

# Reduced Neuronal Size and Glial Cell Density in Area 9 of the Dorsolateral Prefrontal Cortex in Subjects with Major Depressive Disorder

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Reductions in glial cell density and neuronal size have been described recently in major depressive disorder (MDD). Considering the important trophic influence of glia on neurons, we hypothesized that this glial cell deficit is more prominent close to neurons. In this investigation we have characterized neuronal and glia cytoarchitecture in prefrontal area 9 using spatial point pattern techniques and two-dimensional measures of cell size and density. In post-mortem brain tissue of subjects with MDD, schizophrenia, bipolar disorder (BPD), and normal controls (15 subjects per group), we examined the laminar location and size of all neurons and glial nuclei in a 500  $\mu\text{m}$  wide strip of cortex extending from the pia to the grey-white matter border. In MDD, we observed reductions in glial cell density (30%;  $P = 0.007$ ) in layer 5 and neuronal size (20%;  $P = 0.003$ ) in layer 6. We also found that glial cell density (34%;  $P = 0.003$ ) was reduced in layer 5 in schizophrenia, while neuronal size was reduced in layers 5 (14%) ( $P = 0.006$ ) and 6 (18%;  $P = 0.007$ ) in BPD. The spatial pattern investigation of neurons and glia demonstrated no alteration in the clustering of glia about neurons between control and patient groups. These findings confirm that glial cell loss and neuronal size reductions occur in the deeper cortical layers in MDD, but provide no support for the hypothesis that an altered spatial distribution of glia about neurons plays a role in the development of these changes.

## Introduction

Recent stereological investigations of post-mortem brain from subjects with major depressive disorder (MDD) have demonstrated that reduced cortical glial cell density, reduced mean neuronal size and/or reduced density of large neurons are features of the disorder (Ongur *et al.*, 1998; Rajkowska *et al.*, 1999a; Cotter *et al.*, 2001a). Similar cytoarchitectural changes are present in bipolar disorder (BPD) where reduced cortical glial cell density, and unchanged or reduced neuronal density are reported (Ongur *et al.*, 1998; Cotter *et al.*, 2001a; Rajkowska *et al.*, 2001). In schizophrenia, neuronal size is reduced (Rajkowska *et al.*, 1998; Pierri *et al.*, 2001) but neuronal density is increased (Selemon *et al.*, 1995, 1998). Glial cell deficits have also been described in schizophrenia [for a review see Cotter *et al.* (Cotter *et al.*, 2001b)], but less consistently than has been the case with MDD. Considering the important trophic influences exerted by glia on neurons (Connor and Dragunow, 1998; Magistretti *et al.*, 1999; Coyle and Schwarz, 2000) and on synaptic development (Ullian *et al.*, 2001), it is conceivable that the reduced neuronal size reported in these disorders is a consequence of diminished glial cell support.

The dorsolateral prefrontal cortex (DLPFC) is implicated in mood disorders and in schizophrenia by neuropsychological (Goldberg *et al.*, 1993), neuroimaging (Buchsbam *et al.*, 1982; Weinberger *et al.*, 1986; Baxter *et al.*, 1989) and cytoarchitectural investigations (Harrison, 1999; Cotter *et al.*, 2001b). In this investigation of Brodmann's area 9 (BA 9) of the DLPFC we have set out to characterize neuronal and glial cell spatial organ-

ization, size and density in control human brain, schizophrenia, BPD and MDD. Based on the assumption that glia that are proximate to neurons have a greater influence on them, we hypothesized that MDD would be characterized by a deficit in perineuronal glia and reduced neuronal size. We also hypothesized that this would lead to an altered spatial pattern of neurons and glia, such that there would be reduced clustering of one about the other.

## Methods and Materials

### Human Subjects

Human brain specimens from BA 9 were obtained from the Stanley Foundation Brain Consortium (Torrey *et al.*, 2000). The sample consisted of 60 subjects (15 normal controls, 15 schizophrenics, 15 BPD and 15 MDD). Diagnoses were made according to Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria (American Psychiatric Association, 1987). Detailed case summaries were provided on demographic, clinical and histological information (see Table 1 for group summary details). All brains underwent clinical neuropathological examination and none demonstrated evidence of neurodegenerative changes or other pathological lesions.

### Tissue Sampling and Staining

A series of 10  $\mu\text{m}$  thick paraffin sections, which contained DLPFC (Brodmann's areas 9 and 46) were obtained from the Stanley Foundation Brain Bank, from which one section was selected for investigation. All sections were stained for cresyl violet using standard methods.

### Identification of Region of Interest and Image Analysis

Prefrontal cortex area 9 was selected for assessment in this investigation according to the cytoarchitectural criteria outlined previously (Rajkowska and Goldman-Rakic, 1995). The assessments were carried out by a single investigator (D.M.), blind to group status. Tissue sections were viewed at 40 objective (NA 0.65) using a Leica DMLB microscope, a Hitachi 3CCD colour camera (HVC20), and a Marzhauser 100  $\times$  100  $x,y$  motorized stage. Using the software Image-Pro Plus 4.0 (Media Cybernetics, MD, USA), we obtained a series of contiguous black and white images of the cortex from pia to the grey-white matter border from which a single composite image was formed. As only a proportion of the cells were within the focal plane of the tissue at 40 objective magnification, we used a multiplanar imaging tool to obtain three images of each frame separated by two steps of 3  $\mu\text{m}$  in the  $z$ -axis. The software incorporated all focused objects into a single image from which the larger composite image was made. The size of the composite field was standard in the  $x$ -axis (500  $\mu\text{m}$ ) but varied in the  $y$ -axis according to cortical width. After identifying the laminar boundaries on these composite images, the area of the layers was measured and the width of each cortical layer was calculated (Table 2).

### Cell Areal Density, Size and Spatial Data Acquisition

A two-step image editing process was undertaken. Firstly, we used a semi-automated thresholding method to identify and outline all darkly stained cells in the composite images. The light intensity level for thresholding was selected for each image so that the glia and the neurons were best outlined, i.e. so that the border which outlined the cells was placed just on the edge of the cell. A time-consuming editing process

followed in which the images were carefully manually edited to distinguish neurons from glial cells, and to ensure that cell outlines were correct, and that non-glial and non-neuronal material was excluded. Neurons were identified by the presence of a cresyl violet-stained cytoplasm, and their generally larger shape and non-spherical outline. Glia were identified by the absence of stained cytoplasm, the presence of a thicker nuclear membrane and more heterogeneous chromatin within the nucleus. From each sampled image the number and size per layer of neurons and glial nuclei (referred to as glial cells in the text), and their  $x$  and  $y$  coordinates were obtained. For the spatial pattern analysis of each set of coordinate data, we identified four pairs of coordinates that together defined a rectangular boundary reaching from the pia to the grey-white matter border. Cell clustering was analysed within these boundaries.

### Statistical Analysis

We analysed neuronal and glial cell nuclear size, their two-dimensional density and their spatial arrangement. The objective of each of these main statistical analyses was to compare the outcome in each of the three patient groups with that of the controls. Cell size and cell density data was available by cortical layer and the analyses were carried out within layers.

### Spatial Arrangement of Cells

We employed spatial pattern analysis to compare the spatial arrangement of neurons and glia between the patient groups and the control group. Spatial pattern analysis was carried out by investigation of the K-function, which is essentially a combined count-distance measure (Ripley, 1976). Briefly, the sampled cells of a case are represented as a point pattern by their  $x$  and  $y$  coordinates. The K-function assesses how many cells of a given type occur within a distance of a particular cell as the distance changes. This is performed for all the sampled cells for a range of separation distances whose maximum is typically chosen as a quarter of the smallest length of the sampling frame. When the cells counted are of the same type as those sampled, the K-function is referred to as univariate; when cells are of two different types, it is referred to as a bivariate K-function. In our analysis, the maximum distance was set 125  $\mu\text{m}$  and K-functions evaluated in steps of 2  $\mu\text{m}$ .

Spatial pattern analysis typically assesses the spatial arrangement of a single cell pattern (Diggle, 1983). Here we concentrate on comparing the spatial cell pattern between groups of cases. To compare univariate cell pattern we followed a procedure suggested by Diggle *et al.* for comparing univariate K-functions across groups (Diggle *et al.*, 1991). Their test procedure uses bootstrapping of exchangeable (univariate) K-function residuals to derive a statistical test for an overall effect of group membership. To compare bivariate patterns (the spatial interaction between neurons and glia) across groups we applied an extension of Diggle *et al.*'s (Diggle *et al.*, 1991) procedure, which modified their bootstrapping approach to facilitate the comparison of bivariate K-functions (S. Landau *et al.*, submitted for publication). The extended procedure also allowed for planned comparisons between groups. We specifically compared the cellular spatial patterns and the interaction between glia and neurons between the control group and the patient groups. To explore the relationship across groups of clustering of glia and neurons that were located close to one another we repeated this bivariate analysis using a maximum cell separation distance of 50  $\mu\text{m}$ . Finally, as a post-hoc exploratory assessment, we also examined the bivariate spatial pattern relationship of neurons and glial nuclei on the basis of tissue from layers 5 and 6 only. This was undertaken because our analysis of these layers demonstrated significant neuronal size reductions in two of the disorder groups (see subsequent results sections). These layers might therefore be expected to show more overt changes on neuronal glial clustering should such a relationship influence neuronal size. For each bootstrap test the data were resampled 999 times. The spatial pattern analyses were carried out in S-PLUS 2000 (MathSoft, 1999) using the S+ Spatial Stats module (Kaluzny *et al.*, 1998), the Splancs library (Rowlingson and Diggle, 1993) and S-PLUS functions written in-house.

### Cell Size and Densities

#### Identification of Variables to be Adjusted For

All group comparisons of cell sizes and cell densities were adjusted for

gender differences since the Stanley Foundation sample consisted of two subsamples drawn in each group; a subsample of nine males from the male patient population and one of six females from the female population. The demographic and histological variables, including brain hemisphere, listed in Table 1 were considered potential confounders of group differences if they differed between the psychiatric groups and the control group according to  $t$ -tests or chi-squared tests at the 10% significance level. In addition, for each cell type and outcome a forward selection procedure with 10% inclusion threshold was employed to identify further demographic and histological variables that could be shown empirically to predict the outcome. Group comparisons of cell sizes and cell densities were then also adjusted for potential confounders and empirical predictors identified in this way. In order to account for six layer-wise comparisons of cell density and size we compared the  $P$ -values of the group tests with the Bonferroni-adjusted significance level of  $0.05/6 = 0.0083$ .

### Cell Size

In our experimental data the cases constituted the (independent) primary sampling units. Cell size was recorded for 127 572 cells yielding ~964 neuronal size measurements and 1162 glial cell measurements per case. Consequently, we employed regression modelling using robust standard errors to compare cell sizes between groups. The advantage of this statistical method is that its validity is not affected by correlations between cell size measurements from the same case, while standard regression would assume that all cell size measurements are independent and hence would lead to underestimates of  $P$ -values. Because of their positively skewed distributions we summarized the size data by their medians (Table 2) and analysed them on the log-scale where empirical distributions were well approximated by normal distributions.

As mentioned above, the analysis of the cell sizes was carried out in two stages. In the first stage a forward selection procedure was employed on the complete data set to identify variables which could be shown empirically to predict cell sizes. The regression models used during the forward selection always included layer as a covariate because the size of neurons is known to vary between cortical layers (cf. Table 2). In the second stage, groups were compared within each layer after adjusting for relevant histological and demographic variables (Table 1). The adjustment was achieved by including the selected adjustment variables in the respective model together with dummy variables whose parameters represented the cell size ratios between each of the three psychiatric groups and the control group. The robust model fitting was carried out in Stata 7 (Stata Corporation, 2001).

In addition, we employed robust regression modelling to test for an interaction between group differences and brain hemisphere on cell size in each layer since hemisphere-specific effects have been implicated in schizophrenia (Harrison, 1999). More precisely, we added further terms to the model which represented hemisphere-specific cell size ratios and tested their significance. We found no statistical evidence for the existence of such group interactions either for neuronal or glial sizes (see subsequent results section) and therefore excluded interaction terms from the final model and consequently assessed group differences on the basis of all 60 cases irrespective of whether the case's brain tissue was from the right or left hemisphere.

### Cell Density

We employed Poisson modelling (McCullagh and Nelder, 1989) to compare cell densities between groups. This allowed us to take account of the discrete (non-continuous) nature of the count data. In the selection procedure all layer-wise cell counts were modelled simultaneously employing a log-link Poisson model which used the two-dimensional size of a case's search area as an offset. A dispersion parameter was introduced to account for the spatial clustering within sections. In addition, a random effect for subject was included to account for layer-wise densities of the same subject being more similar than densities from different subjects. Layer was always included as a factor in the model since neuronal densities are known to vary across cortical layers. The random effects Poisson models were fitted using the procedure GLMM in the statistical package Genstat 5 (Payne *et al.*, 1993), which employs Schall's method (Schall, 1991) to fit a generalized linear mixed model.

Having identified demographic and histological variables for which to adjust cell densities, separate log-link Poisson models, using two-dimensional size of search area as an offset and allowing for overdispersion due to spatial clustering, were fitted to the cell count data in each layer. For each cell type and layer a model contained the adjustment variables and dummy variables whose parameters represented the density ratios between each of the three psychiatric groups and the control group. Accumulated analysis of deviance (McCullagh and Nelder, 1989) using the experimental method was employed to test for differences between the patient groups and the control group. This Poisson modelling was again carried out using Genstat 5 (Payne *et al.*, 1993).

As for cell sizes, we employed the layer-wise Poisson models to test for an interaction between group differences and brain hemisphere on cell density since hemisphere-specific effects have been implicated in schizophrenia (Harrison, 1999). We again found no statistical evidence for the existence of such group interactions either for neuronal or glial densities (see subsequent results section) and therefore excluded interaction terms from the final model.

As areal densities might provide biased estimates of the true three-dimensional densities with the size of the estimation bias depending on the cell sizes (Abercrombie, 1946), we re-analysed glial and neuronal cell densities across groups using 3D densities calculated according to the Abercrombie correction.

## Results

### *Spatial Arrangement of Cells*

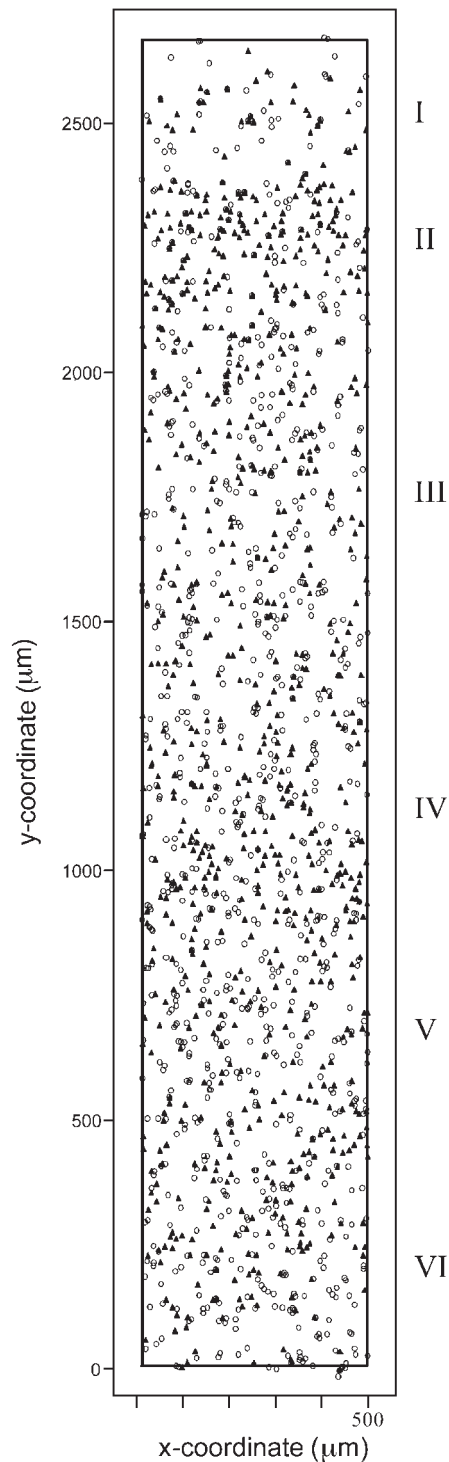
Two categories of cellular spatial pattern analysis were undertaken: first univariate analysis of individual cell populations to assess whether their spatial arrangement varies with group identity; and second, bivariate analysis to determine whether the spatial relationship between neurons and glia alters in psychiatric disorder.

### *Univariate Analysis*

The comparison of univariate K-functions of the control group and the psychiatric groups showed no significant differences in the clustering of either the glial cells (controls versus MDD:  $P = 0.93$ ; schizophrenia:  $P = 0.67$ ; BPD:  $P = 0.67$ ), or of the total neuronal population between control and schizophrenia or BPD groups (controls versus schizophrenia:  $P = 0.17$ ; BPD:  $P = 0.11$ ). However, subjects with MDD showed reduced neuronal clustering (controls versus MDD:  $P = 0.049$ ).

### *Bivariate Analysis*

In this section of the analysis the potential interaction between glial cells and neurons in terms of their physical locations to one another were compared between groups using all cortical layers as the search area (Fig. 1), and after restricting the search area to layers 5 and 6. Across all layers, the comparison of the observed bivariate K-functions between each of the psychiatric groups and the control groups did not show any significant differences in the degree of spatial interaction between glia and neurons ( $P$ -values all group comparisons  $> 0.5$ ). Nor were there any significant group differences in the observed bivariate K-functions when the cell separation distance was reduced to  $50 \mu\text{m}$  ( $P$ -values all group comparisons  $> 0.3$ ). As cell size reductions and reduced glial cell density were most prominent in layers 5 and 6 (see subsequent results sections), we repeated the bivariate analysis specifically within these layers. Group mean bivariate K-functions remained fairly stable and, again, we found no group differences in the observed bivariate K-functions between controls and any of the psychiatric groups (controls versus MDD:  $P = 0.94$ ; schizophrenia:  $P = 0.9$ ; BPD:  $P = 0.088$ ).



**Figure 1.** Neuronal and glial positions and boundary boxes employed from a representative control case in the bivariate analysis of spatial patterns across the cortex.

### *Cell Size*

Summaries of the neuronal and glial cell sizes are shown in Table 2.

### *Identification of Variables to be Adjusted For*

At the 10% level mean fixation times for schizophrenics ( $P = 0.009$ ), those with BPD ( $P = 0.001$ ) and those with MDD



**Table 1**

Group summaries of demographic, histological and clinical information on the brains donated by the Stanley Foundation Brain Consortium

Variable	Group				
	Controls	SCZ	BPD	MDD	
Demographics	Age at death in years (mean $\pm$ SD)	48.1 $\pm$ 10.7	44.5 $\pm$ 13.1	42.3 $\pm$ 11.7	46.5 $\pm$ 9.3
	Gender (male, female)	9M, 6F	9M, 6F	9M, 6F	9M, 6F
Histological	Fixation time in months (mean $\pm$ SD)	10.4 (3.9)	17.2 (8.5)	15.7 (3.6)	14.4 (6.6)
	Post-mortem interval in hours (mean $\pm$ SD)	23.7 (9.94)	33.7 (14.6)	32.5 (16.1)	27.5 (10.7)
	Brain hemisphere (right:left)	7:8	6:9	8:7	6:9
	pH (mean $\pm$ SD)	6.27 $\pm$ 0.24	6.16 $\pm$ 0.26	6.18 $\pm$ 0.24	6.2 $\pm$ 0.23
Clinical	Cause of death	13a:2b	F7a:7c:1d	3a:9c:1e:1f:1 g	7a:1b:4c:1d:1e:1 h
	Duration of disorder in years (mean $\pm$ SD)	0 $\pm$ 0	21.3 $\pm$ 11.4	20.1 $\pm$ 9.7	12.7 $\pm$ 11.1
	Fluphenazine mg equivalents (minimum:median:maximum)	0:0:0	0:35 000:200 000	0:7500:60 000	0:0:0
	Past alcohol/drug abuse or dependence (no:yes)	13:2	12:3	12:3	14:1
	Current alcohol/drug abuse or dependence (no:yes)	15:0	12:3	11:4	12:3
	Treated with antidepressants at death (no:yes)	15:0	10:5	9:6	6:9
	Lithium treatment at death (no:yes:mood stabilizer)	15:0:0	12:2:1	5:4:6	13:2:0
	Family history of disorder (none:SCZ:BP:MDD:?)	15:0:0:0:0	10:3:0:2:0	8:1:3:3:1	3:0:1:8:3
	Death by suicide (no:yes)	15:0	11:4	6:9	8:7

SCZ represents schizophrenia, fluphenazine mg equivalents is lifetime neuroleptic dose in fluphenazine milligram equivalent dose. Cause of death is categorized under the following headings: a – cardio-pulmonary; b – road traffic accident; c – suicide; d – alcohol intoxication; e – pneumonia; f – subdural haematoma; g – malnutrition; h – accidental drowning. Note that one subject with BPD had a family history of BPD and of MDD.

( $P = 0.055$ ) were significantly higher than for controls. Mean post-mortem interval for schizophrenics ( $P = 0.038$ ) and those with BPD ( $P = 0.085$ ) were also significantly higher than for controls. No significant group differences were detected for the remaining demographic and histological variables in Table 1. Thus all modelling of cell sizes was adjusted for gender and the potential confounders fixation time and post-mortem interval. For neurons no further empirical predictors of cell size were found. For glial cells, tissue pH was found to affect glial cell nuclear size [ $t$ -test based on robust standard errors:  $t(59) = -4.58$ ,  $P < 0.001$ ]. Hence for glia the layer-wise group comparisons were also adjusted for tissue pH.

#### Group Comparisons

Tests for a dependence of group effects on brain hemisphere show that there was no statistical evidence for the existence of hemisphere specific effects from our data (all  $P$ -values  $> 0.05$ ; for neuronal size, schizophrenia versus controls; all  $P$ -values  $> 0.5$ ). Group comparisons were therefore carried out on the basis of all cases, irrespective of whether their tissue was from the right or left hemisphere. The results of the comparisons of the neuronal and glial cell sizes between the psychiatric groups and the control group are shown in Table 2. We identified reductions in neuronal sizes in BPD in layers 3 ( $P = 0.023$ , estimated decrease 10%; confidence intervals for cell size ratios (CI) = 0.82–0.98), 5 ( $P = 0.006$ , estimated decrease 14%; CI = 0.78–0.96) and 6 ( $P = 0.007$ , estimated decrease 18%; CI = 0.7–0.94) and in MDD in layer 6 ( $P = 0.003$ , estimated decrease 20%; CI = 0.69–0.92). Of these, the reductions in the deeper layers 5 and 6 remained statistically significant after adjusting for six layer-wise comparisons. We also detected an increase in neuronal cell size in schizophrenia relative to controls in layer 1 ( $P = 0.043$ , estimated increase 13%; CI = 1.0–1.27), but this finding did not remain significant after adjusting for six layer-wise comparisons. For glia no significant changes in nuclear sizes were found for any of the psychiatric groups relative to controls in any of the layers at the single test level of 5% (Table 2).

#### Cell Density

Summaries of the neuronal and glial densities are shown in Table 2.

#### Identification of Variables to be Adjusted For

As for cell sizes, all modelling of densities was adjusted for gender and the potential confounders fixation time and post-mortem interval. For neurons, no further demographic and histological variables were detected to be predictors of neuronal density. For glia, tissue pH was found to empirically predict density [Wald test: Chi-squared(1) = 5,  $P = 0.025$ ; estimated increase in density of 3.6% per 0.1 increase in pH, 95% CI from 0.4% to 6.9%]. Hence for glia the layer-wise group comparisons were also adjusted for tissue pH.

#### Group Comparisons

Tests for a dependence of group effects on brain hemisphere show that there was no statistical evidence for the existence of hemisphere-specific effects from our data (all  $P$ -values  $> 0.1$ ; for neuronal density, schizophrenia versus controls, all  $P$ -values  $> 0.4$ ). Group comparisons were therefore carried out on the basis of all cases, irrespective of whether their tissue was from the right or left hemisphere. The results of the comparisons of the neuron and glial densities between the psychiatric groups and the control group are also included in Table 2. At the single test 5% level we detected decreased neuronal density in MDD relative to controls in layer 2 ( $P = 0.029$ ; estimated decrease 21%; confidence interval for cell density ratios (CD) = 0.64–0.97), but this finding did not remain significant after adjusting for six layer-wise comparisons. For glial cells, we found reduced density in schizophrenia in layers 5 ( $P = 0.0033$ , estimated decrease 34%; CI = 0.5–0.87) and 6 ( $P = 0.04$ , estimated decrease 26%, CI = 0.56–0.99). We also found reduced density in BPD in layer 6 ( $P = 0.044$ , estimated decrease 25%; CI = 0.57–0.99) and in MDD in layers 5 ( $P = 0.0067$ , estimated decrease 30%; CI = 0.55–0.9) and 6 ( $P = 0.025$ , estimated decrease 27%; CI = 0.55–0.96). Of these, the reductions in layer 5 in schizophrenia and MDD

**Table 2**Observed median cell densities (measured in cells/mm<sup>2</sup>), median cell sizes (measured in μm<sup>2</sup>) by group and layer

Patient group	Cortical layer	Neurons									Glial							
		Size			Density						Size				Density			
		Width	Median	t(59)	P-value	Est. ratio	Median	F(1,53)	P-value	Est. ratio	Median	t(59)	p-value	Est. ratio	Median	F(1,52)	p-value	Est. ratio
SCZ	1	248.6	47.8	2.07	0.043	1.13	297	0.04	0.84	1.04	18.4	1.19	0.24	1.07	610.7	0.32	0.57	1.08
	2	208.1	69.3	-0.06	0.95	1	951.2	0.83	0.37	0.9	19.9	0.85	0.4	1.05	412.6	0.07	0.79	0.96
	3	901.4	106.6	-0.76	0.45	0.95	550.8	1.01	0.32	1.11	19.6	-0.32	0.75	0.98	557.9	0.02	0.89	1.02
	4	260.3	74.9	-0.49	0.63	0.96	839.5	0.06	0.81	0.97	19.9	0.21	0.83	1.01	670.8	0.17	0.68	0.95
	5	1217.3	103	-1.14	0.26	0.93	538	0.01	0.92	1.01	20.2	0.65	0.52	1.03	545.2	9.46 <sup>a</sup>	0.0033	0.66
	6	473	88.4	-1.21	0.23	0.89	328.4	0.27	0.61	0.92	21	0.16	0.87	1.01	790	4.44	0.04	0.74
BPD	1	266.7	46.8	1.79	0.078	1.12	297.1	<0.01	0.94	0.99	18.3	0.87	0.39	1.04	481.3	0.76	0.39	0.88
	2	213.5	64.8	-0.85	0.4	0.94	785.3	2.53	0.12	0.84	19.8	0.51	0.61	1.02	443.2	0.61	0.44	0.88
	3	986.7	97.2	-2.33	0.023	0.9	494.4	0.02	0.89	1.01	20.2	0.95	0.35	1.04	569.9	0.36	0.55	0.93
	4	260.2	70.4	-1.31	0.2	0.91	837.6	0.09	0.77	1.04	20.6	0.92	0.36	1.04	654.9	<0.01	0.97	1.01
	5	1373.4	97.5	-2.84	0.006 <sup>a</sup>	0.86	460.4	0.42	0.52	1.08	20.7	1.43	0.16	1.06	629.4	2.51	0.12	0.82
	6	489	83.3	-2.78	0.007 <sup>a</sup>	0.82	304.8	0.21	0.65	0.93	21.3	0.31	0.76	1.01	736.1	4.28	0.044	0.75
MDD	1	289.8	43.2	0.49	0.63	1.03	229.6	0.11	0.74	1.06	17	-0.45	0.65	0.98	392.7	3.61	0.063	0.77
	2	251.7	62.3	-1.44	0.16	0.91	728.2	5.02	0.029	0.79	17.8	-1.18	0.24	0.94	344.5	3.57	0.064	0.73
	3	997.9	102.1	-1.42	0.16	0.92	461.2	2.65	0.11	0.87	18.2	-1.46	0.15	0.94	457.3	3.92	0.053	0.77
	4	279.8	73	-0.72	0.48	0.96	785.2	0.44	0.51	0.92	18.7	-1.47	0.15	0.94	571.1	2.91	0.094	0.82
	5	1373.1	104	-1.72	0.091	0.92	478.6	0.09	0.77	0.97	19.9	-0.01	0.99	1	551.4	7.97 <sup>a</sup>	0.0067	0.7
	6	444.3	81.5	-3.14	0.003 <sup>a</sup>	0.8	311.4	0.15	0.7	0.95	19.7	-0.96	0.34	0.95	705.6	5.35	0.025	0.73
Controls	1	296.7	41.5	-	-	-	247.3	-	-	-	16.4	-	-	-	575.2	-	-	-
	2	31.6	66.6	-	-	-	991.5	-	-	-	18.1	-	-	-	485.9	-	-	-
	3	966.2	108.1	-	-	-	517.5	-	-	-	18.7	-	-	-	624.7	-	-	-
	4	268.7	76	-	-	-	781.4	-	-	-	19.4	-	-	-	664.3	-	-	-
	5	1217.3	113.6	-	-	-	515.6	-	-	-	19.5	-	-	-	718.6	-	-	-
	6	368.3	104	-	-	-	312.3	-	-	-	21	-	-	-	850.9	-	-	-

t-tests based on robust standard errors for comparing cell sizes, and approximate F-tests for comparing cell densities between the control group and patient groups are presented. Cell size and density ratios are also included. For cell size and density, all comparisons are adjusted for gender, fixation time and post-mortem interval. Comparisons for glial size and density were also adjusted for pH

<sup>a</sup>Significant at the experiment-wise significance level of 5%, adjusting for six layer-wise comparisons (single test level of 0.83%).

remained statistically significant after adjusting for six layer-wise comparisons.

Using the Abercrombie correction to estimate three-dimensional neuronal densities increased the estimated density ratios in layers 2–6 by up to 8% but did not lead to any significant group differences (all *P*-values > 0.05). In fact, the decreased neuronal density in MDD in layer 2 was no longer significant even at the single test level of 5%. Application of the Abercrombie correction to glial densities changed estimated density ratios by less than 2% and had little effect on *P*-values. After Abercrombie correction we estimated the density of glial cells in layer 5 to be reduced by 35% in patients with schizophrenia [ $F(1,52) = 9.63$ ;  $P = 0.003$ ] and by 29% in patients with MDD [ $F(1,52) = 7.71$ ;  $P = 0.008$ ] in comparison with controls.

## Discussion

This investigation of area 9 of the DLPFC is the first investigation of the spatial pattern relationship of glial cells to neurons in psychiatric disorders. On the basis of previous work showing glial cell loss in MDD, and in the light of the important roles of glia in supporting normal neuronal function and synaptic development, we hypothesized that there would be reduced clustering of glia about neurons in MDD. We found no evidence for this. We have, however, replicated the findings of reduced glial cell density in subjects with MDD and schizophrenia (Ongur *et al.*, 1998; Rajkowska *et al.*, 1999a; Cotter *et al.*, 2001a). The estimated sizes of these reductions, which were most marked in layer 5, are 30% and 34%, respectively (Table 2). These findings add to the growing literature indicating that abnormalities of glial cell function may undermine cortical

function and predispose to major psychiatric disorder. We also found neuronal size reductions in layer 6 in MDD, and in layer 5 and 6 of BPD (Table 2). The estimated sizes of these reductions were 20, 14 and 18%, respectively.

## Methodological Issues and Potential Confounding Variables

The major advantages of this study are the presence of three psychiatric groups, good sample size, clinical detail, the careful pathological characterization, and the unique quantitation of the spatial pattern organization of the neuronal and glial cytoarchitecture. However, there are several methodological issues specific to the spatial pattern analysis that may have affected the results. First, three cell types constitute the glial cell population and each of these has different effects on neurons. Consequently, in assessing the spatial pattern relationship between neurons and all glial cell populations we may have diluted and lost effects present between neurons and specific glial cell populations. This is important as we do not know which of the three glial cell populations is predominantly responsible for the glial cell deficit seen in MDD. Secondly, inherent clustering of neurons due to the laminar organization of the cortex may have made it difficult to identify subtle alterations in neuronal–glial spatial pattern. Furthermore, if the proposed alteration in neuronal–glial pattern exists only within specific cortical layers in MDD this would have diminished our chances of detecting changes across groups. However, examination of the data (Table 2) indicates that reductions in glial density in MDD are present in most cortical layers, as are reductions in neuronal size. Furthermore, examination of the bivariate spatial pattern relationship between glia and neurons

specifically within layer 5 and 6, the layers that show the most prominent reductions in glial cell density and neuronal size, found no group differences in clustering.

The most important methodological issue relates to the absence of unbiased estimates of three-dimensional cell density (Gundersen *et al.*, 1988). Due to the constraints of the spatial pattern analysis we calculated a two-dimensional cell density (Benes and Lange, 2001). However, larger objects are over-counted in two-dimensional counting (Saper, 1996) and can create a biased elevation in cell density. Consequently, the reductions in neuronal size (though not necessarily always statistically significant reductions), which we observed in each disorder indicate that the neuronal density values were potentially biased and lower than they would have been had cell size been equal. We found that glial cell nuclear size was greater than or equal to that of controls in all groups and layers in which glial density measures were found to be reduced, with the exception of layer 6 in MDD. Consequently, we believe that our findings of reduced glial cell density in MDD and schizophrenia in layers 5, and trend reductions in BPD and schizophrenia in layer 6 are not due to biased counting methods. Nonetheless, in order to correct for this potential bias in cell density estimates due to group differences in cell size, we undertook additional analyses of glial and neuronal density, using the Abercrombie correction (Abercrombie, 1946), which corrected for such changes. The results of this analysis did not differ from those obtained using the unadjusted data.

Another methodological issue concerns our criteria for identification of neurons and glia. Glia were identified by standard criteria, whereas neurons were identified on the basis of their generally non-spherical outline, larger appearance and Nissl-stained cytoplasm. We did not identify neurons on the basis of a nucleolated nucleus, and as a result our values for neuronal area are smaller than typical area measures obtained at the level of the centrally located nucleolus. This is because thin tissue sections, such as we have used, sample through axes other than that which contains the greatest neuronal profile. As a result, there are many more smaller profiles than are obtained in stereological studies which select the focal plane at which the neuronal profile is largest (Rajkowska *et al.*, 1998). While this does not create biases in cell size measures across groups, for each group is affected equally by these assessment methods, it does lead to an underestimation of neuronal size in two-dimensional studies.

Other potential confounders relate to the tissue preservation and processing methods. It is possible that changes in cell density or size could be affected by factors independent of psychiatric disorder, such as tissue fixation period, tissue pH or post-mortem interval. Consequently, in our analysis we identified any of these variables which had predictive effects on cell density and controlled for them where appropriate. Because alcohol abuse at the time of death may have influenced our results we examined the median cell size and density data in each group among non-alcohol abusing subjects and compared them to alcohol abusing subjects. No significant differences between these groups were observed (data not shown), suggesting that alcohol abuse was not responsible for the abnormalities in this study. Also, because tissue was available only from one hemisphere of each subject, in this investigation we were unable to assess laterality effects within our subjects. Nor was there evidence in our data that hemisphere influenced cell density or size, or of an interaction between diagnosis and brain hemisphere on cell density or size.

## Comparison with Previous Studies

### Spatial Pattern Analysis

We found no differences in the bivariate assessments of neurons and glia between groups. Consequently, our data provide no support for the hypothesis that an altered pattern of glial-neuronal positions is present in the MDD. This is consistent with the glial cell deficit in MDD being independent of neuronal position. From this, it can be inferred that oligodendroglia are unlikely to be responsible for the glial cell deficit in MDD, for such a reduction in oligodendroglia, many of which satellite around neurons (Peters *et al.*, 1991), would be reflected in reduced neuronal-glial clustering at smaller distances, and this was not observed. To this extent, the data supports previous investigations indicating that the glial deficit in MDD is astrocytic in origin (Johnstone-Wilson *et al.*, 2000; Miguel-Hidalgo *et al.*, 2000; Webster *et al.*, 2000a), although the possibility of microglial involvement cannot be ruled out. The univariate analyses demonstrated reduced overall neuronal clustering in MDD, but did not detect changes in the other groups. This finding probably reflects the reduction in neuronal density in layer 2 in this group, for this would make neuronal clustering less prominent (see Table 2). In keeping with previous work (Benes *et al.*, 1987) our univariate K-functions show that the spatial distribution of glial cells is closer to complete spatial randomness than that of neurons.

The bivariate spatial pattern analysis method has been applied previously to the investigation of HIV-associated dementia, where it was observed that astrocytes and non-pyramidal neurons appear increasingly clustered with increasing degrees of dementia (Roberts *et al.*, submitted). Univariate spatial pattern methods have also been applied to HIV-associated dementia where an alteration in the pattern of neuronal organization correlating with the onset and progression of the disease was demonstrated (Asare *et al.*, 1996). Applied to schizophrenia, these methods have demonstrated abnormal spatial pattern in the anterior cingulate cortex (ACC) (Benes and Bird, 1987; Diggle *et al.*, 1991) and the entorhinal cortex (Arnold *et al.*, 1997). However, as we did not distinguish pyramidal from non-pyramidal neurons in our study, and as non-pyramidal neurons in layer 2 may demonstrate the most disturbed spatial organization in schizophrenia (Benes and Bird, 1987b), our spatial analysis of the total neuronal population may not have been sensitive to such changes. However, in keeping with our findings, other recent studies have failed to demonstrate altered cortical neuronal organization in this disorder (Akil and Lewis, 1997; Krimer *et al.*, 1997; Bernstein *et al.*, 1998).

### Glial Cell Density

Previous stereological investigations of MDD (Ongur *et al.*, 1998; Rajkowska *et al.*, 1999a; Cotter *et al.*, 2001a) support our current findings of reduced glial cell density in this disorder. In the same series of brains as studied in the current investigation, Ongur and colleagues (Ongur *et al.*, 1998) found reduced glial cell density in the subgenual ACC, and we described a similar reduction in the supragenual ACC (Cotter *et al.*, 2001a). Reduced glial cell density has also been described by Rajkowska and colleagues (Rajkowska *et al.*, 1999a) in the prefrontal cortex area 9 and the caudal orbitofrontal cortex. As in our previous investigation (Cotter *et al.*, 2001a), this glial cell deficit was present in all cortical layers deep to layer 2, though the changes were most prominent in the deepest layers.

We also found a 34% reduction in glial cell density in layer 5 of schizophrenia with a trend for such a reduction in layer 6 (Table



2). While contrasting with some studies (Selemon *et al.*, 1995, 1998; Ongur *et al.*, 1998), this is in keeping with previous work showing glial cell reductions in the orbitofrontal (Rajkowska *et al.*, 1999b), anterior cingulate (Benes *et al.*, 1986, 1991; Cotter *et al.*, 2001a) and primary motor cortices (Benes *et al.*, 1986) in this disorder. Reductions in the levels of glial fibrillary acidic protein (Johnston-Wilson *et al.*, 2000) which labels astrocytes, and myelin basic protein (Honer *et al.*, 1999) which labels oligodendrocytes have also been described in the anterior frontal cortex in schizophrenia and MDD. These findings point to a potential cellular basis of the glial cell deficit described in our study. Our data have shown no significant reductions in glial cell density in BPD, though trend reductions were seen in layer 6. This data contrasts with that of Rajkowska *et al.* (Rajkowska *et al.*, 2001) who found reduced glial cell density in sublayer 3C of the prefrontal area 9 in BPD.

#### Neuronal Density

We found no significant differences in neuronal density between controls and patient groups. However, because we found that neuronal size was generally reduced in the patient groups, these findings relating specifically to neuronal density are potentially biased and give underestimates of neuronal cell density in these groups. This may explain why our findings differ from those previous stereological studies which found increases in mean neuronal density in the prefrontal cortex in schizophrenia (Selemon *et al.*, 1995, 1998). The same argument may also explain why Akbarian and colleagues (Akbarian *et al.*, 1995), who also used two-dimensional counting methods, found no significant differences in neuronal density in schizophrenia. It is worth noting that the application of the Abercrombie correction to our neuronal density data elevated neuronal density in the disorder groups by up to 8%, but these differences did not attain statistical significance.

#### Cell Size

Reductions in mean neuronal size in prefrontal area 9 have been described previously in MDD in layers 3 and 6 (Rajkowska *et al.*, 1999a). This finding of reduced neuronal size in layer 6 is replicated in our current investigation. We also report that neuronal size is reduced in BPD in layers 5 and 6, with a trend for a similar effect in layer 3. These results mirrors our previous findings in the ACC where reductions of 10% and 20% were found in neuronal size in BPD in layers 5 and 6 respectively (Cotter *et al.*, 2001a). The only other previous investigations to assess neuronal size in BPD also sampled the ACC, had no layer-specific data, and found no alterations in neuronal size.

Previous investigations of neuronal size in the prefrontal cortex have found reduced mean neuronal size in schizophrenia compared with controls (Rajkowska *et al.*, 1998; Pierri *et al.*, 2001). Rajkowska and colleagues (Rajkowska *et al.*, 1998) found that neuronal size was reduced in layer 3 by 7%, with trend reductions in layer Va. This finding is supported by the observation that pyramidal neuronal size is reduced in deep layer 3 in schizophrenia (Pierri *et al.*, 2001). In contrast to these findings, we have found no significant reductions in mean neuronal size in any cortical layer. However, thin tissue sections such as used in this investigation do not always cut through and sample neurons at their greatest diameter, and underestimate the size of the sampled population. Additionally, as it can be difficult to discriminate pyramidal from non-pyramidal neurons using these methods, we assessed both populations together. These factors may have influenced our results and may explain our failure to replicate these previous studies of schizophrenia. Nonetheless,

we note that mean neuronal sizes in our study are reduced by 5%, 7% and 11%, respectively, in layers 3, 5 and 6 (Table 2), and that these overall reductions are consistent with those previously described.

#### Possible Explanation

The biological mechanism underlying these changes of glial cell deficit and neuronal size reductions are unknown. It has been proposed that alterations in the levels of neurotrophic factors or in cell survival pathways the may contribute to these changes (Manji *et al.*, 2000). We have previously proposed that stress-related glucocorticoid-mediated toxicity may be implicated (Cotter *et al.*, 2001b). Elevated levels of glucocorticoids (Sapolsky, 1994; McEwen, 1997; Brown *et al.*, 1999) are consistent with much of the shared macroscopic and microscopic neuroanatomy described in mood disorders and in schizophrenia (Harrison, 1999) and include reduced neuronal size (McEwen, 1997; Brown *et al.*, 1999) and reduced astrocyte activity and function (Crossin *et al.*, 1997). Furthermore, reduced levels of the messenger RNA for the glucocorticoid receptor are reported in the frontal cortex and hippocampus of subjects with MDD, BPD and schizophrenia (Webster *et al.*, 2000b). Consequently, glucocorticoids may act directly on neurons, or indirectly through glia to undermine neuronal and cortical function in these disorders.

#### Implications

This study replicates the findings of previous studies of glial cell deficit and reduced neuronal size in MDD (Ongur *et al.*, 1998; Rajkowska *et al.*, 1999a; Cotter *et al.*, 2001a) and provides convincing evidence for relatively widespread glial cell deficit in the prefrontal cortex in MDD. In keeping with previous studies, these changes are not specific to MDD, but are also present to varying degrees in schizophrenia and BPD. Due to the important roles of glial cells in synaptic function (Araque *et al.*, 1999; Ullian *et al.*, 2001), and in providing metabolic support for neurons (Magistretti *et al.*, 1999), the consequences of this glial cell loss are likely to be profound. Elucidation of the mechanisms underlying these changes will be critical to our understanding of the basis of these diseases and to the development of strategies of neuroprotection (Manji *et al.*, 2000).

#### Notes

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