Increase in HLA-DR Immunoreactive Microglia in Frontal and Temporal Cortex of Chronic Schizophrenics

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Abstract. Glia play a major role in neuronal migration, synapse formation, and control of neurotransmission in the developing and mature nervous system. This study investigated whether chronic schizophrenia is associated with glial changes in 3 regions of the cerebral cortex: dorsolateral prefrontal cortex (Brodmann’s area 9), the superior temporal gyrus (area 22), and the anterior cingulate gyrus (area 24). In a blind study, astroglia and microglia were identified immunocytochemically in frozen sections from postmortem schizophrenic and control brains. Astroglia and microglia were identified using antibodies to glial fibrillary acidic protein (GFAP) and class II human leucocyte antigen (HLA-DR) respectively. They were then quantified for each cortical layer. Significant differences were found in HLA-DR+ microglial numerical density in 2 of the areas. A 28% increase (p < 0.05) was found in area 9 in 8 schizophrenics (115 ± 9 cells/mm²) compared with 10 controls (89 ± 5 cells/mm²), when combining all cortical layers and both cerebral hemispheres. For area 22, there was a 57% increase (p < 0.01) in microglia in 7 schizophrenics (139 ± 6 cells/mm²) compared with 10 controls (88 ± 5 cells/mm²). In area 24 the same trend was evident, but the results did not reach significance. Microglial number was further analyzed for each cortical layer, which confirmed the overall pattern. For all areas, numerical density of astroglia showed no significant differences between schizophrenics and controls. Cortical thickness was measured in all areas and total neuronal numerical density was estimated for area 22. Again, no significant differences were found between schizophrenics and controls. This study demonstrates a specific increase in the numerical density of HLA-DR+ microglia in temporal and frontal cortex of chronic schizophrenics, not related to aging, which might be implicated in possible changes in cortical neuropil architecture in schizophrenia.

Key Words: Astroglia; Cerebral cortex; Glia; Immunohistochemistry; Microglia; Schizophrenia.

INTRODUCTION

The aetiology of schizophrenia remains obscure. Proposed hypotheses attempting to explain dysfunction in cortico-limbic pathways of the schizophrenic brain include neurodegeneration, aberrant neurodevelopmental processes, viral infections, and metabolic disorders. Much evidence suggests that schizophrenia is associated with disturbed brain development (1–4). If the disorder is developmental, the disease process is likely to be active before, rather than in parallel with, the clinical progression of the disease. Also, genetic and environmental factors would act in utero or in early childhood (4). Neuromotor, behavioral, and intellectual abnormalities in children who developed schizophrenia in adult life (5–7) suggest that brain structure and function may be impaired long before the onset of overt schizophrenia and support a neurodevelopmental hypothesis.

Computerized tomography and magnetic resonance imaging (MRI) have indicated cerebral ventricular enlargement, reduced cortical asymmetry, and a loss of cortical volume, particularly in the temporal lobe. Some neuroimaging data suggest that these abnormalities may be

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fully developed by, or before, the onset of illness (8). They appear to be nonprogressive in most patients, suggestive of an early developmental anomaly, as opposed to a lesion incurred at the time of symptom onset. However, there are contradictory reports of progressive changes in brain morphology over a 5-year period, more consistent with a neurodegenerative aetiology (9–12). This process may occur throughout life, attributable to periodic activation of defective genes that regulates cerebral growth (13). Such dysfunction would cause prenatal maldevelopment and then be reactivated during adolescence. As the brain matures and cortical connections are established, such maldevelopment could result in aberrant cellular interactions via improper neuronal migration, proliferation, or cell death, leading to dysfunctional intracortical communication. Overall, the evidence suggests a neurodevelopmental disturbance in association with a progressive, possibly neurodegenerative process with age in schizophrenia.

There is increasing evidence for synaptic pathology in schizophrenia (14–18). Although this is consistent with a neurodevelopmental abnormality, the origin of synaptic pathology could equally be attributed to neurochemical or viral mechanisms. Evidence from postmortem studies on schizophrenic brain suggests a number of subtle cortical neuronal abnormalities affecting many brain regions. However, there are contradictory findings even within the same brain regions and neuronal populations. Areas that consistently show significant findings are the hippocampal formation, and the frontal and cingulate cortex, where
alterations in cytoarchitecture, reduced neuronal soma size, and cytoskeletal abnormalities have been described (15, 19–26). In particular, a subset of inhibitory gamma-aminobutyric acid (GABA)-ergic interneurons are reportedly lost from specific cortical layers, or migrate abnormally into the cortex. These findings suggest a possible defect in inhibitory GABA transmission. There are, however, inconsistencies regarding this data (21).

Recent studies have shown that the number of dendritic spines is reduced on cerebral cortical pyramidal cells in temporal and frontal cortex (15, 27). These subtle alterations of cortical architecture could account for the synaptic disturbances implicated in this disorder. Overall, it is likely that the main structural changes in schizophrenia are in the neuropil rather than at the level of neuronal number (15, 28).

Few studies have investigated abnormalities in glial cells in schizophrenia. If subtle neuronal abnormalities occur, one might expect them to be reflected in one or more of the glial cell types since they are implicated in many important functions in the CNS. Attempts to demonstrate glial pathology in schizophrenia have largely depended on histological studies for the presence of astrogliosis. Harrison (3) suggested that the absence of astrogliosis, in the presence of neuronal and cytoarchitectural abnormalities, is one of the main indicators of a developmental as opposed to a degenerative process. Many studies have found contradictory evidence regarding gliosis but the overall conclusion is that there is no increase in astrogliosis (19, 24, 29–32). Much of the discrepancy in the data may be attributed to a lack of neuropathological screening of the cases under study. Recent studies have used more sensitive and discriminating techniques, such as immunocytochemistry rather than histological stains, for identifying specific glial cells. It is likely that the issue of glial changes in schizophrenia has been hampered by the difficulty of discriminating these cell types in postmortem tissue, simply classifying them into either one category or inadequately into 2 groups (astroglia, and oligodendrocytes with microglia) (24).

The present study was designed to detect any changes in astroglia and microglia in cortical areas implicated in schizophrenia. Glial cells and neurons function as a unit, the integrity of which is of fundamental importance to many aspects of neural structure and function. For example, neurotransmitter receptors on astroglia extend the potential networks of communication in the brain and thus alter the concept of an entirely neuronal synaptic circuitry. If neuronal abnormalities are subtle and glia act to modulate neuronal function, one might expect glial changes in the affected areas. We have focused on 3 of the regions most prominently implicated in schizophrenia research by in vivo PET and postmortem studies. These are the dorsolateral prefrontal cortex (Brodman’s area 9), the superior temporal gyrus (Brodman’s area 22), and the anterior cingulate cortex (Brodman’s area 24). We demonstrate for the first time a specific increase in HLA-DR* microglia in 2 of these regions, areas 9 and 22, although no significant change was observed in cingulate cortex. The increases occurred in both hemispheres and in all cortical layers, and were not attributable to cohort variables such as age, sex, or postmortem delay. In contrast, no significant changes were observed in astroglial number in schizophrenics compared with controls.

MATERIALS AND METHODS

Tissue Preparation

Brain material, both schizophrenic and control, was obtained through the Charing Cross Prospective Schizophrenia Study including cases from Charing Cross Hospital, London, and the State Psychiatric Hospital, Wiesloch, Germany. Institutionalized schizophrenic patients were recruited as donors with the cooperation of family and clinicians, and the approval of local ethical committees. Control material was obtained from routine autopsies or donated bodies. Clinical histories and neuropsychological assessments of the schizophrenic patients were available. DSM III-R criteria were used to diagnose schizophrenia. The patients studied here were all chronic schizophrenics of a predominantly paranoid type. Access to the clinical data and diagnosis was only permitted once the quantitative work was complete to ensure that the work was carried out “blind” to avoid observer bias. A standardized protocol for the dissection of brains was followed. Twenty-three brains were collected and used in this study, 12 from schizophrenics and 11 from controls. There were no significant differences in age amongst the groups. The mean age of the schizophrenics was 80 ± 8, and of the controls 72 ± 13 (Table 1). However, there was a significant difference between the postmortem interval (PMI—the time from death to fixation of the brain specimens) for the schizophrenic and control groups. Therefore, the effect of PMI on the quantification of glial cells was investigated.

A routine neuropathological examination was carried out during and after the dissection. Parkinson and Huntington diseases were excluded on clinical history, and by examination of the substantia nigra and caudate nucleus. The presence of Alzheimer disease (AD) was assessed by clinical history, macroscopic examination of the brain, and a modified CERAD procedure (33), using immunocytochemical screening of sections from cingulate, hippocampal, frontal, occipital, and parietal areas of the cerebral cortex for plaques and tangles with antibodies against β-amyloid protein (1E8; SmithKline Beecham) and Tau protein (AT8; Immunogenetics). This data is presented for areas 9 and 22 in the Table 1. Three of the brains had histological evidence of AD. Two were from the schizophrenic group and had clinical dementia, with global dementia scores of 6 and 4. The third was from the control group, although clinical dementia was not recorded. These cases were excluded from the study since glial changes are a prominent feature of AD (34, 35).

Cortical blocks were removed at dissection and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Two blocks were removed from areas 9, 22, and 24 of right and left
Immunohistochemical assessment of β-amyloid protein deposition and neurofibrillary tangles in the dorsolateral frontal cortex (area 9) and temporal cortex (area 22) of schizophrenic, control and Alzheimer disease brains. The range is 0 = no deposition, to +++ = very large numbers of plaques. The classification of Alzheimer disease was made following modified CERAD criteria.

Immunocytochemistry

Sections were immersed in 0.1 mM PBS and then treated with cold methanol for 5 minutes (min). To block endogenous peroxidase activity, sections were incubated (2 × 20 min) in a solution of 3% hydrogen peroxide in 0.1 mM PBS. They were then washed (2 × 5 min) in PBS, and treated for 40 min with a 1.5% serum solution (goat or horse) in 0.1 mM PBS containing 0.2% Triton X-100 (TPBS). Excess serum solution was removed before incubating overnight at room temperature with the primary antibody solution in TPBS. A polyclonal rabbit antibody against glial fibrillary acidic protein (GFAP, Dako, 1:5) for microglia. Slides were washed (2 × 5 min) in PBS and incubated for 1 hour (h) at room temperature in a biotinylated secondary antibody solution (Vectastain; 1:200 in TPBS + 1.5% serum) and then washed again (2 × 5 min). They were then incubated for 1 h at room temperature with a peroxidase-conjugated biotin-streptavidin complex (Vectastain; 1:50 in 0.1 mM PBS). The reaction product was visualized with a solution of 3,3′-diaminobenzidine (0.05%), cobalt chloride (0.25%), and hydrogen peroxide (0.1%) in 0.1 mM PBS for 5 to 8 min. The reaction was stopped by washing twice in PBS. Sections were then dehydrated and mounted using DPX.

Separate sections from each region were stained with cresyl fast violet in order to determine cortical cytoarchitecture and thickness. Additionally, sections of superior temporal cortex were incubated for 5 min with 4′, 6-diamidino-2-phenylindole (DAPI) to label all cell nuclei for the estimation of total numerical cell density.

Image Capture and Analysis

For the determination of glial numerical density, images were captured by light microscopy at low power using the ×10 objective of an Olympus BHS microscope connected to a JVC 3-CCD color video camera. Final magnification to the camera was ×50. For each slide, 3 cortical strips were chosen midway between the depths of a sulcus and the crest of a gyrus, where cortical organization was most uniform. Along each strip, 1 image per layer was captured. The laminar organization was determined by comparison with an adjacent cresyl violet section. In total, 18 images were captured per slide. Thus, 54 images per hemisphere were obtained for each area. For glial

### Table: β-Amyloid Deposition and Neurofibrillary Tangle Scores for the Brain Cohort

<table>
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<th>Patient no.</th>
<th>Age (yrs)</th>
<th>Area 9 β-amyloid</th>
<th>Area 9 NF tangles</th>
<th>Area 22 β-amyloid</th>
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Hemispheres and stored at 4°C for approximately 2 months, after which they were cryoprotected in 30% sucrose in PBS for 24 h. Sections were cut at 20 μm and floated on 0.1 mM PBS. They were then mounted on coated slides and allowed to dry. Slides were stored frozen at −20°C.
Changes in glial numerical cell density with fixation delay time. Blocks of superior parietal cortex (Brodmann’s area 7) were fixed in 4% paraformaldehyde at varying times postmortem. Three sections from each block were immunolabelled for microglia using the LN3 anti-HLA-DR antibody (a) and for astroglia using an anti-GFAP antibody (b). Eighteen images \((\times 50)\) were captured per slide and the glia were counted for each image (as described in the Materials & Methods section). The values represent the average counts across cortical layers I–VI ± S.E.M. and \(n = 9\) (*\(p < 0.01\) compared with 14 h).

For determining total cell numerical density, images were captured from DAPI labelled sections by fluorescence microscopy (UV filter channel) at \(\times 20\) magnification (final magnification to camera: \(\times 100\)). Three cortical strips per slide were chosen midway between a sulcus and a gyrus, as before. Images were then captured consecutively from layer VI to the pial surface. The average number of images to capture the whole strip was 17. Total cell density was then determined by counting the stained nuclei.

For measuring cortical thickness, images were captured from the cresyl violet sections at \(\times 1\) magnification (final magnification to camera: \(\times 5\)). Two images, each including all cortical layers, were captured per slide. Measurements were made midway between a gyrus and a sulcus, as for glial quantification. A marker was placed on the pial surface and a line drawn perpendicularly to the white matter interface. Measurements were repeated 3 times for each image and the mean taken as the cortical thickness.

RESULTS

Effect of Postmortem Interval on Quantification of Glia

Before determining if glial changes occurred in schizophrenics, it was important to exclude factors unrelated to the disease process itself that might affect these changes. To ensure that GFAP and LN3 antibodies could be used reliably to quantify glia, it was necessary to establish whether the antigens were stable over the PMI range of our cohort. Samples with a very short PMI of 4 h were kept in PBS and then fixed in 4% paraformaldehyde at various times after dissection to reproduce the effects of increasing PMI. Sections were then immunostained with LN3 and GFAP antibodies, as described above, and a comparison of tissue quality and glial densities was made for each time point. HLA-DR⁺ microglial numerical cell density did not vary substantially over a 5 to 39 h fixation delay, although a slight increase was observed at 21 h (Fig. 1a). At postmortem delays of over 31 h, the quantification of microglia was unreliable due to deterioration in section quality and cell morphology. Since microglial density was not significantly different from 5 to 31 h fixation delay, it is reasonable to assume that the HLA-DR antigen is stable over an equivalent PMI. There was also no significant change in astroglial numerical density after fixation delays of 5 to 31 h (Fig. 1b).

Astroglial Changes

GFAP immunoreactivity prominently revealed astroglial cell bodies and thread-like processes (Fig. 2a, b). The cells in control and schizophrenic cases were similar in morphology. Long processes were particularly obvious traversing layer II. Regional variations in astroglial number were evident in controls and schizophrenics. Significantly more astroglia were present in the grey and white matter of the cingulate cortex than in frontal and temporal cortices (51% more in grey matter and 54% more in white matter). There was a trend towards increasing astroglial numerical density in the order temporal < frontal < cingulate cortices. No significant differences were observed in average astroglial numerical density between control and schizophrenic groups in any of the 3 cortical regions (Fig. 3).
INCREASED MICROGLIA IN SCHIZOPHRENIA

Fig. 2. Astroglial and microglial immunohistochemistry. Immunoperoxidase labelling of 20 μm cryosections with an anti-GFAP antibody for astroglia (A, B) and with an anti-HLA-DR (LN3) antibody for microglia (C, D). Tissue is from the superior temporal cortex of control (A, C) and schizophrenic (B, D) subjects. Images are taken from layer III of the cortical grey matter and the scale bar represents 40 μm.

Since no significant differences were evident between schizophrenic and control groups, astroglial numerical density was further analyzed in individual cortical layers for each area. Again no significant differences were seen between the 2 groups (Fig. 4). The pattern of astroglial distribution between layers was also similar. Substantially elevated astroglial numbers were seen in layer I compared with other layers and the white matter. Slightly increased numbers were also evident in layer VI and the white matter compared with layers II–V. The greater astroglial density in the cingulate cortex was mainly attributable to larger numbers in layers I and Va.

The laminar distribution of astroglia in frontal and temporal cortices resembled the distribution in cingulate cortex. Again, no differences were found between control and schizophrenic groups for each layer and increased astroglial density was observed in layers I and VI and in the white matter, compared with layers II–V. The schizophrenic group exhibited a similar astroglial distribution to the control group in all areas.

Microglial Changes

Microglia in both control and schizophrenic brains were found to label intensely with antibodies to the HLA-DR antigen (Fig. 2c, d). The laminar distribution of microglia (as shown by numerical density of microglia in the controls) was similar for all areas examined, showing a similar density across cortical layers I–V and a significantly greater density of cells in layer VI. The number of microglia tended to increase with the transition from grey to white matter. Although microglia were not quantified in the white matter, a qualitative comparison showed that more HLA-DR immunoreactivity was observed in white matter than in grey matter.

An increase in the average HLA-DR<sup>+</sup> microglial number across layers I–VI in the schizophrenics was seen in all 3 areas (Fig. 5). In the cingulate cortex, this difference did not reach statistical significance. In contrast, in areas 9 and 22, significant increases in microglial number were observed in the schizophrenic group. Neither tissue destruction nor inflammatory cell infiltration was detectable.
Fig. 3. Comparison of the average astroglial numerical cell density. GFAP$^+$ astroglia were counted on 6 cryosections per case using 18 fields ($\times$50) per slide for each cortical region. Cells were counted for each cortical layer in both cerebral hemispheres. Estimates from both cerebral hemispheres were averaged to give a mean astroglial numerical cell density per cortical layer. Values in this figure represent the average cell density across cortical layers I–VI ± S.E.M. The number of cases for each group were as follows: anterior cingulate cortex—control $n = 5$, schizophrenic $n = 8$; dorsolateral prefrontal and superior temporal cortex—control $n = 10$, schizophrenic $n = 8$. No significant differences were observed.

Fig. 4. Cortical distribution of astroglia. Six cryosections per case were immunolabelled with an anti-GFAP antibody. GFAP$^+$ astroglia were counted manually in 21 fields ($\times$50) per section. Cells were counted in each cortical layer and in the white matter for both cerebral hemispheres. Values represent the mean astroglial density per cortical layer, from both cerebral hemispheres ± S.E.M. Numbers ($n$) for each group are given in Figure 2.

in any area. In area 9 there was a 28% increase ($p < 0.05$) in microglia in schizophrenics ($115 \pm 9$ cells/mm$^2$) versus controls ($89 \pm 5$ cells/mm$^2$). In area 22, the increase in microglia in schizophrenics was even greater at 57% ($p < 0.01$; $139 \pm 6$ cells/mm$^2$ in schizophrenics versus $88 \pm 5$ cells/mm$^2$ in controls).

A comparison of HLA-DR$^+$ microglial number was made for layers I–VI for each area studied (Fig. 6) to determine whether the increases in microglia in schizophrenic cortex occurred throughout a cortical area or specifically in individual layers. In area 24, although there was a general trend for increased microglial density in layers I–VI in schizophrenics, there were no significant differences between schizophrenics and controls for any given layer. In area 9, an increase in microglia was evident for all layers in the schizophrenic group, and was not layer-specific. The increases in microglial density in schizophrenics reached significance in layer I (28%), layer II (29%), layer III (41%), and layer IV (34%). In area 22, increases in microglial density in the schizophrenic group were evident for all layers, as in the frontal cortex, and were highly significant (67% for layer I, 68% for
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Fig. 5. Comparison of the average microglial numerical cell density. HLA-DR+ microglia were counted on 6 cryosections per case using 18 fields (×50) per slide for each cortical region. Cells were counted for each cortical layer in both cerebral hemispheres. Estimates from both cerebral hemispheres were averaged to give a mean microglial numerical cell density per cortical layer. Values in this figure represent the average cell density across cortical layers I–VI ± S.E.M. The number of cases for each group were as follows: anterior cingulate cortex—control n = 5, schizophrenic n = 8; dorsolateral prefrontal and superior temporal cortex—control n = 10, schizophrenic n = 8. Significant differences were determined using Student’s T-test for independent variables where *p < 0.05 and **p < 0.01.

layer II, 70% for layer III, 65% for layer IV, 53% for layer V, and 35% for layer VI).

Resting ramified microglia, with small oval cell bodies and multiple long, thin processes, were evident throughout the cortex of control cases (Fig. 7a–c), demonstrating the constitutive expression of MHC class II molecules on human microglia. Microglia in schizophrenic cortex resembled those seen in the controls, although their processes were typically more ramified (Fig. 7d–f). The greatest increase in microglial number in schizophrenics was in the temporal cortex where the cell density was equivalent to that for the AD cases. Such an increase in microglial density in the temporal cortex in AD is well documented (35). However, in AD the microglia have an “activated” morphology (Fig. 7g, h). Activated microglia possess an enlarged cell body with thickened, elongated processes. Most microglia in the Alzheimer cases had this morphology. Thus, although the number of microglia is increased in the schizophrenic group, their morphology is not drastically altered. The immunoreactivity for HLA-DR in the schizophrenic group is slightly more intense which may suggest an up-regulation on these microglia.

Fig. 6. Cortical distribution of microglia. For each cortical hemisphere, 3 cryosections per case were immunolabelled with anti-HLA-DR antibody. LN3+ microglia were manually counted in 18 fields (×50) per section. Cells were counted for each cortical layer in both cerebral hemispheres. Values represent the average cell density across individual cortical layers I–VI ± S.E.M. Numbers (n) for each group are given in Figure 2. Significant differences were determined using Student’s T-test for independent variables (*p < 0.05, **p < 0.01, ***p < 0.001).
Fig. 7. Morphology of HLA-DR+ microglia. The microglia in control tissue predominantly expressed a ‘resting’ ramified morphology characterized by a small cell body and multiple long and thin processes (A–C). The microglial cell processes can
and there was a tendency towards an increased number of processes. However, a full densitometric analysis would be required to substantiate these observations. Microglia in both control and schizophrenic tissue were often closely associated with neuronal cell bodies.

It has been suggested that schizophrenia may be associated with differential effects in the 2 hemispheres. However, our results showed no differences in microglial density or laminar distribution between hemispheres for either control or schizophrenic groups.

**Correlation of Microglial Changes with Confounding Variables**

To validate whether the increased microglial numerical density in schizophrenia was due to the disorder, the estimates were correlated with other cohort parameters, which might have accounted for the differences. These variables included age, PMI and β-amyloid deposition.

**Effect of Age on Microglia:** Previous studies have reported conflicting views with regard to the association between microglial numerical density and age. Rogers et al (36) reported a linear increase of HLA-DR reactivity in white matter microglia with age, whilst Mattiace et al (37) found only a slight increase with age. We therefore tested for an association of age and microglial density for temporal and frontal cortices. In the control group, a slight increase in density is evident with increasing age ($r^2 = 0.55; p < 0.05$ in temporal and 0.20 in frontal cortices), but no correlation between microglia and age was found in schizophrenics ($r^2 = 0.02$ and 0.00, respectively).

**Effect of PMI on Microglia:** Because there was a significant difference in PMI between schizophrenic and control, HLA-DR+ microglial numerical cell densities in temporal and frontal cortex were compared with PMI. No significant correlation between microglial density and PMI for either the control or schizophrenic groups was found (all $r^2$ values are <0.12). This is in accord with our findings that the HLA-DR antigen was stable with postmortem delays of up to 30 h (Fig. 1a).

**β-Amyloid Deposition and Microglia:** Increases in microglial numerical density have been associated with amyloid deposition in the brain (34, 38). Therefore, microglial densities in frontal and temporal cortex of schizophrenics and controls were compared with β-amyloid deposition in the same areas (Fig. 8 and see the Table 1 for β-amyloid deposition data). In the control group (Fig. 8a), the association between amyloid deposition and microglial density was very weak for frontal cortex ($r^2 < 0.01$) and moderate for temporal cortex ($r^2 = 0.34$). There was more of an association for the schizophrenic group (Fig. 8b; $r^2 = 0.40$ and 0.44, for frontal and temporal cortex, respectively), but this failed to reach significance. The association between amyloid deposition and age for schizophrenics was also weak ($r^2 = 0.24$ and 0.14 for frontal and temporal cortex) indicating that, although increased numbers of microglia appeared to be associated with amyloid deposition in the brains, this was not linked to age and could therefore be attributable to the schizophrenia itself. For the control group, amyloid and age were more closely associated ($r^2 = 0.57$ and 0.55 for frontal and temporal cortex; $p < 0.05$) which suggests that in the normal case, microglia are associated with amyloid deposition and that both variables increase slightly with age. However, the increase observed in microglial number in schizophrenics was diffuse throughout the cortex and not associated with plaques. The amyloid deposition in schizophrenic cortex did not correlate well with the global deterioration score for the patients ($r^2 = 0.09$ and 0.04, for frontal and temporal cortex). Likewise, there was no correlation between age and global deterioration ($r^2 = 0.00$) or microglial number and global deterioration in either temporal or frontal cortex ($r^2 = 0.01$, $r^2 = 0.09$).

**Neuroleptic Treatment:** The difference in microglial number between schizophrenics and controls may have been due to chronic neuroleptic treatment of the former. The neuroleptic dosage was recorded in the clinical data as a chlorpromazine equivalent. No significant correlations were observed between neuroleptic dosage (as chlorpromazine equivalents) and microglial number in any brain region. However, all patients were treated with neuroleptics for over 30 years during which dosage regimens and drug type are likely to have changed. Therefore, it is not possible to exclude an effect of neuroleptics on microglia.

**Multiple Linear Regression Analysis:** To determine whether a combination of variables could account for differences in microglial number, multiple regression analysis was employed to define a model to describe the contribution of all independent variables to microglial density estimates. The variables assessed were age, PMI, amyloid deposition, sex, and diagnosis. In superior temporal cortex, the diagnosis of schizophrenia contributed clearly be seen to envelop or make contact with neighboring neuronal cell bodies (arrow). The cells in the schizophrenic cortex were highly ramified (D–F) and appeared to be intermediate between ‘activated’ cells, as described in Alzheimer disease (AD) and the ‘resting’ ramified morphology seen in the control cortex. Qualitatively, the cells appeared to have more secondary and tertiary processes than the ‘resting’ ramified cell type. ‘Activated’ microglia are characterized by their swollen cell body and thickened or protracted processes (G–H). Cells may also ‘clump’ together and can be difficult to distinguish. Scale bar = 10 μm.
Fig. 8. Correlation between β-amyloid deposition and HLA-DR+ microglial number. Values represent the microglial numerical cell density versus the β-amyloid deposition rating (Table 1) for frontal (area 9) and temporal (area 22) cortices. See the Table 1 for n-values. Correlations were determined using a linear regression analysis: \( r^2 \), 0.01 for control area 9; \( r^2 \), 0.34 for control area 22; \( r^2 \), 0.40 for schizophrenic area 9; \( r^2 \), 0.44 for schizophrenic area 22. Correlations did not reach statistical significance.

Fig. 9. The effect of schizophrenia on cortical thickness of area 22. Cryosections of each regional hemisphere were stained with cresyl-violet. Two images (5×) were captured from each section and cortical thickness was measured in triplicate for each image. These measurements were averaged to give a mean cortical thickness per hemisphere. Values represent cortical thickness in mm ± S.E.M. Numbers (n) for each group are given in Figure 2. Significance of results was determined by Student’s t test for independent variables (*p < 0.05, **p < 0.01).

Neuropil Changes

Cortical Thickness: Any change in numerical density of microglia in a given cortical area could result from a number of mechanisms. Firstly, microglial cell division, in the absence of changes in other cell populations or in the neuropil, would give rise to an increase. Alternatively, shrinkage of the neuropil without change in total cell number would also give rise to increased numerical density. Shrinkage of the neuropil would be reflected in a decrease in cortical thickness, between the grey/white matter interface and the pial surface. Overall, no consistent significant differences were observed in cortical thickness between control and schizophrenic cortex (Fig. 9). In area 22, where the greatest change in microglial number was observed, no significant differences in cortical thickness between control and schizophrenic cortex were evident for either hemisphere. In addition, no differences were observed in cortical thickness between the 2 hemispheres, within each group, for all areas. Our data

most significantly to the microglial numerical density estimate since its coefficient was by far the greatest, whereas age and PMI had the least effects. Overall, the increased microglial density in schizophrenic temporal cortex was highly likely to be due to the schizophrenia (p < 0.001). In the frontal cortex, the diagnosis also contributed most to microglial density. However, in this area amyloid deposition also had a fairly high coefficient and thus correlated well with microglial density. Age and PMI had a minimal influence on microglia. From an analysis of variance, it was clear that the influence of diagnosis on microglial density was diminished by the effects of one or more other variables. Of all the variables, the significance of diagnosis on microglial number was only lost when analyzed with amyloid deposition. Amyloid deposition did influence, to some degree, microglial number in frontal cortex, although diagnosis still had the predominant effect (p < 0.05).
INCREASED MICROGLIA IN SCHIZOPHRENIA

Total cell density

![Graph showing total cell density](image)

**Fig. 10.** The effect of schizophrenia on total neuronal cell density of area 22. Cryosections of each temporal hemisphere were stained with DAPI to visualise all the cell nuclei present. Cell nuclei were counted along 3 cortical strips per cryosection (approximately 17 fields (×100) depending on the thickness of the cortex). The cortical strips covered layers I–VI of the grey matter. Total cell estimates from 9 strips were averaged to give a mean total cell density per hemisphere. Values represent the total cell density across cortical layers I–VI ± S.E.M. The number of cases for each group were: control n = 10, schizophrenic n = 7. Results were not significantly different as determined by Student’s t-test for independent variables.

do not support the idea of a significant reduction in neoprol from other clinical conditions.

**Total Cell Density:** Total cell density in area 22 was quantified to further assess whether other cellular changes might account for an increase in microglial density in schizophrenics. Only area 22 was assessed since the most significant alterations in microglia were in this area. There were no significant differences between schizophrenics and controls in total cell density in either hemisphere (Fig. 10). The mean total cell densities for schizophrenics were 17,020 ± 1,220 cells per strip and 16,662 ± 980 cells per strip, for left and right hemispheres, respectively. For the control group, the values were 18,720 ± 559, for left and 18,311 ± 559, for right hemisphere (Fig. 10). The cortical strips covered layers I–VI of the grey matter. The mean total cell estmates from 9 strips were averaged to give a mean total cell density per hemisphere. Values represent the total cell density across cortical layers I–VI ± S.E.M. The number of cases for each group were: control n = 10, schizophrenic n = 7. Results were not significantly different as determined by Student’s t-test for independent variables.

**DISCUSSION**

This study investigated whether there are astroglial or microglial changes associated with the pathology of schizophrenia. Until now, the study of giosis in schizophrenia has centred largely on histological studies, which have classed all glial subtypes together, or have focused on astrogliosis. In contrast, our study categorized subtypes individually using immunocytochemical techniques. From our observations, previously reported negative findings relating to glia in schizophrenia should be interpreted with caution. We describe for the first time a specific increase in the number of HLA-DR+ microglia in frontal and temporal cortex of schizophrenics compared with controls, with more marked increases in the temporal. Microglia are sensitive markers for the condition of neurons in their close proximity (39). Their morphology can indicate subtle alterations, injury, or aberrant functioning of neurons. Tissue damage produces microglial activation characterized by a graded series of responses. They alter their morphology to become activated cells, up-regulating the expression of MHC class I and II molecules and cell adhesion molecules, increasing enzyme activities, and possibly cytokine release, and ultimately may become amoeboid and phagocytic. Microglia also may proliferate at the site of injury. Increases in the number or activation state of microglia have been reported in a number of degenerative conditions including Alzheimer disease, Parkinson disease, Creutzfeldt Jakob disease, multiple sclerosis, HIV encephalopathy, temporal lobe epilepsy, and Down syndrome (39, 40). In a number of these conditions microglial changes are most noticeable in grey matter, leading to the suggestion that diffuse activation of HLA-DR+ microglia in grey matter might be a general phenomenon in chronic CNS conditions. Therefore, clinical factors must be considered in determining the significance of HLA-DR immunoreactivity in cortical microglia (37). For example, numerical density of microglia in control brains may vary according to the clinical condition at the time of death (the agonal state). In our cohort it was difficult to determine whether there was an association between microgli al density and agonal state. In the control group 7/10 patients died suddenly from cardiovascular disease or pulmonary emboli, compared with 4/8 in the schizophrenic group. It is thus unlikely that coronary heart disease was responsible for the increased microglial numbers in schizophrenia. With the small sample numbers used in this study it is not possible to exclude a contributory role for other clinical conditions.

The present results could be explained by either the up-regulation of HLA-DR expression on microglia that had not previously expressed the antigen, or by an increase in the total number of microglia. There is now a general consensus that microglia in the human brain constitutively express HLA-DR molecules (41, 42) and that previous studies reporting microglia as HLA-DR negative used less sensitive immunostaining techniques and archival paraffin embedded material. The present study used freshly collected and prepared material for cryosectioning because preliminary experiments revealed that paraffin embedded material did not provide reliable and sensitive enough HLA-DR immunostaining. Therefore, it is likely that our results represent an increase in the total number of microglia in Brodmann areas 9 and 22 of schizophrenic brains. The question of whether there is ongoing microglial proliferation in schizophrenia cannot be answered by the present study.

Microglial activation and proliferation may be a common feature of all neural damage regardless of the initial cause. In schizophrenics, an increased microglial number in frontal and temporal cortices could be attributed to several factors. One, which is consistent with a developmental hypothesis of schizophrenia, is a response to perinatal injury, following which increased numbers of microglia might remain in the adult grey matter. One may speculate as to why their number should remain elevated. They could serve a continued neuroprotective function for neighboring neurons. They might be associated with the maintenance of altered synaptic connectivity, such as the decreased number of dendritic spines on pyramidal cells in schizophrenia (15), since microglia have been shown to remodel the CNS (43). An important question is whether remodelling of dendritic spines is a continuous process in schizophrenia or merely the reflection of an earlier event.

The increase in HLA-DR+ microglia might be attributed to an effect of chronic neuroleptic treatment. The patients in this study were chronic schizophrenics and had been receiving anti-psychotic treatment for many years. Comparisons of microglial numerical density in young schizophrenics would therefore be desirable. Neuroleptic treatment induces a mild gliosis in monkey prefrontal cortex (43). Significant differences were found in glia in layer I after typical neuroleptics and in layer III after atypical neuroleptics. The inference is that glia respond to altered transmitter metabolism in response to blockade of dopamine and/or serotonin receptors. The direct or indirect effects of these drugs on glia are not known, although glia also possess neurotransmitter receptors (45, 46) and may modulate neurotransmission in a number of ways (47, 48).

Our results clearly show a tendency towards increased numbers of microglia in temporal and frontal cortex of brains that showed an increased β-amyloid deposition, although this correlation did not reach significance. Multiple linear regression analysis confirmed these results and showed that the diagnosis of schizophrenia was the only significant determinant of the increase in microglia. Although there are numerous reports of increased microglial numerical density and increased HLA-DR expression in AD, the increase is not generally distributed but clustered around β-amyloid plaques and neurofibrillary tangles. The increase seen in the present study was of a general nature and not localized to any cortical layer or to β-amyloid deposits, suggesting that microglia in schizophrenic cortex have responded to a more diffuse stimulus. The finding that HLA-DR+ microglial number was elevated in all schizophrenic cases relative to control, irrespective of age, raises the possibility that this may represent a premature diffuse ageing phenomenon. The lack of correlation between age and β-amyloid deposition in schizophrenia also agrees with this possibility. Therefore, the possibility that increased β-amyloid deposition is a feature of chronic schizophrenia should be explored further.

Since excitotoxicity is implicated in schizophrenia (49), the role of microglia in regulating extracellular levels of glutamate in the CNS is highly relevant. Cultured microglia take up and release glutamate and also produce a glutamate analogue, quinolinic acid, an endogenous excitotoxin produced via tryptophan metabolism (50). The excitatory actions of glutamate mediated by NMDA receptors have been linked to important developmental events such as neuronal migration, neurite outgrowth, and synaptic connectivity in developing brain (51). Excessive activation of glutamate receptors can initiate a series of intracellular biochemical events that cause neuronal damage. It is suggested that uncontrolled glutamate release (neuronal over-activity or loss of GABA/glycine-mediated neuronal inhibition) metabolically compromises neurons possessing NMDA receptors such that they are unable to maintain a normal ionic homeostasis, particularly with regard to calcium flux. Microglia could be involved in contributing to a hyper-glutamatergic state via the production of quinolinic acid, which can be induced in microglia in vitro (52).

Microglia have been implicated in mediating neurotoxic damage (53). However there is little evidence for this role in human brain. Furthermore, if microglia mediated excitotoxic injury in schizophrenia, one would expect to see a response in neighboring astroglia, since they are particularly sensitive to hyperglutamatergic states. Perinatal hypoxic-ischaemic injury induced by cerebral artery ligation or intracortical injection of NMDA induces acute microglial activation and proliferation (51, 53). By 28 days post-injury, the microglia revert to a ramified morphology but a microglial scar is still evident. Microglia may secrete proinflammatory cytokines in the acute postinjury period and participate in the maintenance as well as removal of...
injured neurons (39). However, it is unclear whether an increased number of microglial cells is maintained in these areas into adulthood. Programmed cell death may gradually eliminate activated microglia following CNS injury (54). Both the above mechanisms produce distinct areas of neurodegeneration, which is not the case in schizophrenia. It is also tentatively suggested that suppression of microglial activity could confer neuroprotection after acute brain injury (55). Therefore, an increase in microglia in schizophrenic cortex is consistent with prenatal brain injury or cortical maldevelopment, provided that the microglial response is maintained into adulthood. In agreement with this hypothesis, although an increased number of HLA-DR+ microglia were noted in this study, all of the cells exhibited a predominantly resting ramified morphology rather than an activated one indicating the lack of ongoing neurodegenerative changes.

Thus, microglia may regulate malfunctioning synaptic mechanisms in the cortex in many ways. An important question arising from our results is whether the observed increase in microglia in schizophrenics is present at the onset of the disease or occurs progressively during the course of the illness.

We have demonstrated the absence of significant differences in astroglia in chronic schizophrenics consistent with previous reports (24, 29, 30). There was, however, a nonsignificant trend for increased astroglial numerical density in schizophrenics in all areas, in both white and grey matter. In previous studies, the definition of gliosis was different depending on the type of stain and counting criteria used. The regions examined in the above studies included the hippocampal complex, thalamus, and pons. In studies of GFAP-immunoreactive astroglia, no differences were observed in their number or in optical density in the caudate or brainstem (31), or in the temporal lobe (32). However, Arnold et al (56) reported an increase in GFAP+ astroglia in demented compared to nondemented schizophrenics in layer V of the mid- and orbitofrontal cortices and primary visual cortex. They concluded that the change represented either up-regulation of GFAP in normal astroglia or astrogliosis.

Reactive astrogliosis is a general response to a variety of CNS injuries. The inability of the immature CNS to mount an astroglial response to injury is argued to be the reason why astrogliosis is not seen in schizophrenia (4). It is also used as evidence of a nonprogressive aetiology in schizophrenia. This of course assumes that once astrogliosis has occurred, the signs of it remain long-term. However, the consistently elevated but nonsignificant increases in astrogliosis may indicate a progressive course for minor neurodegenerative changes in the schizophrenic brain. Furthermore, GFAP+ cells will not represent the total astroglial population since GFAP-negative, glutamine synthetase-positive astrogliosis (57) are not accounted for in our study. Preliminary experiments showed that this antigen was adversely affected by postmortem delay and therefore could not be used in this study.

In summary, this study demonstrates a specific increase in microglial cell number in the absence of any astrogliosis in 2 cortical regions implicated in schizophrenia. To determine whether the microglial increase is attributable to developmental or neurodegenerative processes would necessitate repeating this study on younger patients. This would determine whether the increase in microglia is present early in the disease process (perhaps in response to a perinatal injury) or whether the change in microgliosis is incremental as the disease progresses.

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REFERENCES

1. Weinberger DR. Implications of normal brain development for the pathogenesis of schizophrenia. Arch Gen Psychiatry 1987;44:660–69