Gender differences in human cortical synaptic density

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Certain cognitive functions differ in men and women, although the anatomical and functional substrates underlying these differences remain unknown. Because neocortical activity is directly related with higher brain function, numerous studies have focused on the cerebral cortex when searching for possible structural correlates of cognitive gender differences. However, there are no studies on possible gender differences at the synaptic level. In the present work we have used stereological and correlative light and electron microscopy to show that men have a significantly higher synaptic density than women in all cortical layers of the temporal neocortex. These differences may represent a microanatomical substrate contributing to the functional gender differences in brain activity.

electron microscopy | neocortex | neuronal density | sex

It is well known that men and women display different capacities in certain cognitive functions that are unrelated to differences in the general level of intelligence. The most consistently reported differences relate to spatial and language abilities, and whereas men excel in mental rotation and spatial perception, women perform better in verbal memory tasks, in verbal fluency tasks, and in the speed of articulation (1, 2). These differences are not thought to be a only consequence of the influence of sex hormones on brain organization during development but also of genetic factors (3–5). Because higher brain functions are related to the activity of the neocortex, many studies aimed at identifying possible structural correlations for cognitive gender differences have focused on the cerebral cortex, using a variety of anatomical and brain imaging techniques. At the macroscopic level, sexual dimorphism has been reported in the cortical volume of the Wernicke and Broca areas (6), as well as in the frontal and medial paralimbic cortices (7–10), and in the thickness and density of the gray matter in the parietal lobes (for a review see ref. 10). At the microscopic level, differences have been reported in the density of neurons (11–14) and in the complexity of the dendritic arbors as well as in the density of dendritic spines in several cortical areas (15). Nevertheless, the functional significance of these differences remains unknown because no generally valid equation relates neuronal number or morphology to behavioral complexity (13, 15). For example, the intelligence of humans with brains weighing as little as half the average and with no evidence of any compensatory increase in neuron density may be normal or even above the mean.

Understanding how neuronal circuits contribute to the functional organization of the cerebral cortex requires a detailed ultrastructural analysis of neuronal connectivity. However, the difficulties encountered when attempting to apply microanatomical techniques to study the human brain explain why most studies on the structure of the neocortex have been performed at the light microscopic level. The most important problems when performing ultrastructural studies are related to the lack of suitable human brain tissue to study synaptic circuitry, for which the only source of control tissue might be autopsy material (i.e., from individuals that did not suffer brain pathologies or psychiatric illness). Unfortunately, the ultrastructural preservation of postmortem human brain tissue is usually rather poor, and it is generally unsuitable for the detailed quantitative analysis that can be performed on biopsy material. Indeed, this is one of the main reasons for the paucity of data regarding the synaptic circuitry in the normal human brain. Thus, the key question as to whether cortical synaptic circuits differ between men and women remains unsolved.

The purpose of this study was to analyze the possible gender differences in synaptic density, for which we have taken advantage of biopsy material obtained during neurosurgical treatment for epilepsy. This resected tissue represents an excellent opportunity to study the human brain at the electron microscope level, in part because the resected tissue can be immediately immersed in the fixative. Undoubtedly, this is why the quality of the immunocytochemical staining at both the light and electron microscopy levels in human biopsy material has been shown to be comparable to that obtained in experimental animals (e.g., ref. 16). Hence, using correlative light and electron microscopy coupled to stereological techniques, we show that there is significant sexual dimorphism in the density of synapses in all cortical layers of the temporal neocortex. These differences may represent a microanatomical substrate that contributes to gender functional differences in brain activity.

Results

Light Microscopy Analysis. We analyzed the thickness and neuronal density in layers I, II, IIIA, IIIB, IV, V, and VI of 100-μm Nissl-stained sections from the tissue obtained. No significant differences were found between men and women regarding the neuronal density (Table 1 and Fig. 1*A*) as previously reported in the temporal neocortex (13). However, other reports have shown greater neuronal density in the posterior temporal neocortex of women when compared with men (12). The discrepancy between the study of Pakkenberg and Gundersen (13) and our present results when compared with those obtained by Witelson *et al.* (12) may be attributed to the cytoarchitectonic differences of the regions examined. Indeed, we examined the anterior part of the middle temporal gyrus, corresponding to area 21 of Brodmann [supporting information (SI) Fig. S1] (17), whereas they analyzed the superficial surface of the posterior part of the superior temporal gyrus, also denominated the TA1 area (18) or area 22 by Brodmann (17).

Furthermore, the cell body (glia and neurons), neuropil, and blood vessel volume fractions (V_v) were also examined in each of these cortical layers in 2-μm-thick semithin sections stained with toluidine blue (Fig. S2). Again, no significant differences were found in any of the parameters examined (Fig. 1*B*). In summary, no cytoarchitectonic differences could be observed in the tissue obtained from men and women.

Ultrastructural Analysis. We have studied the morphology and density of synapses in each cortical layer, and the ultrastructure

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Table 1. Number of neurons per cubic millimeter (mean ± SEM) and the percentage of synaptic junctions per layer

	Neuronal density		Percentage of synapses			
			Women		Men	
Layer	Women	Men	% AS	% SS	% AS	% SS
I	11,558 ± 1,200	11,034 ± 850	76	24	72	28
II	54,915 ± 3,200	49,127 ± 2,900	80	20	83	17
IIIa	18,112 ± 510	17,279 ± 560	85	15	92	8
IIIb	15,869 ± 430	15,907 ± 220	84	16	86	14
IV	49,754 ± 3,200	47,758 ± 970	86	14	89	11
V	26,393 ± 1,200	24,070 ± 830	88	12	92	8
VI	16,520 ± 430	16,291 ± 380	88	12	91	9

AS, asymmetric synapses; SS, symmetric synapses.

of the neuropil was indistinguishable in women from that in men (Fig. 2 *A* and *B* and Fig. S3 *a* and *b*). The types of synaptic junctions were classified into three categories: asymmetric, symmetric, and uncharacterized (Tables 1 and 2). In the first two types, the synaptic cleft could be visualized and synapses were identified based on the morphology of the postsynaptic density. Thus, asymmetric synapses had a prominent postsynaptic density whereas symmetric synapses had a thin postsynaptic density (Fig. 2 *C* and *D* and Fig. S3 *a* and *b*) (19–21). In the uncharacterized synapses, the synaptic cleft could not be visualized because of the oblique plane of section. In this study, uncharacterized synapses were included in the final estimate of the total synaptic density. Furthermore, uncharacterized synapses in Fig. 1 were included as asymmetric and symmetric types according to the frequency of both types of synapses. Therefore, the proportion of each type of synapse in this work is an estimate of the real ratio (see ref. 22).

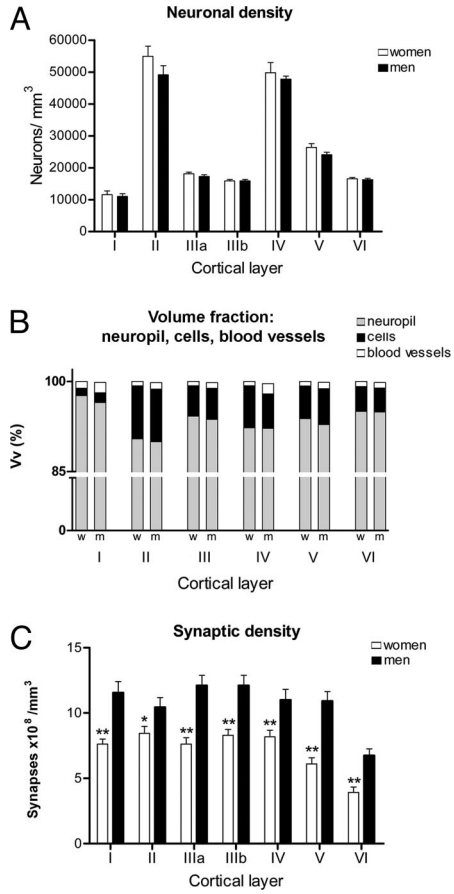

Fig. 1. Graphs of the neuronal densities, volume fraction (V_v), synaptic densities, and synapses per neurons in men and women in each cortical layer. (*A*) Graph showing the neuronal densities (mean ± SEM) in each cortical layer demonstrating that there are no significant differences between men and women. (*B*) Comparison of the V_v between men and women, calculated for the neuropil, cell bodies (including those from glia and neurons), and blood vessels in each cortical layer. Note that the neuropil represents between 90% and 98% of the volume, for which no significant differences were found between men and women. (*C*) Graph showing a comparison of synaptic density (mean ± SEM) between men and women in each cortical layer. w, women; m, men. *, $P < 0.05$; **, $P < 0.01$.

When the mean cross-sectional lengths of asymmetric, symmetric, and uncharacterized synapses were analyzed, no significant differences were found between men and women in any layer (Table 2).

Synapses were quantified in the neuropil (i.e., avoiding the neuronal and glial somata, blood vessels, large dendrites, and myelinated axons) (23), and we found men to have a higher synaptic density in all layers (Fig. 1*C*). The smallest difference in density was found in layer II, in which the synaptic density was 18% higher in men than in women (Fig. 1*C*), whereas the greatest difference was found in layer V, where the synaptic density in men was 52% higher than in women (678 million synapses per cubic millimeter plus). Considering all layers, men also have a significant higher average synaptic density of 12.9×10^8 per cubic millimeter, whereas in women it was 8.6×10^8 per cubic millimeter. Thus, there was a 33% difference in synaptic density between men and women.

Nevertheless, the proportion of asymmetric and symmetric synapses when considering all layers together (Tables 1 and 2) was similar in men and in women, 86% and 14% in men and 84% and 16% in women, respectively.

Discussion

The most striking finding from the present study is that despite the well known anatomical and functional interindividual variability in the brain (e.g., refs. 24 and 25), we consistently observed a lower synaptic density in women in all cortical layers of the temporal neocortex. Because we examined only relatively few cases (four women and four men), we consider that these differences must therefore be very robust in the general population.

Nevertheless, we would caution the reader that the main limitation in this kind of study is that we have virtually no data about the synaptic density in biopsy samples of the strictly normal human neocortex. Indeed, it is well known that synaptic reorganization occurs in the epileptic brain, although these changes occur in regions with neuronal loss and gliosis such as

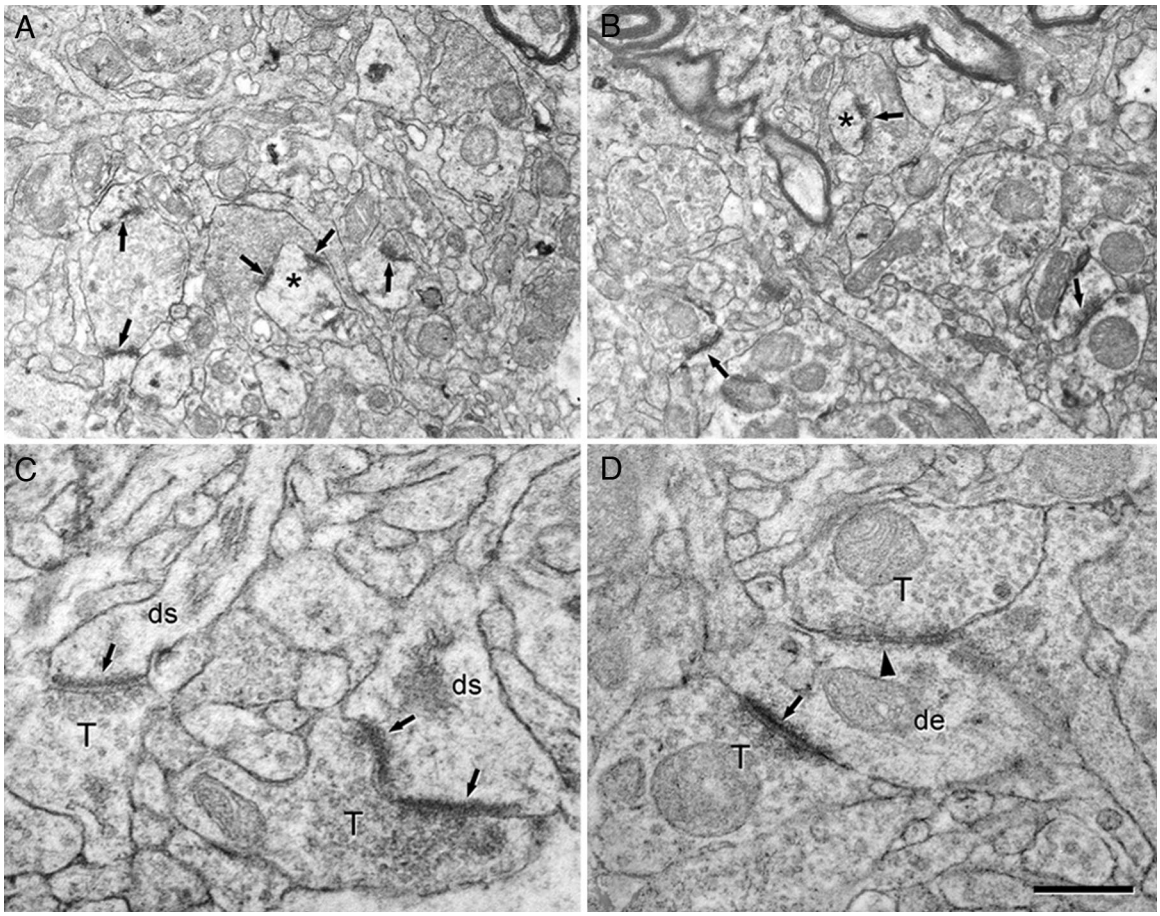

Fig. 2. Electron micrographs to illustrate the ultrastructure of the human temporal neocortex. (*A* and *B*) Low-power electron micrographs showing the neuropil from layer IIIb of the temporal neocortex from a woman (*A*) and a man (*B*). Some synapses are indicated by arrows, and the asterisks illustrate two dendritic spines that are also shown at higher magnification in Fig. S3. (*C* and *D*) High-power electron micrographs showing the two major morphological types of synapses in the neuropil. Asymmetric synapses (arrows) had a prominent postsynaptic density, whereas symmetric synapses (arrowhead) had a thin postsynaptic density. de, dendritic shaft; ds, dendritic spines; T, axon terminals. [Scale bar (in *D*): 0.9 μm for *A* and *B* and 0.4 μm for *C* and *D*.]

the sclerotic hippocampus (e.g., ref. 26) or the peritumoral or dysplastic cortex (e.g., refs. 27 and 28). The eight biopsies used in the present study can be considered to be close to what would be expected as normal conditions for the following reasons: first, the epileptic activity was clearly of mesial origin; second, the whole neocortex in all of these patients displayed non-spiking activity; and, third, they presented normal cytoarchitectonic and ultrastructural characteristics. In addition, although we cannot rule out that synaptic changes may also occur in the neocortex, there is no reason to believe that the differences in synaptic density observed between men and women was due to the epileptic condition because all of the subjects were epileptic. Thus, it is likely that these differences are truly due to sex differences.

Importantly, no differences in cytoarchitecture were observed. More specifically, no significant differences were found between men and women regarding the thickness of the gray matter, the volume fraction of cortical elements (neuropil, cells, and blood vessels), and neurons per volume as previously reported in the temporal neocortex (13). As a consequence, the number of synapses in each layer was greater in men than in women, and, thus, in this particular region of the neocortex the general connectivity in men appears to be more extensive than in women. Accordingly, gender appears to influence synaptic connectivity, and this phenomenon is regulated independent of other cytoarchitectonic features.

If we consider the columnar organization of the input connections, the differences in connectivity between neighboring neurons, and the combinations of the interlaminar connections of both pyramidal and nonpyramidal neurons, it is clear that neurons in different layers do not process the same information (29, 30). Furthermore, pyramidal neurons located in different layers project to different cortical and subcortical nuclei (31–33).

Table 2. Accumulated data when considering all cortical layers

	Mean cross-sectional length, μm, of:			Mean no. ($\times 10^8$/mm^3) of:			Percentage of:			*Vv*		
Sex	Asymmetric synapses	Symmetric synapses	All synapses	Asymmetric synapses	Symmetric synapses	All types of synapses	Asymmetric synapses	Symmetric synapses	No. of neurons/mm^3	Cell bodies (glia and neurons)	Blood vessels	Neuropil
Women	0.30 ± 0.09	0.21 ± 0.10	0.29 ± 0.06	3.17 ± 2.08	1.06 ± 1.84	7.17 ± 3.29	86	14	27,589 ± 16,854	5.3	0.8	93.9
Men	0.30 ± 0.08	0.20 ± 0.09	0.27 ± 0.05	4.23 ± 2.59	1.42 ± 2.00	10.61 ± 4.97	84	16	25,924 ± 15,110	5.2	1.2	93.3

Data (mean ± SD) of all the synapses include asymmetric, symmetric, and uncharacterized synapses.

Hence, it is likely that the differences in synaptic density between men and women observed in all cortical layers represent a microanatomical substrate for sex differences in the fine-tuning of several functions.

The larger number of synaptic connections in men does not necessarily mean that all cortical circuits in this region are more complex than in women. Rather, specific circuits may be more complex in the male brain. The temporal lobe is a complex, associative, and multiintegrative cortical region (ref. 17; for a review see ref. 34). Therefore, the functional consequences of the differences in synaptic circuitry observed here are particularly difficult, if not impossible, to correlate with specific functions related to men or women.

Interestingly, a recent study on synaptic density carried out in the monkey prefrontal cortex seems to indicate that there are no differences between males and females (35). However, many studies have shown variations between species and cortical areas in terms of density, proportion, and types of neurons, as well as in the density of synapses (e.g., ref. 22). Thus, whether these gender differences are unique to the human cerebral cortex or whether similar conditions arise in monkeys and great apes should be specifically analyzed in each species and cortical area. Finally, and in line with this consideration, we would advise the reader to exercise caution in extrapolating the present data to the whole brain. Indeed, it was reported that the anterior commissure, which connects several regions of the frontal and temporal lobes, is 12% larger in women than in men, suggesting that women would have more commissural associative connections (36). Further work will be necessary to examine whether synaptic density is similar or different in other cortical areas.

Materials and Methods

Human postoperative brain tissue was obtained from eight patients suffering pharmacoresistant mesial temporal lobe epilepsy secondary to hippocampal alterations. In each case the patient's consent was obtained in accordance with the Helsinki Declaration (37), and all protocols were approved by the ethical committee at the Hospital de la Princesa (Madrid). Tissue was obtained from four women of 26, 31, 31, and 41 years of age and from four men of 24, 27, 32, and 36 years of age, and this material has been used in previous studies (38–40). Video EEG monitoring of bilateral foramen ovale electrodes was indicative of left mesial temporal lobe epilepsy in all patients. Furthermore, during surgery the epileptogenic regions were identified through subdural recordings with a 20-electrode grid (lateral neocortex) and a four-electrode strip (uncus and parahippocampal gyrus). Intraoperative electrocorticographic recordings revealed spiking activity localized in the mesial structures, whereas the lateral neocortex of all these patients displayed normal activity. That is, no spikes, sharp waves, or slow activities were observed during intraoperative electrocorticography. All of the patients were right-handed, and they had normal IQs.

The lateral neocortex of all these patients was non-spiking, displaying normal activity in intraoperative electrocorticography, although a small portion of the anterior part of the left temporal lobe had to be removed to access the altered hippocampus. Biopsy tissue was immediately immersed in the fixation solution. Then, the lateral neocortex and mesial structures were subjected to standard neuropathological assessment. The surgical outcome of epileptic patients was evaluated after 18 months, and the patients were classified following the Engel scale as grade I (41).

The anterolateral temporal cortex tissue (T2), corresponding to area 21 of Brodmann (17) (Fig. S1), was cut into 1.5-cm-thick coronal slices and immersed in a cold solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 24–36 h. Coronal sections (100 μm) were cut with a Vibratome and collected in series in PB. Some sections were Nissl-stained to reveal the laminar boundaries and to carry out the histopathological assessment. All of the lateral neocortical biopsies were histologically normal.

Estimation of Neuronal Density. Neuronal density was estimated by using optical dissectors as described by West and Gundersen (ref. 42; see also ref. 43) and with the aid of StereoInvestigator software (version 7.0; MicroBrightField). Optical dissectors were performed on every cortical layer from each case using the Nissl-stained sections adjacent to those used to count the synapses. After a starting point was randomly selected, five sections were selected at equally spaced intervals in the same cortical area. Optical dissectors were made with an oil immersion ×100 objective on a surface of 900–1,600 μm^2 and with a depth of 22–25 μm, rendering a study volume of 19,800–40,000 μm^3 per optical dissector. To provide the systematic area offset the movement of the stage was controlled through StereoInvestigator software (MicroBrightField). A neuron was counted only if the nucleolus was clearly identified in the height of the optical plane along the *z* axis.

Estimation of the Volume Fraction (V_v) of the Neuropil. Semithin sections (2 μm) stained with toluidine blue were used to estimate the volume fraction (V_v) occupied by the neuropil, which excluded blood vessels and cell bodies (including those from neurons and glia). This was accomplished by point counting and by applying the Cavalieri principle using the integrated StereoInvestigator stereological package (see Fig. S2).

Electron Microscopy. Sections adjacent to those used for Nissl staining were processed for electron microscopy. These sections were postfixed in 2% glutaraldehyde in PB for 1 h, treated in 1% osmium tetroxide, dehydrated, and flat-embedded in Araldite resin. Plastic-embedded sections were studied by a correlative light and electron microscopy method described in detail elsewhere (44). Briefly, sections were photographed under the light microscope and then serially cut into semithin (2-μm-thick) sections with a Reichert ultramicrotome. The semithin sections were stained with 1% toluidine blue in 1% borax, examined under the light microscope, and photographed to locate the area and layers of interest. Selected semithin sections were resectioned into serial ultrathin sections with a silver-gray interference color corresponding to a thickness of ≈60–70 nm (45). The ultrathin sections were collected on formvar-coated, single-slot grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope. Using this correlative light and electron microscopy method, it was possible to determine the exact region of the neuropil that was analyzed by electron microscopy, and, therefore, we could accurately identify the layer analyzed. Photographs were taken randomly at ×30,000 with a digitalizing image system (Mega View III Side-mounted TEM Camera; Soft Imaging System) and by using imaging acquisition software (analiSIS 3.2; Soft Imaging System). At least 30 micrographs at a magnification of ×30,000 were obtained per layer and case (for a detailed description on the estimation of synapses see ref. 23).

The two major morphological types of cortical synapses were clearly identified in the cortical tissue analyzed, these being type I and type II according to Gray (19) or those denominated asymmetric and symmetric by Colonnier (ref. 20; for review see refs. 46 and 47). The synapses in which the synaptic cleft and associated membrane densities could not be visualized clearly (because of the oblique plane of section) were considered as uncharacterized synapses.

Synaptic density per unit area (N_A) was estimated from electron microscopy samples of the neuropil from each cortical layer (for a detailed description see ref. 23). The density of synapses per unit volume of the neuropil was calculated by using the formula $N_V = N_A/d$ where N_A is the number of synaptic profiles per unit area and d is the average cross-sectional length of synaptic junctions. The cross-sectional length of synaptic junctions was measured by using the Image J analysis program (Scion).

Estimation of Tissue Shrinkage. To obtain homogeneous estimates of neuronal density and synapses, tissue shrinkage was evaluated by using StereoInvestigator software to measure the cortical surface and volume in sections before and after processing for Nissl staining or electron microscopy. Initially, the surface area of the nonprocessed Vibratome sections and the thickness were measured at six random points to estimate shrinkage along the *z* axis (i.e., section compression). Thereafter, the sections were Nissl-stained or processed for EM, and the same measurements were taken again. As a result, the cortical tissue was estimated to have shrunken 68.7% in volume when processed for Nissl staining and 42.6% when processed for electron microscopy. These shrinkage values were taken into consideration when estimating the thickness of the cortical layers and neuronal and synaptic density, as well as for the estimation of the ratio of the number of synapses per neuron.

Statistical Analyses. Statistical comparisons between the two groups (men and women) were performed by using the unpaired Student *t* test or the Mann–Whitney nonparametric *U* test, depending on whether the datasets fitted a normal distribution and passed the test for homogeneity of variances (48). All statistical studies were performed with the aid of the Prism statistical package (Prism 4.0; GraphPad). To assure a blind analysis of the material, all of the cases were coded, and only later were the codes broken for the statistical analyses.

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