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Reduced minicolumns in the frontal cortex of patients with autism

D. P. Buxhoeveden*†, K. Semendeferi‡, J. Buckwalter§, N. Schenker‡¶, R. Switzer** and E. Courchesne§¶

*Departments of *Anthropology and †Psychology, University of South Carolina, Columbia, SC, Departments of ‡Anthropology and §Neurosciences, University of California at San Diego, CA, ¶Children's Hospital of San Diego, CA, and **Neuroscience Associates, Inc., TN, USA*

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Cell minicolumns were shown to be narrower in frontal regions in brains of autistic patients compared with controls. This was not found in primary visual cortex. Within the frontal cortex, dorsal and orbital regions displayed the greatest differences while the mesial region showed the least change. We also found that minicolumns in the brain of a 3-year-old autistic child were indistinguishable from those of the autistic adult in two of three frontal regions, in contrast to the control brains. This may have been due to the small size of the columns in the adult autistic brain rather than to an accelerated development. The presence of narrower minicolumns supports the theory that there is an abnormal increase in the number of ontogenetic column units produced in some regions of the autistic brain during corticoneurogenesis.

Keywords: autism, columns, frontal cortex

Introduction

The frontal lobe is involved with the kind of higher-order cognitive, language and socio-emotional functions that are impaired in autism. However, until recently, there has been a paucity of developmental anatomical evidence to support a distinctive role for the frontal cortex. The first such evidence is based on *in vivo* MRI studies of 2- to 4-year-old autistic children [1,2], and shows that frontal lobes undergo a developmentally early period of abnormal overgrowth delimited to dorsal frontal, mesial frontal and temporal cortices (in order of deviance). Other regions, including orbital, primary motor, parietal and occipital parietal cortices, were not significantly enlarged. One study on minicolumn organization reported a reduction of minicolumn size that included the frontal region and temporal regions but results on the frontal region were not reported separately from temporal cortical areas nor were they analysed for age effects [3]. The last few years have witnessed an interest in the role of minicolumns in cortical organization [4–9] as well as the clinical setting [3,10,11]. The size of minicolumns may be an important indicator of their physiology so that evidence of reduced minicolumn size has functional implications [12–14]. Some of the ways in which minicolumns may become narrower than the normal configuration (i.e. for a given species, area, age, etc.) include reduced serotonin levels [15–17] as reported in autism [16] or any number of aberrations affecting embryonic cell migration and the formation of ontogenetic columns [18,19].

Although limited by sample size, the current study is the first to examine developmental differences in autism at

Correspondence: Daniel P. Buxhoeveden, Hamilton College, Room 317, 1512 Pendleton Street, The University of South Carolina, Columbia, SC 29803, USA. Tel: +1 803 777 4460; Fax: +1 803 777 0259; E-mail: buxhoevede@gwm.sc.edu

the level of the microvertical organization, and represents the most comprehensive analysis to date of minicolumns in the frontal lobe. A recent fMRI study demonstrated that primary visual cortex (V1, or Brodmann's area 17) is organized functionally normally in the brains of autistic individuals and that any difference in function may arise from higher-level cognitive areas [20]. Thus, it is possible that autism does not involve neural defects in visual cortex. In addition, cell minicolumns in area 17 have been examined in Down syndrome (DS) cases as well and found to be unaffected [21]. We felt that primary visual cortex would be a good control region for this study.

Case reports

Case 1

A 3-year-old boy diagnosed with autism. His mother reported on the Autism Diagnostic Inventory-Revised (ADI-R [22]) that the child was able to speak in simple two to tree word phrases from the age of 15–24 months at which time he regressed and his speech was mainly unintelligible. He exhibited limited eye gaze and social smiling, and showed no pretend play skills. He had no interest in interacting with peers, preferring to engage in solitary activities such as playing on the computer, looking at books or putting together puzzles. It was also reported that he did not offer comfort to his parents if they were sad, hurt or ill, and at times would respond inappropriately such as by giggling. He demonstrated extreme negative responses to several sensory stimuli, and was particularly sensitive to clothing texture and noises such as the hair dryer and electric toothbrush. He also engaged in stereotyped behaviours such as repeatedly rubbing his thumb and forefinger together, head banging and other aggressive and self-injurious actions. The ADI-R [22] also revealed that his father experienced depression, his paternal uncle had hyperactivity and a seizure disorder, and his maternal grandfather exhibited depression and rage. The cause of death was cardiac arrest following drowning.

Case 2

A 41-year-old man diagnosed with autism at age 2. He also had mental retardation. He resided in a residential facility from the time he was 8 years old until his death. His therapy team described his speech as repetitive and echolalic. He was also described as aggressive and would head bang, bite, scream, pull hair and hit. His mother reported on his *post mortem* ADI-R [22] that he would ignore others as if they were objects and did not offer comfort to others. He did not exhibit imitation skills, did not use conversational or instrumental gestures, and did not smile. The ADI-R [22] also revealed a family history of psychiatric disorders, which included a maternal aunt with social problems, a maternal great aunt who was institutionalized, and a maternal second cousin with schizophrenia. He was prescribed various antidepressive, antiseizure and antianxiety medications throughout his life including Ativan, Haldol, Dalmane, Tegretol, Nozinan, Loxepac, Flurazepam, Synthroid, Chloral Hydrate, Epival, Zyprexa, Carbamazapine and Cogentin. He died from food asphyxiation.

Methods and materials

The age of the control brains was 2, 21, 34, 44 and 75 years. All were male and measures were taken from the left hemisphere. Two of the control cases (ages 21 and 75) had been embedded in paraffin, cut serially at coronal sections 20 microns thick, and stained with a modification of silver stain for neuronal perikarya [23]. The rest of the control and pathological cases were cryoprotected in 20% glycerol–2% DMSO for 1 week; they were then cast in a gelatin matrix that was cured for 4 days, and then the block containing the brain was rapidly frozen in a mixture of dry ice and 2% methyl butane. The frozen block was mounted and cut in the coronal plane at 80 microns. The sections were Nissl stained by cresyl violet. We examined three cortical regions of the frontal cortex; dorsal, orbital, mesial and primary visual cortices (Brodmann's area 17). Nearly 400 micrograph images were used so that the mean number of images examined per region per brain was close to 18. This constituted an estimated average of 400–500 minicolumns per region, or 1600–2000 minicolumns per brain. This compares with the use of only several images and approximately 50 minicolumns for each brain used in a previous analysis [3]. A one-way ANOVA and Tukey's post-test were used for statistical analysis.

Photographs of brains were taken at 100× total magnification and digitized. The digitized images were sent to a Dell PC Intel IV processor. Imaging was performed using software developed in our laboratory that is based on ImageJ software, which is a PC-based version of NIH image. We modified the program to perform semi-

automated measures of cell columns. The program allows for standardization of analysis between observers so that results are highly reproducible between individuals. Cells were segmented from the background by thresholding and converted into a binary image. Watershed was used as a technique for edge detection to separate overlapping cell borders that would otherwise be counted as one cell. All this was automated by the computer. The only input by the operator was to choose a threshold level for cell size and to outline the region of interest (ROI) within the image to be digitized.

The ROI was limited to layer IIIb and care was taken to omit artifacts or blood vessels from measurements. Previous studies of minicolumns in autism have focused on layer III as well [3]. Layer III was chosen because of the following: (i) it is the most linear and thus preferable layer in which to detect minicolumns. The detection method assumes cell columns are one cell wide in lamina III [24]. (ii) Minicolumns are vertical arrays subsuming all the layers. If cell columns in layer III are closer together then it must follow for the other layers as well. In addition, the long apical dendrites from layer V pyramidal cells pass through layer III where the pyramidal cells in layer III add their apical dendrites to these bundles before ascending into layers II and I. Therefore, they become part of the pyramidal cell module as described by Peters and Sethares [25] and there is a unification of the supragranular and infragranular layers. (iii) It is a very important layer in regards to its associational functions within a minicolumn. (iv) We have assembled a large data bank based on layer III minicolumn, so the use of this layer standardizes results with previous studies from other cortical regions.

In this instance, cells smaller than 50 pixels in area were not counted. We wanted to focus on the larger pyramidal cells that constitute lower layer III. The measurement of spacing distances and number of cell columns per unit area were based on pixel density in the *y* dimension collapsed onto the *x* or horizontal plane measured over multiple levels of the image. Horizontal lines of one-pixel depth were run throughout the entire ROI and the final results were summed and displayed. Parameters measured included spacing distance between cells (cell spacing or CS) in the horizontal domain as they descended in the vertical plane. This was based on edge-to-edge measures. Neuropil space (NS) was measured as the amount of nonpixel dense space (or 'the noncell space') between cell edges in the horizontal axis. CS includes both the NS and the area size of the cell, and is therefore always greater. The grey level index (GLI) is the total ratio of the space occupied by the pixel-dense elements to the total space available in the ROI, and is based on a method that has been used extensively for decades [26]. It is an estimator of the density of cell soma within a given region that includes cell number as well as cell size. All things being equal, if cell columns are closer together, the GLI can be expected to increase; if cell columns were further apart, the opposite would be the case. However, this need not be the case as changes in cell numbers and size can offset changes in column spacing distances.

The program does not assume the presence of vertical units. Rather it measures horizontal spacing of cells as it descends in the vertical plane. The premise, based on multiple studies [9,21,24], is that cell columns in layer III are one pyramidal cell wide. CS is synonymous with the separation of cell minicolumns, and results obtained with this software are very similar to those obtained with previous methods [21]. Therefore, the terms 'cell spacing' and 'minicolumn spacing' are used interchangeably in this paper.

Results

Autistic adult *vs.* control adult

Regional abnormalities in frontal cortex in autistic adult Figure 1 shows examples of cell minicolumns from the dorsal frontal cortex of the 41-year-old autistic man and the 44-year-old control man. Figure 2 shows results from the 41-year-old autistic adult compared with all control adults. Whether statistically compared one to one against each control or to the three controls as a group, the autistic adult had statistically significantly ($P < 0.05$) reduced minicolumn spacing, reduced neuropil spacing and increased GLI in dorsal, mesial and orbital cortices. Differences from control values were greatest in dorsal and orbital cortices. For example, in the autistic adult as compared with the average of the three controls, minicolumns were reduced by 23% in dorsal and 24% in orbital cortices; neuropil spacing was reduced by 27% in dorsal and 28% in orbital cortices.

Visual area V1 not abnormal in autistic adult No differences between the autistic adult and the controls were found in V1 (Figure 3). Minicolumn spacing was 33.1 microns for the autistic adult and averaged

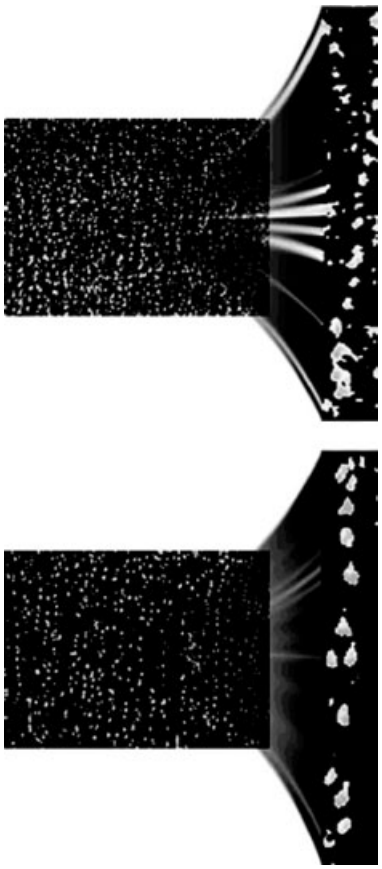

Figure 1. Top: autistic adult. Bottom: control adult. Both are dorsal frontal region taken at 100× total magnification. Cells appear to be smaller in the autistic cortex.

Table 1. Measures Of Cell Column Spacing Distances

Frontal gortex		
Autism		
Child (n = *1*)	*Adult* (n = *1*)	*Child % of adult value*
Dorsal (Mean/SD)		
40.8 μm/6.4 μm	43.2 μm/5.5 μm	94
Orbital		
38.3 μm/3.3 μm	42.1 μm/4.7 μm	91
Mesial		
45.7 μm/6.7 μm	52.9 μm/9.6 μm	86
Control		
Child (n = *1*)	*Adults* (n = *4*)	*Child % of adult value*
Dorsal (Mean/SD)		
43.7 μm/1.2 μm	50.8 μm/8.6 μm	86
Orbital		
39.7 μm/2.5 μm	54.0 μm/11.2 μm	74
Mesial		
42.5 μm/3.8 μm	58.2 μm/12.5 μm	73
Area 17		
Autism		
Child (n = *1*)	*Adult* (n = *1*)	*Child % of adult value*
29.8 μm/1.3 μm	33.1 μm/6.6 μm	90
Control		
Child (n = *1*)	*Adults* (n = *3*)	*Child % of adult value*
30.9 μm/1.3 μm	34.4 μm/8.7 μm	90

34.7 microns for the three control adults, and neuropil spacing was 24.4 *vs.* 24.7 microns respectively. These minicolumn values for visual cortex are similar to those previously reported for that cortex in other samples of humans and other primates [9].

Developmental comparison

Autistic child (3 years) vs. autistic adult (41 years) The values for minicolumn size and neuropil spacing were not statistically differentiated between the two brains ($P > 0.05$, Tukey's post-tests) in two of three frontal regions (dorsal and orbital) (Table 1, Figure 4). However, in mesial cortex the CS and NS were statistically smaller in the autistic child. The GLI was higher in all three regions and most differentiated in mesial cortex. This suggests that columns are more densely packed in the autism child than in the adult. Other elements such as cell number and size, cannot be ruled out however.

Control child (2 years) vs. control adults A comparison of cell columns for a 2-year-old control resulted in the finding of differences in CS and NS for all three regions (P-values <0.05 to <0.001) (Table 1, Figure 4). The NS was notably different in each region (dorsal, $P < 0.05$; mesial, $P < 0.001$; orbital, $P < 0.01$: Tukey's post test) and mesial cortex displayed the greatest differences, especially for NS. This implies that minicolumns in mesial cortex undergo relatively more enlargement during development. The GLI was much higher in all three regions and statistically different in the 2-year-old compared with adults, and the dif-

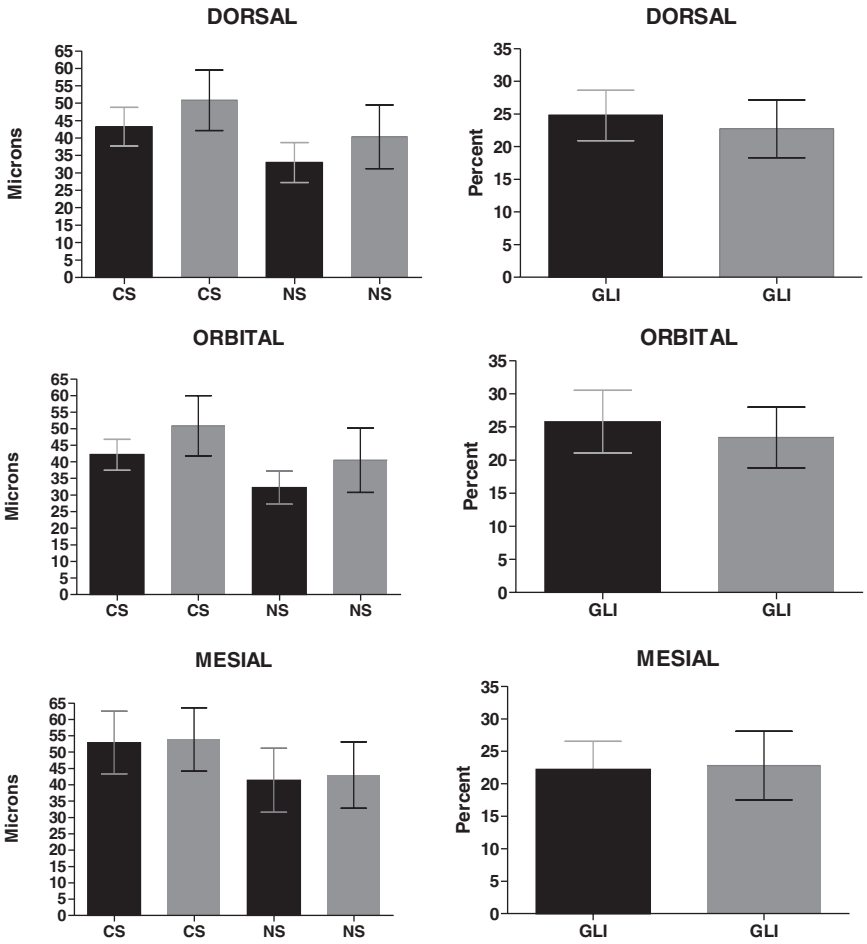

Figure 2. Minicolumns in adult autistic and control frontal regions. Left: CS and NS are significantly smaller in autism than in control cases within dorsal and orbital cortices. Right: GLI is greater in autism than in control cases for dorsal and orbital cortices. Black = Autism. Grey = Control. CS = column spacing. NS = neuropil space. GLI = grey level index. Error bars = SD.

ference between child and adults was much larger than for the autistic brains (Figure 4). A major reason for this is because the GLI in the adult controls was much lower than in the autistic adult brain.

Primary visual cortex (V1) Minicolumn spacing distance for the 3-year-old autistic child (29.8 μm) and 2-year-old control child (30.9 μm) were not statistically distinguishable. The relative size of the columns were 90% of the mean adult values for both the autistic and control brain.

Discussion

These results are consistent with Casanova *et al.*'s [3] study of autism in other regions that include temporal lobe and prefrontal cortex, where minicolumn spacing was reported to be 46.8 microns for $n = 9$ autistic cases and 52.8 microns for their $n = 9$ controls. The values in our study for dorsal cortex – 45.5 and 56.2 microns for the autistic adult and the controls respectively – fit well with that published study. Thus, minicolumn abnormality may be a consistent and reliably measured cytoarchitectural defect in autism. Furthermore, an abnormal increase in the number of ontogenetic column units would explain the presence of larger than normal frontal lobe grey and white matter enlargement in autistic children [1,2].

Our investigation, though small, extends information about minicolumn abnormality in autism in three ways. First, we found regional differences within the frontal cortex in minicolumn abnormality with dorsal and orbital

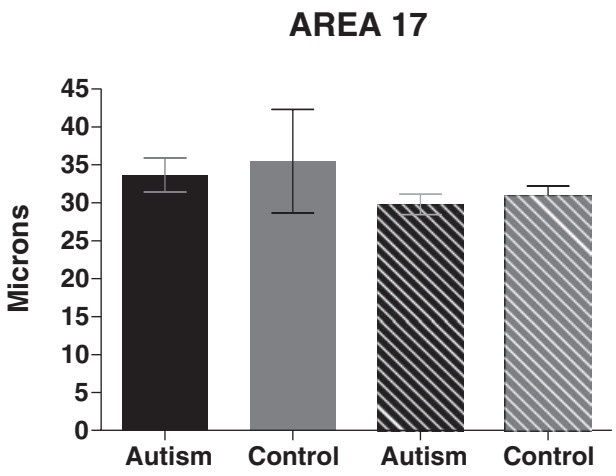

Figure 3. Cell column spacing in Area 17. No statistical difference was established between the autism and control groups, or adult and children groups in Area 17. Solid bars are adults. Striped are children. Error bar = SD.

cortices displaying the greatest abnormality and mesial the smallest. It is unclear why mesial cortex was the least changed in our small sample, and we will have to await greater sample sizes to see if this remains a consistent finding. The underdevelopment of minicolumns in frontal cortex likely signals the failure of the normal emergence of a diversity of highly specialized functional units that are necessary for the refined processing of and learning about information critical to higher-order functions.

Second, we did not find minicolumn abnormality in primary visual cortex in our autistic cases. This important result is consistent with the view that the neurobiological causes of autism impair cortices that mediate higher-order functions but spare those that mediate basic level lower-order sensory ones. Thus, the presence of *normal* minicolumns in primary visual cortex may signal that the autistic brain retains the capacity for detailed and refined processing of lower-level visual information as reported elsewhere [19].

Third, across multiple regions and measurements, minicolumns in the child with autism were very close in size to that of the adult with autism, and could not be distinguished statistically in two of three frontal regions. We were able to contrast this with a 2-year-old control that displayed significant differences in all three regions. In another developmental study of minicolumns in Brodmann's area 22, controls brains of children were also smaller than and statistically different from those of adults, while minicolumns in V1 were found to be much closer to adult size in both normal and DS children [11], a finding which we duplicated in this study for the autistic individual and the control. It is estimated that minicolumns attain adult size at about the same rate as the cortical volume [11], which would be by middle to late teens.

A comparison of the actual column size between the autistic and control brain suggests that the apparent similarity in minicolumn size to the adult found in the autistic individuals is the result of the adult having smaller cell columns and not evidence of accelerated growth. Should these results prove sustainable, this would suggest a failure of continued columnar growth.

Minicolumn abnormality has been reported in some other disorders, but a closer analysis demonstrates that the pattern of abnormality in autism may be distinctive. The potential variations of minicolumn size (or the amount of neuropil spacing between them) is limited to being normal, larger or smaller. By itself this does not supply much potential for specificity in neurobiological or clinical assessments, and so it is necessary to consider column size in a greater context. The significance of column size has to do with the fact that the size of cortex is the product of the number of ontogenetic cell columns created during neurogenesis [18,19], and their continued expansion during development [21,27,28]. Therefore, column number and size (which share a relationship as well) are intimately interrelated to cortical surface area and postnatal development, and must be considered as a dynamic system.

In addition to the number of columns, their size also affects cortical volume, so that all things being equal, a brain (or cortical region) with the same number of columns, but smaller ones, will have a smaller cortex. This is not what is seen in autism. When considered more closely, the discovery of smaller than normal columns represents interesting causal issues.

In Rett's syndrome, the finding of smaller columns was statistically significant only in area 21 but not in other association areas, and it was concluded that the regional nature of the changes as well as differences in mean CS differentiated the abnormal minicolumnar morphometry of Rett's syndrome from that of autism [29]. Cell columns in Asperger's were also smaller in areas 9, 21, 22 [30]. Asperger's syndrome is considered part of the autistic spectrum disorder so the findings may represent a fundamental similarity. However, direct comparisons cannot be made until more is known about their development, and

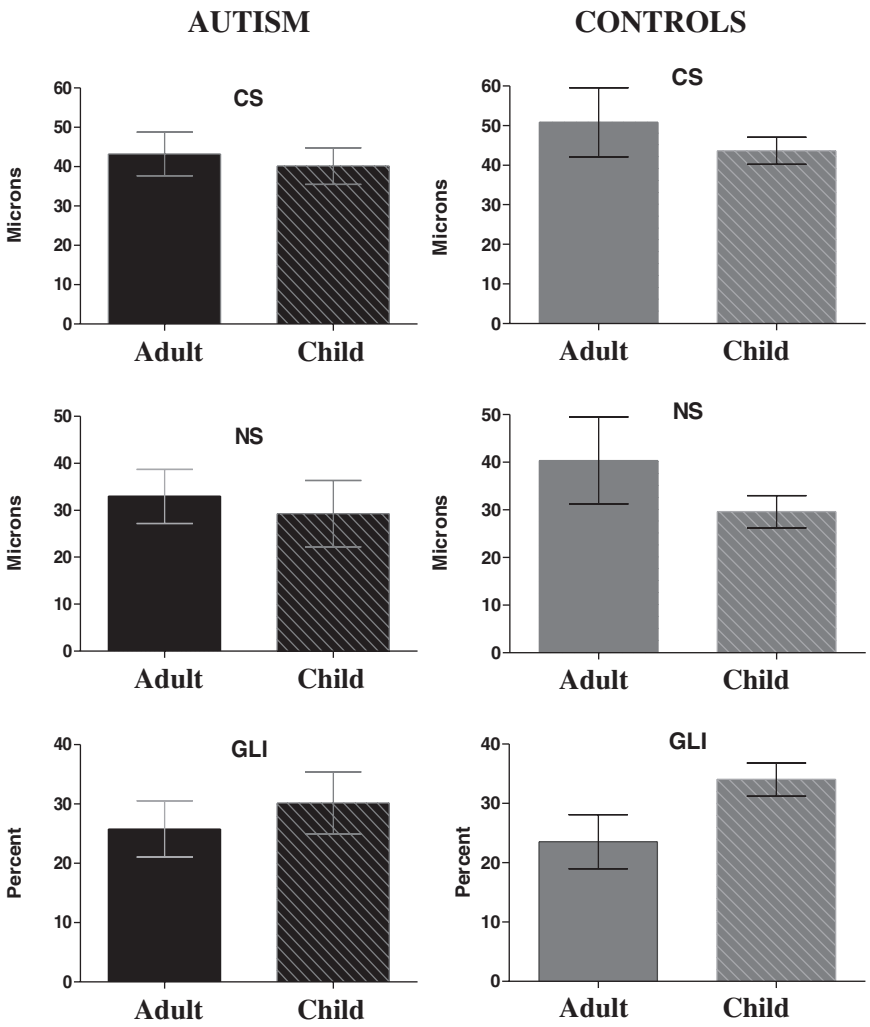

Figure 4. Dorsal frontal cortex in the developing autistic and control brain. Left: Autism. Solid black = autistic adult. Striped black = autistic child. While the columns are smaller in the child, a Tukey's Post-test found no statistical differences between them. Error bars = SD. Right: Controls. Solid grey = Control adults. Striped grey = 2-year-old control. Differences between the control brains are much greater than in the autistic group, and all parameters were statistically significant based on the measurement of hundreds of columns per brain.

of course, more cases are studied. To date, only two Asperger's cases have been studied, and we have no data on brain size or age differences.

The only other developmental disorder in which age and brain volumes were examined is DS [11,31]. In this instance they show a very different pattern. Columns in adults were normal in size but with suggestions they contained fewer cells per column. The attainment of adult size in the DS children compared with adult DS differ in an important way from autism. In DS children, the absolute size of the columns in selected regions were significantly larger than in controls of the same age. On the other hand, the absolute size of the columns in the autistic child may be similar to controls (from other regions) and appear relatively large only because those of the autistic adult are smaller than that of normal. Incidentally, the absolute size of the minicolumn in the autistic child shows them to be within range of minicolumns in Brodmann's area 22 for normal children [11]. Among the many possible interpretations, it may be that the cell columns are normal in size at this time but there are more of them which accounts for the noted increase in cortical volume [1,2]. Consequently, their continued maturation is slowed significantly compared with controls, resulting in permanently smaller

than normal cell columns in the frontal cortex. The apparent lack of larger frontal cortical volumes in the autistic adult would lend support to this interpretation [1,2]. The findings in autism are intriguing and demonstrate a unique pattern between column size, maturation rates and cortical volume that have not been described anywhere else.

Conclusion

The physiological significance of narrow vertical microorganization has been suggested to enhance specialization and reduce generalization [17]. The initial pattern of cerebral grey and white matter overgrowth reported in the earliest stages of autism [1] is predictable on the basis of an increased number of ontogenetic cell units [18]. However, the failure to continue growing suggests the normal processes associated with cortical enlargement has been stunted. As the spacing distance between cell columns essentially reflects the amount of NS between them, the small size of these columns infers that expansion of neuropil is the primary part of the column that has failed to develop [21].

We add a cautionary note that despite the intensity of our study, which measured thousands of columns in each brain, these results must be considered preliminary because of the small sample sizes and biological variability involved. The acquisition and thorough examination of large sample sizes will be needed to conclusively document these findings.

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