all other mammals studied to date suggests the potential evolution of a greater capacity for cortical plasticity in these species. This may represent a neural substrate that supports a greater capacity for learning and behavioral flexibility in great apes and humans.

Interestingly, the morphological specializations observed in all species—pericellular baskets in macaques and chimpanzees and coils/clusters in chimpanzees and humans—are formed by type 2 serotonergic axons that originate from the median raphe nuclei. There is evidence of an increase in serotonergic cell body number in the median raphe of cats and primates relative to rodents (Jacobs et al. 1984; Azmitia and Gannon 1986). The putative increase in median raphe cell number may be correlated with the incidence of pericellular baskets, as this feature has not been detected in rodents (e.g., Audet et al. 1989). A comparative study among mammals would elucidate whether this is a phylogenetic increase in 5HT cell bodies within the median raphe and whether this increase scales with brain or neocortex size. Due to the formation of distinctive morphological features (i.e., pericellular arrays), it may be suggested that the type 2 axons support a specialization in cortical inhibitory circuits, although Smiley and Goldman-Rakic (1996) did not detect differences in synaptic morphologies between type 1 and type 2 axons. Finally, future studies of coils/clusters in chimpanzees and humans are necessary to determine if these features surround or are associated with inhibitory interneurons.

The present research makes a significant contribution to our understanding of the structure of the human cerebral cortex in comparison with other primate species. Although there is a paucity of comparative data concerning cortical organization, recent work in this field has yielded promising results. For example, a unique neuronal subtype (spindle cells) is found in great apes and humans in a restricted set of cortical areas involved in emotional and cognitive processes (Nimchinsky et al. 1999). These spindle-shaped neurons (or Von Economo neurons) are projection neurons that are enriched with dopamine D3 receptors and 5HT 2b receptors (Allman et al. 2005). In light of their localization and biochemical phenotype, spindle cells may play a critical role in mediating intuitive processes, a key component of TOM (Allman et al. 2005). Interestingly, spindle cells have recently also been found in humpback whales and other large-brained cetaceans (Hof and Van der Gucht 2007).

In addition, other species differences in cortical organization have been described among primates at the microanatomical and molecular level. Histologically, humans differ from other apes in displaying a distinctive alteration of dendrites and interneurons in layer IVA of primary visual cortex (Preuss and Coleman 2002). Humans might also be distinguished from other primates in having increased population-level asymmetry of neuropil across several cortical areas, including area Tpt and the primary motor cortex representation of the hand (Buxhoeveden et al. 2001; Sherwood et al. 2007). Other studies have identified potential species differences in the functional biochemistry of the cortex using genomic and molecular approaches. For instance, several genes, including a subset of dopamine and acetylcholine receptor genes, demonstrate rapid evolution in protein-coding sequence domains in primates relative to rodents (Dorus et al. 2004). In addition, a novel copy of glutamate dehydrogenase that is expressed by astrocytes,

GLUD2, evolved in the great ape and human lineage (Burki and Kaessmann 2004), suggesting a role for the enhancement of cognitive functions by regulating glutamatergic transmission. Such adaptations for increased neuronal activity and energy production might have been further refined in humans through the evolution and upregulation of genes involved in the aerobic metabolic pathway (Cáceres et al. 2003; Uddin et al. 2004) and the proliferation of glial cells (Sherwood et al. 2006). In sum, although comparative studies are relatively few in number, they have the potential to contribute to our understanding of the neuroanatomical substrates that underlie species differences in functional capacities.

Conclusions

The present study represents the first comparative analysis of cortical serotonergic innervation between humans and nonhuman primates. These data are critical to our understanding of the specific roles that 5HT plays in cortical functions and how these systems may have evolved within the primate order. Contrary to our prediction that humans would have an expanded and denser serotonergic innervation in cortical areas relevant to higher cognitive functions, our findings indicate that the evolution of the human brain and its functions did not require a greater overall contingent of serotonergic afferents. Nonetheless, our analysis revealed intriguing species differences that may represent the evolution of specific cortical functions in apes. Humans and chimpanzees have a denser SERT ALv/Nv than macaques, exclusive to the infragranular layers of cognitive cortical areas, suggestive of a specific shift in the role that 5HT plays in shaping cortical output. Additionally, humans and chimpanzees display a unique morphological axon feature (clusters/coils) that may be relevant to plasticity.

The inclusion of chimpanzees in the present study was instrumental in elucidating more precisely the evolutionary history of changes in cortical serotonergic innervation that have led to the human condition. Traditionally, only a few model species are analyzed to infer the functional significance of physiological features. Oftentimes in neuroanatomy, differences between humans and monkeys are interpreted to represent significant changes that occurred only in the recent terminal lineage of humans. Such differences, however, may have arisen at any point since humans and monkeys last shared a common ancestor. In this context, the results of the current study highlight the importance of including species not traditionally considered in neuroanatomical studies, such as the great apes, to determine more specifically the characteristics that make humans distinctive versus those that represent shared similarities with our close cousins.

In summary, an evolutionary event appears to have taken place at some point after the Old World monkey–hominoid split approximately 25 million years ago that affected the anatomical position of 5HT-containing axons in the cortex that might have implications for cognitive functions, specifically in cortical output and possibly in cortical plasticity. Future studies analyzing the occurrence of coils/clusters in other hominoids, colocalization with other cell markers, and distribution within the cerebral cortex and subcortical structures will help in identifying the specific functions of this morphological feature.

Notes

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