A Microscopic Study of Language-Related Cortex in Autism
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Abstract: Impaired language function is a principle criterion for the diagnosis of autism. The present study of brain from age-matched autistic and control subjects compared brain regions associated with the production and processing of speech. Wernicke's area (Brodmann 22, speech recognition), Broca's area (Brodmann 44, speech production) and the gyrus angularis (Brodmann 39, reading) from autistic subjects (7-44 years of age) and control subjects (8-56 years of age) were examined microscopically. Striking differences in the density of glial cells, the density of neurons and the number of lipofuscin-containing neurons were observed in the autistic group compared with the control group. The mean density of glial cells was greater in the autistic cohort than controls in area 22 (p<0.001), area 39 (p<0.01) and area 44 (p<0.05). The density of neurons was lesser in autism in area 22 (p<0.01) and area 39 (p<0.01). The autistic group exhibited significantly greater numbers of lipofuscin-containing cells in area 22 (p<0.001) and area 39 (p<0.01). The results are consistent with accelerated neuronal death in association with gliosis and lipofuscin accumulation in autism after age seven. Production of lipofuscin (a matrix of oxidized lipid and cross-linked protein more commonly associated with neurodegenerative disease) is accelerated under conditions of oxidative stress. Area 22 in autism evidenced the greatest glial increase, the greatest neuronal decrease and the greatest increase of non-specific cells containing lipofuscin, which itself may contribute to greater free-radical generation in brain.

Key words: Autism, cerebral cortex, gliosis, lipofuscin, oxidative stress

INTRODUCTION

Autism, a developmental neuropsychiatric disease, is diagnosed in early childhood and features social and communication deficits, restricted and repetitive behaviors and interests and a characteristic course. Relatives of autistic subjects are more likely to manifest social, cognitive and psychiatric deficits and a milder form of autism is unassociated with mental retardation. It is strongly suggested that outside the formal diagnosis of autism, there exists an unexpectedly large population with milder, but significant, social and communication deficits.

While functional alterations in cortical auditory and language processing in autism are reported, language-related cortex of autistic subjects has not been subjected to systematic microscopic examination. Language is a complex brain function, involving numerous subcortical and cortical areas, including areas for primary and secondary auditory processing. For this study we analyzed three higher-order cortical areas involved in the processing of language and production of speech: Wernicke's area, Broca's area and the angular gyrus in the brains from autistic and control subjects.

Wernicke's area (Brodmann area 22) is a supplementary-auditory section of neocortex in the left temporal lobe which mediates the understanding of auditory words. Wernicke's aphasia, an inability to decipher the meanings of the speech sounds, results from damage to area 22. Broca's area is a premotor region of the neocortex located in the left frontal lobe (Brodmann areas 44 and 45) which mediates the production and control of human speech. Lesions to this area (Broca's aphasia) produce speech problems, but do not affect semantic or basic syntactic abilities. Wernicke's and Broca's areas are connected by the arcuate fasciculus; conscious language output originating in Wernicke's area is carried via the arcuate fasciculus to Broca's area, where a detailed and coordinated program for vocalization is compiled and sent to the facial zone of the motor cortex. The angular gyrus (Brodmann area 39) is a small region behind Wernicke's area which contains regions of the occipital, parietal and temporal lobes. Area 39 facilitates
interaction of visual and auditory brain cortex and has been implicated in alexia, dyslexia\textsuperscript{[8]} and agraphia.

Hypothetically, structural alteration of one or more of these cortical areas might contribute to the communication impairment found in autism. The purpose of this study was to assess the microscopic anatomy of Wernicke's area, Broca's area and the angular gyrus in preserved brain of accurately-diagnosed autistic subjects in comparison to age-matched controls. The pattern of cortical layering, relative thickness of cortical layers and neuronal and glial densities for each layer was determined. Glial density, neuronal density and number of nonspecific lipofuscin-containing cells was determined for each subject. The data allowed assessment of each parameter as a function of subject age.

**MATERIALS AND METHODS**

**Human subjects:** The tissue required for this study was obtained from the Harvard Brain Tissue Resource Center, the Human Tissue Bank at the University of Miami and the NICHD Brain and Tissue Bank for Neurodevelopmental Disorders at the University of Maryland, as facilitated by the Autism Tissue Program. Medical history and documentation of autism by Autism Diagnostic Interview (ADI-R) was provided for each subject. Formalin-preserved blocks from the left-hemisphere were from eight autistic and seven control subjects:

| Age | Sex  | Identifier | Age | Sex  | Identifier |
|-----|------|------------|-----|------|------------|
| 7   | male | UMB-797    | 8   | male | UMB-662    |
| 9   | male | B-4925     | 17  | male | B-2234     |
| 10  | male | BTB-3714   | 22  | male | B-4981     |
| 14  | male | B-4323     | 30  | male | B-4211     |
| 25  | male | BTB-3711   | 46  | male | B-4192     |
| 26  | male | B-5000     | 52  | male | BTB-3692   |
| 31  | male | B-4871     | 56  | male | B-4503     |
| 44  | male | B-4541     |     |      |            |

**Tissue preparation:** Formalin-fixed postmortem tissue blocks from areas 22, 39 and 44, were stored in 10% phosphate-buffered formalin. Portions of each block were immersed in 20% sucrose cryoprotectant, then serially sectioned in perpendicular orientation to the pial surface with a sliding microtome, at 100 µm. Adjacent series of sections were stored at -20°C in a solution made of 3% glycerol, 3% ethylene glycol, 1% phosphate buffer (PB) and distilled water; alternate sections were stained with cresyl violet (0.1%) in order to visualize laminar boundaries. For lipofuscin study, sections were coverslipped with Fluormount G (Aname 17984-25). All sections were coded and randomized to conform with strict double-blind methodology.

Sample preparation for cell-counts involved several steps. Blocks were divided to provide a portion for microdissection and post-fixation with 2% \textsubscript{4}OsO\textsubscript{4} in 0.9% NaCl containing 1.5% potassium ferricyanure (Sigma, St Louis, MO), for 1 h. Tissue was dehydrated with propylene oxide and flat-embedded in Epon; 1 µm-thick sections were produced with a diamond knife, then dried on glass slides and stained with basic toluidine blue (borate buffer, pH 11).

**Immunohistological staining:** Astrocytes were stained with polyclonal antibody to glial fibrillary acidic protein (GFAP; DAKO, Denmark). The sections were rinsed initially with TBS (0.05 M Tris and 0.15 M NaCl), pH 7.5, then pre-treated with a solution of 50% ethanol and 1% hydrogen peroxide (Sigma, St Louis, MO) in phosphate-buffered saline (PBS) for 30 min in order to remove endogenous peroxidase activity, then rinsed in PBS and subsequently incubated in 1:5 normal goat serum for 30 min in order to block non-specific staining. Then primary anti-GFAP antibody was applied at 1:200 dilution for 2 h at 4°C, followed by rinse with PBS and incubation with biotinylated goat anti-mouse antibody 1:200 in PBS for 1 h at room temperature and subsequent processing by the avidin-biotin-peroxidase method (Vectastain ABC immunoperoxidase kit: Vector, Burlingame, CA). DAB (3,3’-diaminobenzidine tetrahydrochloride: Sigma, St Louis, MO) was used as the chromogen. Sections were mounted on glass slides, dehydrated, cleared in xylene and coverslipped.

**Morphometric analyses:** An optical fractionation method was employed to estimate cell density. This procedure is based on a random systematic choice of cortical tissue samples and performed by means of a regular, previously-designed grid of counting frames (100x100 µm). An average of 4 fields per cortical layer was counted in each section, resulting in an average of 24 fields for each sample in every cortical area studied. The cells were counted only if their somata fell entirely within the frame area. Cell density was calculated by dividing the total number of cells in each layer by the surface area of the frame (or in the examination of cortical columns, division by the area of the cortical column). These data were expressed as number of cells per mm\textsuperscript{2}. 
The randomized and codified sections were observed with a 100× oil immersion lens on a Leica DMRB light microscope. 200 µm-wide columns, spanning pia to white matter, were examined and plotted on paper with respect to cortical layers. Neurons and glial cells were distinguished carefully. Neurons—usually, but not always larger than glial cells were distinguishable by their non-spherical shape, stained cytoplasm and invisible nucleolus. In contrast, glial cells were identified by the absence of cytoplasmic staining, small size and round shape.

Thickness of the cortical mantle was assessed with a Leica PL FLUOTAR lens, A.N. 0.12, which was used to represent a column through the entire extent of the cortical mantle on graph paper. The total cortical thickness was noted to vary noticeably among different regions of a given cortical area, thus precluding direct comparisons of absolute values. Hence, cortical thickness was reported as percentage of total cortical thickness.

Lipofuscin identification and quantification: Lipofuscin-containing cells are readily identifiable by autofluorescence. A Zeiss III RS fluorescence microscope with an HBO 50W AC super pressure mercury lamp was utilized for determination of number of cells containing lipofuscin. Both 365 and 450 nm excitation filters were employed and cell counts and cell-density calculations were performed as previously described for glial and neuronal cells.

Reproduceable photographs of intracytoplasmic lipofuscin aggregates were obtained using a confocal laser-scanning microscope (Leica TCS SL) equipped with a Hellion/Neon laser and a 60×oil objective (Leica Plan Apochromat). The tissue sections were excited at 488 and 568 nm wavelengths and the image was reconstructed from a stack of 10 consecutive confocal planes.

Quantitative analysis of the size of the intracytoplasmic lipofuscin aggregates was performed on single optical images for each brain section. By employing a 60xoil lens in a random-systematic manner, a region of interest (ROI) was selected by computer and aligned over layer I. The sampling ROI was moved vertically through all cortical layers and every deposit of lipofuscin contained within the select ROI was outlined with a mouse cursor. The quantification of the lipofuscin area was accomplished with an Image Analysis System (Soft Imaging System GmbH, Münster, Germany) program.

Statistical analysis: Cell counts across hexalaminar cortical columns were obtained in an uninterrupted series of counting boxes that spanned the entire depth of cortex from the pial surface to the underlying white matter. Four distinct cortical probes were obtained for each brain area. The cell density for each case was determined by dividing the number of cells counted in each sample by the total area of the chosen region. The data were expressed as the Mean±SD of the sample.

The statistical Student’s t-test was used to compare the mean values from individual morphometric parameters between autistic and control subjects of matching ages. The confounding potential influence of postmortem delay and fixation interval were evaluated using the analysis of covariance, which demonstrated no significant relationship.

RESULTS

Analysis of cortical laminar thickness: The thickness of cortical layers in relation to total cortical thickness in the three brain areas examined were not significantly different between autistic and control cohorts (Table 1).

Longitudinal analysis of control data: Density of glial cells: In controls, the density of glial cells in full-depth cortical sections increased in all three brain areas as a function of age (Fig. 1a-c). Thus, in each brain area, glial density was approximately twice as high in sixth-decade subjects, as compared to the youngest subject, age 8. A steep rise in glial cells was measured in area 39 at age 30 (p = 0.0212) and in area 44 at age 30 (p = 0.0007) (Fig. 2a-c). Generally, the decrement in glial density was most pronounced in layers III, V and IV (Table 2).

Neuronal density: In controls, a significant decline in neuronal density was evident in full-depth cortical sections of area 22 (by 40% from age 8 to age 56, p = 0.0187), but not in area 39 or 44 (Fig. 2d,e,f) Laminar analysis (Table 3) demonstrated age-related declines in all cortical layers of area 22, particularly in layers II (reduced 40% from age 8 to age 56, p = 0.0025) and IV (reduced 35% from age 8 to age 56, p = 0.0058). In area 39, a pronounced decrement was observed in layer V (50% from age 8 to 56, p = 0.0057) and in area 44, there was a moderate decline in neuronal density in layers II (reduced 21% from age 8 to age 56, p = 0.0363).

Lipofuscin-containing cells: The lipofuscin cell-count (Table 4) did not differentiate between neuronal and
Table 1: Percentage of total cortical thickness. %: percentage. SD: standard deviation

|       | 8    | 17   | 22   | 30   | 46   | 52   | 56   | 7    | 9    | 10   | 14   | 25   | 31   | 44   |
|-------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Control |     |      |      |      |      |      |      | 8.66 | 6.57 | 7.57 | 8.35 | 6.02 | 7.67 | 7.52 |
| SD     | 2.42 | 1.22 | 0.35 | 0.85 | 1.32 | 1.15 | 0.67 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | I    | 8.66 | 6.02 | 7.67 | 7.52 | 6.57 | 7.57 | 8.35 |
| SD     | 1.45 | 1.55 | 0.59 | 1.19 | 1.88 | 0.58 | 0.48 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | II   | 5.73 | 7.19 | 7.31 | 6.44 | 7.38 | 7.48 | 6.62 |
| SD     | 2.10 | 2.04 | 0.97 | 1.96 | 1.30 | 1.58 | 0.93 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | III  | 25.71| 25.14| 31.28| 27.35| 28.10| 40.89| 32.64|
| SD     | 5.66 | 2.11 | 0.97 | 8.68 | 6.74 | 2.03 | 3.36 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | IV   | 9.56 | 10.19| 10.35| 8.30 | 7.31 | 10.76| 9.29 |
| SD     | 1.33 | 1.54 | 2.46 | 1.75 | 0.92 | 3.50 | 1.12 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | V    | 27.49| 22.50| 21.19| 23.22| 26.16| 16.52| 19.31|
| SD     | 3.71 | 3.40 | 3.96 | 5.96 | 5.37 | 4.22 | 2.23 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | VI   | 22.86| 28.41| 22.29| 26.34| 25.03| 16.68| 24.63|
| SD     | 6.07 | 9.11 | 5.43 | 6.55 | 2.96 | 2.35 | 2.93 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | I    | 9.59 | 10.45| 10.20| 5.48 | 20.16| 11.29| 9.81 |
| SD     | 1.23 | 2.01 | 1.56 | 0.97 | 1.07 | 1.93 | 1.26 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | II   | 8.22 | 10.45| 14.58| 6.85 | 6.73 | 11.29| 6.85 |
| SD     | 0.46 | 0.29 | 1.98 | 0.45 | 0.85 | 1.65 | 0.45 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | III  | 36.99| 26.87| 34.99| 30.14| 33.64| 38.71| 33.86|
| SD     | 5.56 | 12.3 | 2.63 | 7.60 | 2.33 | 4.95 | 1.27 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | IV   | 9.59 | 8.96  | 9.26  | 5.48 | 9.79 | 8.06  | 9.78 |
| SD     | 0.99 | 1.00 | 0.49 | 0.13 | 0.36 | 1.08 | 0.85 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | V    | 20.55| 25.37| 21.87| 38.36| 23.85| 17.74| 27.40|
| SD     | 2.34 | 2.45 | 1.12 | 5.47 | 2.37 | 1.20 | 5.11 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | VI   | 15.07| 17.91| 8.75  | 13.70| 5.29 | 12.90 | 3.34 |
| SD     | 5.77 | 6.21 | 2.50 | 2.36 | 1.57 | 3.98 | 1.87 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | I    | 9.00 | 10.69 | 10.29 | 6.47 | 7.62 | 8.02  | 5.59 |
| SD     | 2.99 | 4.42 | 1.12 | 1.29 | 2.33 | 1.10 | 1.12 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | II   | 7.16 | 6.07  | 7.23  | 4.63 | 4.88 | 5.41  | 4.70 |
| SD     | 0.88 | 3.33 | 1.21 | 0.51 | 0.46 | 1.09 | 0.93 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | III  | 28.21| 30.47| 21.76| 25.62| 29.50| 33.13 | 24.37|
| SD     | 8.72 | 5.04 | 4.83 | 2.58 | 4.87 | 4.36 | 3.53 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | IV   | 6.12 | 5.39  | 7.75  | 6.44 | 11.85| 6.16  | 6.48 |
| SD     | 0.90 | 1.34 | 1.89 | 1.07 | 8.10 | 0.49 | 0.73 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | V    | 21.60| 19.00| 25.79| 25.62| 24.96| 22.47 | 28.25|
| SD     | 6.12 | 9.11 | 7.91 | 3.26 | 7.81 | 4.43 | 1.94 |      |      |      |      |      |      |      |
|       |     |      |      |      |      |      |      | VI   | 27.90| 27.79| 27.19| 31.22| 21.18| 24.81 | 30.62|
| SD     | 6.22 | 8.12 | 6.54 | 0.92 | 1.48 | 1.84 | 2.40 |      |      |      |      |      |      |      |

Fig. 1: Photomicrographs illustrating neurons and glial cells using Nissl staining from Wernicke’s (area 22). Control cases (A, B and C) contrast with autistic cases (D, E and F). As expected with aging, the older control subject (C) demonstrated increased density of glial cells and decreased density of neurons. The changes are accelerated in autism, including the cohort’s youngest subject, age 7 (D)
Fig. 2: Density of glial cells, density of neurons, and density of lipofuscin-containing cells in brain areas 22, 39 and 44. Glial cells (A, B and C), neurons (D, E and F), and cells with lipofuscin (G, H and I) were counted along complete cortical columns. Values for controls subjects are graphed in black, for autistic subjects in gray. The horizontal axes correspond to age in years of the subjects, and the vertical axes represent the number of cells mm$^{-2}$. 

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In all three brain areas from age 8 to age 56 in area II by 770% (p = 0.0015); area 39 by 430% (p = 0.0001); and from age 8 to age 46 in area 44 by 189% (p = 0.0001). At corresponding ages, the lipofuscin cell-count was consistently higher in area 44 than in areas 22 or 39 (Fig. 2g-i). Laminar analysis demonstrated greatest gross number of cells containing lipofuscin in layers II and IV, layers with high cell density. Qualitatively, the size of individual lipofuscin aggregates appeared greatest in layers III and V, also the site of largest pyramidal cells (Fig. 3).

**Longitudinal analysis of autism data**

### Density of glial cells

| BA22     | Control | Autism |
|----------|---------|--------|
| I M      | 106.00  | 94.00  |
| SD       | 48.01   | 36.98  |
| II M     | 969.00  | 944.00 |
| SD       | 108.07  | 151.43 |
| III M    | 419.00  | 431.00 |
| DS       | 122.95  | 83.62  |
| IV M     | 988.00  | 1031.00|
| SD       | 108.25  | 103.64 |
| V M      | 500.00  | 619.00 |
| SD       | 140.31  | 162.57 |
| T M      | 590.00  | 572.00 |
| SD       | 103.00  | 132.00 |

Table 2: Number of glial cells mm⁻². M: Mean. SD: standard deviation. T: total cell density

- **BA39**
- **BA44**

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Fig. 3: Photomicrographs illustrating intracytoplasmic deposits of lipofuscin (arrows) in toluidine blue-stained semi-thin sections. The size of individual lipofuscin aggregates in the cerebral cortex of 17-year-old autistic subjects (B and D) are similar to those in the 52-year-old control subject (A and C). A steep rise in density of glial cells in area 22 between age 7 and 10 (42%) was statistically significant (p = 0.0424) and followed by a plateau from age 10 to age 31.

Laminar analysis (Table 2) demonstrated statistically significant increase in density of glial cells in all layers of area 22; the least increase from age 7 to age 44 was seen in layer V and VI (48%, p = 0.0080; 65%, p = 0.0040). Although full-depth cortical sections did not demonstrate statistically-significant age-related changes in glial density, significant increases were found in layer III of area 39 (136% from age 7 to age 44, p = 0.0026); layers IV and V of area 44 (97% increase from age 7 to age 44, p = 0.0041; 93% increase from age 7 to age 44, p = 0.0005).

Neuronal density: In the autistic cohort, decreased density of neurons over time in full-depth cortical samples was evident in all three brain areas, with a steep early decline followed by essential plateau in density of neurons no later than age 10 (Fig. 1d-f). From age 7 to age 44, density of neurons decreased in area 22 by 39% (p = 0.0324), in area 39 by 43% (p = 0.0144) and in area 44 by 30% (p = 0.0090). The most pronounced decrease in neuronal density from age 7 to age 44 in area 22 was layer VI (39%, p = 0.0351), in area 39, layer II (57%, p = 0.0042) and in area 44, layer II (42%, p = 0.007) (Table 3).

Lipofuscin-containing cells: In full-thickness cortical samples from autistic subjects, the number of lipofuscin-containing cells increased over time in all three areas of brain. Pronounced increases were found between age 7 and age 14 in area 22 (69%, p = 0.014), area 39 (149%, p = 0.0002) and area 44 (45%, p = 0.002) (Fig. 2g-i). In this younger subset, the range of lipofuscin-containing cells was 180 cells mm$^{-2}$ in area 22, 226 cells mm$^{-2}$ in area 39 and 690 cell mm$^{-2}$ in area 44 (Table 4).

Transversal analysis: Comparison of autistic versus control subjects.
Density of glial cells: The density of glial cells in full-thickness cortical samples was greater in autism than age-matched controls and there was no intersection of plotted curves. Collectively, samples from autistic subjects evidenced significantly greater density of glial cells in area 22 (90%, \( p = 0.0002 \)), area 39 (40%, \( p = 0.0032 \)) and area 44 (20%, \( p = 0.0262 \)). Greater mean density of glial cells was evident in autism compared to controls in all six cortical layers. Plotted curves for glial density demonstrated an approximate linear increase in control cohorts in all brain areas and in area 39 of the autistic cohort. For subjects of age 7 through 10, area 22 from autistic subjects evidenced a steep linear increase between age 7 and age 10, then plateau through age 26 and area 44 of the autistic cohort demonstrated a linear decline (Fig. 2a-c). Generally, laminar glial density in autistic subjects was greater than controls of corresponding age, although no difference was found in layer IV (granular) and layers II and III (supragranular) of area 44. It also was noted that the autism-versus-control difference in glial density gradually diminished between age 7 and age 31 in layers V and VI of area 44, while it increased in other layers (Table 2).
### Table 4: Number of cells containing lipofuscin/ mm²

|       | Control | Autism |
|-------|---------|--------|
|       | 8       | 17     | 22 | 30 | 46 | 52 | 56 | 7   | 9   | 14  | 25  | 31  | 44  |
| BA22  | 105     | 177    | 323 | 556 | 677 | 1145 | 1202 | 50.81 | 129.84 | 123.39 | 96.77 | 272.42 | 272.58 |
|       | 42.50   | 63.06  | 104.52 | 145.08 | 150.00 | 349.19 | 463.71 | 52 | 427 | 435 | 621 | 847 | 879 |
|       | 32      | 274    | 411 | 589 | 996 | 1105 | 1097 | 52 | 427 | 435 | 621 | 847 | 879 |
|       | 17.74   | 99.55  | 143.31 | 207.82 | 301.61 | 542.98 | 493.22 | 52 | 427 | 435 | 621 | 847 | 879 |
|       | 97      | 226    | 298 | 516 | 718 | 1097 | 1105 | 93 | 453 | 524 | 637 | 742 | 790 |
|       | 28.23   | 90.32  | 93.71 | 98.23 | 276.61 | 477.42 | 401.45 | 52 | 427 | 435 | 621 | 847 | 879 |
|       | 121     | 218    | 363 | 524 | 758 | 1395 | 1484 | 105 | 556 | 645 | 718 | 847 | 879 |
|       | 50.81   | 88.81  | 240.00 | 340.32 | 508.95 | 504.03 | 49.19 | 114.52 | 108.87 | 237.90 | 397.58 | 244.35 |
| BA39  | 129     | 298    | 419 | 556 | 694 | 1097 | 1089 | 115 | 524 | 645 | 790 | 847 | 879 |
|       | 90.34   | 99.55  | 143.31 | 207.82 | 301.61 | 542.98 | 493.22 | 52 | 427 | 435 | 621 | 847 | 879 |
|       | 97      | 226    | 298 | 516 | 718 | 1097 | 1105 | 93 | 453 | 524 | 637 | 742 | 790 |
|       | 28.23   | 90.32  | 93.71 | 98.23 | 276.61 | 477.42 | 401.45 | 52 | 427 | 435 | 621 | 847 | 879 |
|       | 121     | 218    | 363 | 524 | 758 | 1395 | 1484 | 105 | 556 | 645 | 718 | 847 | 879 |
|       | 50.81   | 88.81  | 240.00 | 340.32 | 508.95 | 504.03 | 49.19 | 114.52 | 108.87 | 237.90 | 397.58 | 244.35 |

**Neuronal density:** Mean neuronal densities for full-thickness cortical samples were significantly reduced in the autistic cohort as compared to controls in area 22 (38% reduction, \( p = 0.0012 \)) and area 39 (24% reduction, \( p = 0.0011 \)), but no significant difference was found in area 44. In relation to controls, decrements in neuronal density were greatest in area 22. Values in autism were lower at all corresponding ages in area 22 and all but age 7 in area 39. Steep initial declines in neuronal density from age 7 to age 10 and essential plateau thereafter in areas 22 and 39 in autism contrasted with gradual linear declines in controls (Fig. 2d-f).

In area 22, the difference in neuronal density grew from 20% fewer neurons at age 10 (\( p = 0.0132 \)) to 42% fewer neurons in adolescence (\( p = 0.0395 \)) (Fig. 2d). The greatest difference in neuronal cell density in area 22 was evident in layers II and IV (Table 3). The neuronal density in area 39 for the control group declined very little with increasing chronological age. Neuronal density in area 39 was equivalent for the youngest autistic (age 7) and control (age 8) subjects. In area 39, a steep decline in neuronal density was evident between age 7 and ages 9/10 in autism (34%; \( p = 0.0282 \)), but density in the eldest autistic subject (age 44), was not substantially lower than at ages 9/10 (Fig. 2e).
Fig. 4: Photomicrographs illustrating glial cells using immunocytochemical staining methods for the glial fibrillary acidic protein (GFAP). A and B demonstrate protoplasmic astrocytes and C and D demonstrate fibrous astrocytes. Conspicuous alterations of the morphology of the glial cells are evident in autistic patients (B and D), compared to control cases (A and C).

Laminar analysis indicated that the decrement of neurons evident in autism relative to controls at age 7 in area 22 was most pronounced in layers V or VI (the infragranular layer), than in layers II or III (Table 3). By age 10, the neuronal density in layer II of area 22 in autism was 53% lower than corresponding control (p = 0.0055). In area 44, lower neuronal density in autism versus controls was found primarily in cortical layers II and III in younger subjects; in older subjects, decrements relative to controls were found in nearly all layers (Table 3).

Lipofuscin-containing cells: The density of lipofuscin-containing cells in full-thickness cortical samples was greater in the autistic cohort than controls: area 22 (50% greater, p = 0.0007) and area 39 (44% greater, p = 0.0096). Other than equivalent values for the youngest of either cohort for areas 22 and 39, autistic subjects showed greater density of lipofuscin-containing cells than controls at all corresponding ages. The youngest (age 7 years) member of the autistic cohort evidenced greater density of lipofuscin-containing cells (46% greater, p = 0.0086) in area 44 than the corresponding 8-year-old control.

Relative to controls, the autistic group evidenced steep increases in the density of lipofuscin-containing cells between age 7 and 14 in all three brain areas. (Fig. 2g-i) For instance, the density of cells with lipofuscin in area 22 in autistic subjects of age 9/10 was equivalent to density in the control subject of age 17; by age 14, the density of cells containing lipofuscin in autism were approximately twice the density in control subject of age 17. (p< 0.0168) (Fig. 2g). Similar sharp rises were apparent among younger members of the autistic cohort in areas 39 and 44. However, significant increases were not observed in area 44 in autism after age 10 (Table 4).

Laminar analysis demonstrated greater density of lipofuscin-containing cells particularly in layers II and IV in all three areas of brain (Table 4). Microscopic
observation imparted the impression that larger lipofuscin aggregates were more concentrated in layers III and V in areas 22 and 39.

**Morphological analysis of glial cells:** Variant astrocyte morphology was observed in autism. In comparison to controls, both the subclass of protoplasmic astrocytes (characterized by short, thick, highly-branched processes) and the subclass of fibrous astrocytes (characterized by long, thin, less branched processes) demonstrated earlier hypertrophy of the perikaryon and processes in autism. (Fig. 4a-d) Further, the subclass of protoplasmic astrocytes exhibited larger size, denser ramifications of fine processes and a fibrinoid aspect in autism that was not appreciated in control specimens (Fig. 4b).

**DISCUSSION**

The present autism study constitutes the first neuroanatomical analysis of diverse regions of the cerebral cortex involved in linguistic processing and production of speech, across a broad age-group. Autistic cortex was markedly altered in comparison to age-matched controls, including increased density of glial cells, decreased density of neurons and increased density of nonspecific lipofuscin-containing cells.

Previous reports suggest neurodevelopmental aberration in autism. Kemper and Bauman (1993) reported a coarse and poorly-laminated cingulate cortex in autism and other authors have presented functional evidence of abnormal connections, as well as a delay in the maturation of some cortical circuits [9-12]. Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI) scans of autistic subjects suggest cortical ectopias, a distinctive feature of altered neuronal migration [10, 13-15].

The present study found no deviation from normal cortical layering pattern, evidence of ectopic cells, nor differences in the relative thickness of cortical layers in areas 22, 39 and 44 of brain from autistic subjects. If the cytoarchitectonic criteria of Benes [16] are valid, these findings argue strongly against the hypothesis of altered neuronal migration in the neuropathogenesis of autism.

**Cortical gliosis in autism:** In all three brain areas (22, 39 and 44), full-thickness cortical samples from autistic subjects of all ages demonstrated greater density of glial cells in comparison to controls (up to more than double). Since thickness of the cortical layers is not altered in autistic subjects, the finding of greater glial density in autism implies a greater gross number of glial cells in each area due to cellular proliferation and not due to reduction of neuropil volume. Proliferation of glial cells as well as the hypertrophic morphological changes in glia reported in this study are consistent with reactive gliosis in autism.

Proliferation of glial cells occurs in diverse pathologies, including ischemia, trauma, neurodegenerative disorders and senescence [17-22]. Glial fibrillary acidic protein (GFAP), a major intermediate structural filament protein expressed predominantly in mature astrocytes, is considered a hallmark of gliosis under conditions of neuronal injury [23-25]. A marked increase in the number of neuroglial cells in the middle frontal gyrus, anterior cingulate gyrus and cerebellum of autistic patients, as well as increased inflammatory cytokines in parenchyma and cerebrospinal fluid (CSF) have been reported in autism [26]. Significantly elevated concentrations of GFAP were reported in the CSF, frontal, parietal and cerebellar cortices of children with autism [27, 28]. The results of the present study present further evidence for widespread gliosis in autism.

A toxic etiology for autism has not been excluded. Many toxins induce gliosis, including metals such as lead, iron and mercury, which specifically induce glial proliferation, degeneration and decreased cellular function in some regions of the brain [29-31]. Neurotoxicity of metals is primarily mediated by increased oxidative stress and both increased metals and increased oxidative stress are reported in autism [32, 33]. Hypothetically, environmental exposure in sensitive subjects might underlie glial proliferation and neuronal death in the pathogenesis of autism.

**Neuronal attrition in autism:** Other investigators have reported reduced numbers of neurons (cerebellar Purkinje and granule cells) [34-36] in brain of subjects with autism, but there are no prior reports of decreased neurons in cerebral cortex. Coleman [37] examined auditory brain regions, including Broca's area, from a single subject with a presumptive diagnosis of autism, but found no differences in neuronal density relative to control subjects. Magnetic resonance imaging of autistic subjects of 3-4 years of age demonstrated less n-acetyl aspartate (NAA) [38], a metabolite produced by neurons, but not glia.

The present study employed a larger cohort of systematically diagnosed autistic subjects and found a striking reduction in neuronal density in area 22 and 39 in autism relative to controls, including lower density of neurons in area 22 from the youngest member of the autistic cohort (age 7). Neuronal density in area 39 was equivalent in the youngest autistic subject and youngest control (age 8), suggesting that the reduced neuronal density in older autistic subjects is not explicable on the
basis of failed genesis, but due to attrition. Loss of
cortical neurons has been described in other
neuropsychiatric pathologies, such as schizophrenia,
bipolar disorder and major depressive disorder, all of
which coexist with different cognitive impairments,
some similar to those observed in autism[41-44].

Decrease in density of neurons in autism was most
pronounced in cortical layers II and IV. While the
present study did not separately tabulate pyramidal cells
and smaller neurons, its findings are comparable to
decreased pyramidal cells in layers II and III and, less
conspicuously, in layers V and VI in frontal and
temporal neocortex in Rett syndrome[45], a
neurodegenerative disease associated with autism[46-48].
The relationship of increased glial cell density and
decreased neuronal cell density varied in autism by
brain area. Increased glial density coincided with
decreased neuronal numbers in area 22 and are 39, but
not area 44. A significant increase in glial density in the
youngest member of the autistic cohort (age 7) was
associated with decreased neuronal density in area 22,
but not in area 39. Determination of glial and neuronal
densities in a larger cohort including younger subjects
is needed to elucidate the temporal and functional
relationship of these two parameters.

**Indications of oxidative stress in autism:** One of the
more interesting findings of the present study is the
significant increase of lipofuscin-containing cells in
autism. Lipofuscin is classically associated with
recognized neurodegenerative diseases and there is no
prior record of previous examination of brain from
autistic subjects for lipofuscin. Increased lipofuscin was
reported previously in brain from subjects with Rett’s
syndrome[49-51].

Lipofuscin is an intralysosomal polymeric material
originated from autophagocytosed cellular components
that cannot be degraded or exocytosed. Biochemical
analysis of lipofuscin reveals a complex aggregated by-
product composed primarily of oxidatively-modified
proteins and lipids[52-55]. In addition, lipofuscin is a
depot for metals, including redox-active and heavy
metals[56]. Lipofuscin accumulation in cells is
accelerated under conditions of oxidative stress[57-59].
Experimentally, lipofuscin itself induces neurotoxicity
via generation of free-radicals[52-54].

Several authors have proposed the involvement of
free radicals and cross-linking reactions by
intralysosomal degradation products, as initial steps in
the formation of lipofuscin[58,60,61]. Higher levels of
lipofuscin may be viewed as a marker for heightened
oxidative processes which are potentially harmful to
brain cells[58], or for greater oxidative damage to brain
cells.

Extensive research has demonstrated that oxidative
stress plays a seminal role in the pathology of several
neurological diseases, including Alzheimer’s
disease[82,83], Down’s syndrome[84], Parkinson’s
disease[85,86], schizophrenia[87,88] and bipolar affective
disorder[89]. Interestingly, recent studies have reported
increased free-radicals, lower levels of antioxidant
proteins and greater oxidatively modified biomolecules
in peripheral samples from autistic children[50-71].

Significantly increased numbers of lipofuscin-
containing cells were found in the three brain areas of
subjects with autism and were most pronounced in area
22, which also featured the greatest increase in glial
density and greatest decrease in neuronal density. Since
the lipofuscin cell-count did not differentiate neurons
and glia, the profile of brain cells with increased
lipofuscin in autism remains undetermined. As noted
earlier, the size of lipofuscin aggregates was judged
greater in layers III and V in areas 22 ad 39, which is
noted to be the cite of the largest pyramidal neurons. In
one area (44), increased lipofuscin was evident in the
youngest of the autistic cohort (age 7), suggesting that
greater lipofuscin as well as differential glial and
neuronal densities may be detectable in younger autistic
subjects in further studies.

The data presented are strictly phenomenological
and do not afford firm conclusions about mechanistic
relationships among the three measured parameters.
While the study did not determine the extent of glial
(versus neuronal) lipofuscin content, it is known that
lipofuscin appears in reactive glial cells[72] and that
oxidative stress in glial cells alters their function[73,74].
Greater lipofuscin in brain of autistic subjects of age
7-44 probably reflects greater oxidative stress in brain,
as suggested by peripheral measurements of oxidative
stress in autism[75,76]. The increased numbers of cells
containing lipofuscin may reflect (a) an
environmentally-induced reactive glial response
injurious to neurons, (b) an environmentally-induced
direct oxidative injury to neurons, or (c) both.

In summary, the present study of language-related
cortical areas 22, 39 and 44 demonstrated a greater
density of glial cells, a lesser density of neurons and an
decrease in density of non-specific lipofuscin-containing
brain cells in autism. Changes in these parameters were
progressive and varied in extent by cortical area and
layer.

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