

ORIGINAL ARTICLE

Altered gene expression in the dorsolateral prefrontal cortex of individuals with schizophrenia

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The underlying pathology of schizophrenia (SZ) is likely as heterogeneous as its symptomatology. A variety of cortical and subcortical regions, including the prefrontal cortex, have been implicated in its pathology, and a number of genes have been identified as risk factors for disease development. We used *in situ* hybridization (ISH) to examine the expression of 58 genes in the dorsolateral prefrontal cortex (DLPFC, comprised of Brodmann areas 9 and 46) from 19 individuals with a premorbid diagnosis of SZ and 33 control individuals. Genes were selected based on: (1) previous identification as risk factors for SZ; (2) cell type markers or (3) laminar markers. Cell density and staining intensity were compared in the DLPFC, as well as separately in Brodmann areas 9 and 46. The expression patterns of a variety of genes, many of which are associated with the GABAergic system, were altered in SZ when compared with controls. Additional genes, including *C8orf79* and *NR4A2*, showed alterations in cell density or staining intensity between the groups, highlighting the need for additional studies. Alterations were, with only a few exceptions, limited to Brodmann area 9, suggesting regional specificity of pathology in the DLPFC. Our results agree with previous studies on the GABAergic involvement in SZ, and suggest that areas 9 and 46 may be differentially affected in the disease. This study also highlights additional genes that may be altered in SZ, and indicates that these potentially interesting genes can be identified by ISH and high-throughput image analysis techniques.

Molecular Psychiatry (2014) **19**, 478–485; doi:10.1038/mp.2013.30; published online 26 March 2013

Keywords: gene expression; *in situ* hybridization; prefrontal cortex; schizophrenia

INTRODUCTION

Schizophrenia (SZ) is a complex psychiatric disorder characterized by disorganized thought processes. It can present with a variety of positive and negative symptoms, including hallucinations, delusions, deficits in speech and behavior, blunted affect, and decline in speech and motivation.^{1,2} Changes have also been noted in a variety of memory and executive functions.^{3–7} Owing to the heterogeneous nature of the clinical symptoms, attempts to define a single underlying pathogenomic lesion have been unsuccessful. Various cortical and subcortical abnormalities have been associated with SZ,^{8–15} as well as alterations at the level of morphology,^{16,17} neurotransmitter systems^{18,19} and neurophysiology.^{20–23} Additionally, polymorphisms in multiple genes have been identified as risk factors for disease development through genome-wide association studies.^{24,25}

The dorsolateral prefrontal cortex (DLPFC) has been identified as one region that may be altered in SZ.^{9,13–15,26} It has been shown to be crucial for verbal memory and fluency as well as working memory, processes that are altered in SZ.^{27–29} A variety of changes have been noted in the DLPFC of schizophrenic patients, including alterations in overall cell density, in the number of specific receptors, changes in gene expression, as well as alterations found using fMRI during specific tasks.^{13–15,30,31}

In this study, we used *in situ* hybridization (ISH) to examine the expression patterns of 58 genes in the DLPFC of individuals who had been diagnosed with SZ prior to death, or individuals with no

history of neuropsychiatric illnesses. The genes examined segregate into those previously implicated as risk factors for the development of SZ, cell type markers (most of which are markers for GABAergic interneurons) or genes that show enhanced expression in a given layer or layers of the cortex. In order to determine if there was any regional specificity to expression patterns, distribution of gene expression was assessed in Brodmann areas 9 or 46, as well as for a combination of the two areas (hereafter referred to as 'DLPFC'). We assessed the density of cells expressing each gene and the intensity of staining within each cell to develop a better understanding of how gene expression may be altered in SZ compared with controls.

MATERIALS AND METHODS

Case selection

Materials from individuals that met DMS-IV criteria for a premorbid diagnosis of SZ were compared with control individuals with no history of neurologic or psychiatric illnesses. Tissue from the DLPFC of the right hemisphere was dissected at the NIMH (Section on Neuropathology, Clinical Brain Disorders Branch, GCAP, IRP), frozen in isopentane, stored at –80 °C and shipped to the Allen Institute on dry ice. Post-mortem interval, pH, cause of death, handedness and information on whether the subjects were smokers or had alcohol or antipsychotics in their tissues at the time of death were collected from the source. Prior to use in the study, each case underwent analysis of tissue quality, RNA quality (RIN) and verification of the presence of the regions of interest (ROIs); only those cases that met

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Received 1 October 2012; revised 24 January 2013; accepted 6 February 2013; published online 26 March 2013

the inclusion criteria were included in the study, resulting in 19 individuals with a diagnosis of SZ and 33 control individuals. A complete list of inclusionary criteria can be found in Supplementary Table 1. With the exception of pH and age, no significant differences were found between the two groups with regard to PMI, RIN values or the presence of alcohol post-mortem (Table 1). Differences in pH have been ascribed to the use of medication and the resulting buildup of lactic acid,³² though other factors may contribute to the difference. As has also been previously noted in literature,³³ the schizophrenic group had a higher incidence of smoking than the control group. However, without longitudinal data to assess how long or how often a subject had smoked, or the types and number of cigarettes smoked per day, smoking was not used in the models for data analysis. Evidence of antipsychotic use at the time of death was not seen in any of the controls, but was documented in 75% of the SZ cases. Owing to the fact that nearly all SZ subjects had evidence of antipsychotic treatment at the time of death, and the likelihood that the remaining SZ subjects were on antipsychotic medication at some point prior to death, no attempt was made to assess whether gene expression levels correlated with antipsychotic treatment.

Once selected for inclusion in the study, the tissue blocks were sectioned at 25 μm on a cryostat. Every 20th section was stained for Nissl substance, and the location of areas 9 and 46 were confirmed and identified when present. Three series of 60 consecutive sections, interspersed by the Nissl-stained slides, were identified as containing the ROIs, and were labeled by ISH for expression of a panel of genes (see below). This yielded three stained sections for each probe, with an interval of no less than 1.5 mm between each section.

ISH and gene selection

The use of a high-throughput ISH platform to generate data on gene expression patterns in human tissue has been previously described.^{34,35} Probes were generated against the human genes of interest and their performance validated in a pilot study. All probes were chosen based on their ability to show specific expression during image analysis/informatics processing. All slides were scanned using an Aperio system (Aperio Technologies, Vista, CA, USA), resulting in digital images with a resolution of 1 μm per pixel. Images were then assessed for quality control, and images that failed quality control metrics were eliminated from the study. All images that passed the quality control metrics are freely available at <http://human.brain-map.org/ish/search>.

Fifty-eight study genes (Supplementary Table 2) were selected based on their inclusion into one or more of the three categories: approximately half of the genes studied were selected as genes previously associated with SZ; ~30% of the genes represented specific cell subtypes (primarily interneurons) and almost 40% were used as laminar markers. Genes included in the SZ-associated group were selected based on previous association and expression studies, or are binding partners or in pathways of SZ candidate genes as identified by the gene list provided by Schizophrenia Forum (<http://www.schizophreniaforum.org/>). SZ-associated genes that are not listed as laminar or cell type markers tended to show pan-cortical labeling patterns.

Image analysis

The presence of areas 9 and 46 were verified by cytoarchitectural analysis, using images from Nissl-stained sections. Areas 9 and 46, corresponding to areas FDm/FDp and FDA respectively, were delineated as described by von Economo and Koskinas.³⁶ Area 9 was identified by a relatively cell-sparse layer, 5b, with clumps of von Economo cells present in layers 3 and 5. Area 46 was identified by a relatively uniform distribution of pyramidal cells throughout layer 5 and absence of a discernible layer 5b, whereas von

Economo cells were rare or absent. The locations of areas 9 and 46, which often occurred in the same section, were transferred from images of Nissl-stained sections to images of matched ISH-stained sections. The ROI, extending from the pia to just beyond the gray matter/white matter boundary, were taken from domains of the cortex in which the plane of section was perpendicular to the cortical surface. Artifacts such as blood vessels, tears in the tissue or debris were avoided while defining ROIs. A minimum of 5-mm linear cortical length was delineated as ROI on each section; for image analysis, each ROI was analyzed at the scanned resolution of 1 μm^2 per pixel (Supplementary Figure 1).

Once ROIs were assigned, they were assessed individually with informatics algorithms^{34,35,37,38} designed to assess two different parameters: cell density (reported as cells mm^{-2}), and the average staining intensity for each stained cell in the ROI. These assessments were made for the entirety of the ROI ('all'), as well as for 100 equal subdivisions parallel to the pia and white matter, with the first subdivision at the pia, and the 100th subdivision lying just under the gray matter/white matter border. The subdivisions were scaled such that in all cases exactly 100 would be applied, regardless of the depth of the cortex. This partitioning allowed analysis of the pattern of distribution across the cortex in a comparable way for all individual ROI, independent of the thickness of the cortex, in a manner similar to the methods developed by Zilles and colleagues.³⁹

Statistical analysis

JMP 8.0.2 (SAS Institute, Cary, NC, USA) and R⁴⁰ statistical programs were used to analyze the data. Histograms for each factor of interest were produced for each gene and area, and outliers were identified as those data points that were greater than the upper quartile + 1.5 * (interquartile range) or less than the lower quartile - 1.5 * (interquartile range). Diagnosis was covaried with pH, age and PMI for all analyses, and compared via an analysis of covariance (ANCOVA). Cell density and average staining intensity were compared between controls and subjects with SZ for areas 9 and 46, and a combination of the two ('DLPCF'), and were compared for both the entirety of the ROIs and for individual layers. Every gene/area combination was tested simultaneously across the layers. *P*-values were corrected across the layers for multiple hypothesis testing using the Benjamini-Hochberg method⁴¹ in R. Statistical significance was set at $\alpha = 0.05$.

RESULTS

In this study, we examined the expression of a variety of genes in areas 9 and 46, as well as in the two areas combined ('DLPCF'). High-throughput image analysis techniques allowed us to analyze thousands of ROIs across 52 individuals and 58 genes. Within each area, ROIs were identified and 100 sublayers were assessed to ascertain gene expression patterns across the depth of the cortex. The expression patterns of laminar marker genes were used to identify which sublayers were contained within the various cortical layers. Cell density and average staining intensity of the reaction product were independently assessed for each gene and compared between control and SZ groups (Supplementary Figure 1). Significant differences were found in 22 of the 58 genes examined; of these, only 5 showed significant differences in area 46. The remaining differences were noted in the DLPCF as a whole or specifically in area 9.

Table 1. Demographics

	Control (mean \pm s.d.)	Schizophrenia (mean \pm s.d.)	Test value, P-value
Gender (M/F; % male)	24/9 (72.7%)	14/5 (73.7%)	$\chi^2 = 0.006$, df = 1, NS
Age (years)	37.21 \pm 9.61 (20–49)	43.74 \pm 8.59 (22–57)	$t = 2.52$, df = 41.22, $P < 0.01$
PMI (h)	29.94 \pm 13.74 (9–68)	31.13 \pm 13.05 (7–51)	$t = 0.311$, df = 39.03, NS
pH	6.43 \pm 0.27 (5.9–7.0)	6.23 \pm 0.12 (6.0–6.4)	$t = -3.60$, df = 46.56, $P < 0.001$
RIN	6.95 \pm 0.73 (5.6–8.4)	6.70 \pm 0.43 (6.0–7.4)	$t = -1.59$, df = 49.96, NS
Smoking (Y/N/NA; % yes)	9/32/1 (27.7%)	16/3/0 (84.21%)	$\chi^2 = 15.714$, df = 2, $P < 0.001$

SZ-associated genes

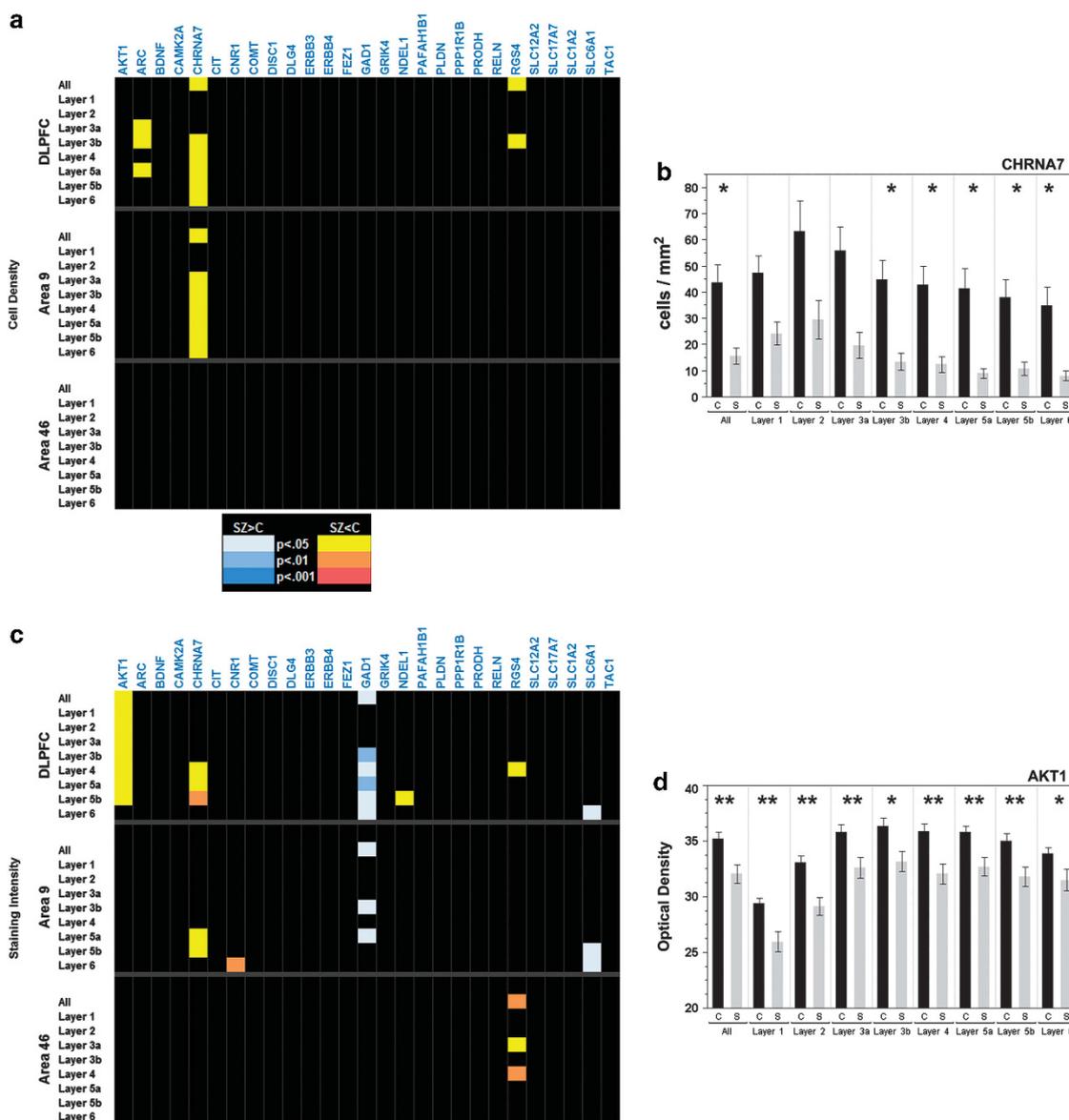
Of the 27 genes chosen for their association with SZ, only 3 (11.1%) showed significant differences in density within the DLPFC. When areas 9 and 46 were examined independently, only one gene, *CHRNA7*, retained significant differences, and only in area 9 (Figure 1a). The significantly lower densities displayed by *ARC* and *RGS4* in the DLPFC did not survive analysis in individual areas, though the trend for lower densities persisted. The most robust differences were found for the gene *CHRNA7*, which showed decreased densities compared with controls in both DLPFC and area 9 alone (Figure 1b).

Changes in staining intensity were found for seven of the SZ-associated genes (25.9%, Figure 1c). The significant differences found for *AKT1*- and *NDEL1*- expressing cells were in DLPFC as a whole, but not in individual areas. *CHRNA7*-expressing cells, which had shown decreased densities, also showed changes in intensity; these changes were found in the DLPFC, but were limited to area 9 when the two regions were examined independently, similar to

the finding for density. Decreased expression of *CNR1* was limited to layer 6 of area 9. Only *RGS4*-expressing cells showed significant intensity alterations in area 46. In general, intensity was decreased in SZ when compared with controls, with the most consistent and significant changes found in *AKT1*-expressing cells (Figure 1d). Within the group of SZ-associated genes, only the interneuron markers *GAD1* and *SLC6A1* had increased expression in SZ. This intensity-specific increase was consistent with other interneuron marker genes (see below).

Interneuron markers

Of the 13 genes chosen to examine interneurons, 5 (38.5%) showed significant differences in density measurements in the DLPFC between controls and SZ subjects (Figure 2a). For these genes, differences were found throughout the depth of the cortex, though less often for layers 1 and 2. Although there was a trend for decreased density in both areas examined, three of these



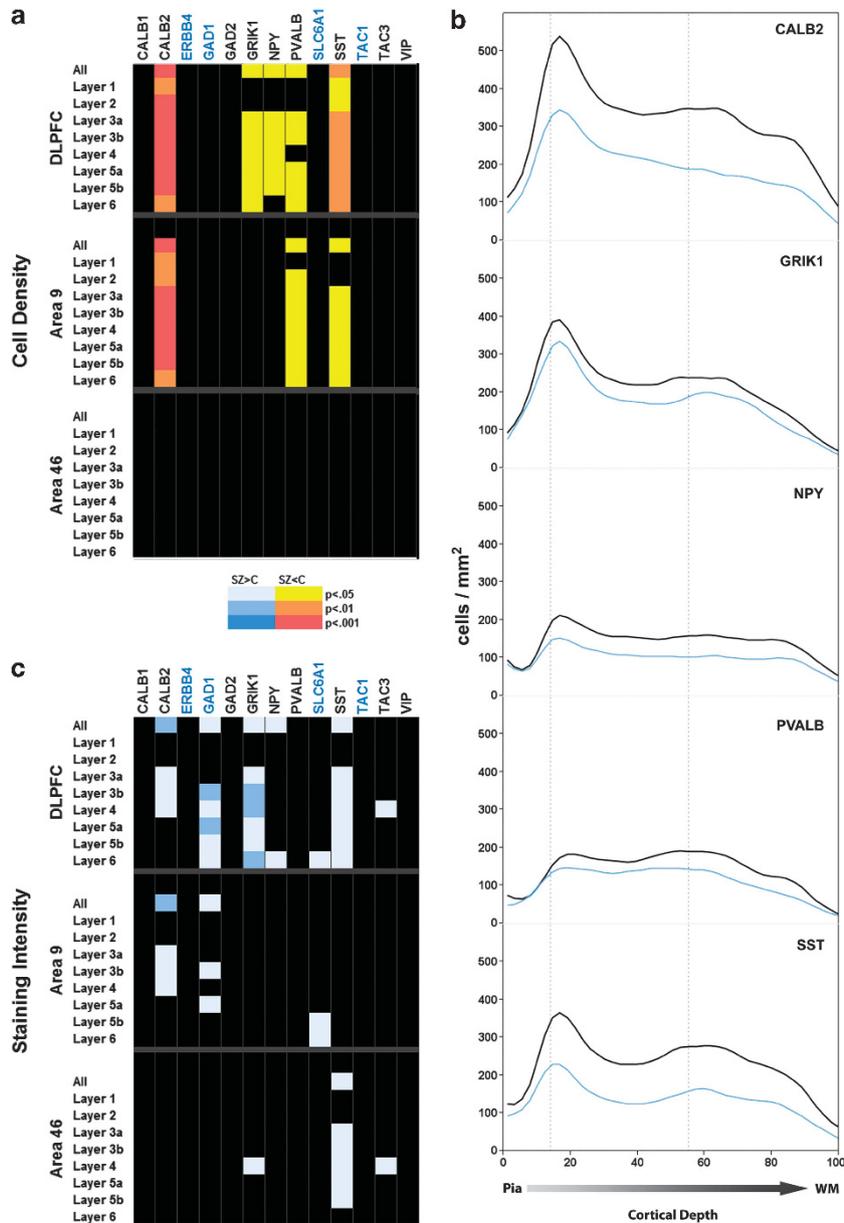


Figure 2. *P*-value charts for cell density (**a**), and staining intensity (**c**) of interneuron marker genes. Genes are listed across the top of the graph (SZ-associated genes are in blue), whereas the areas analyzed and their respective layers are on the left. Corrected *P*-values are denoted by color for each layer as shown in the color key between the two charts. Black, NS. (**b**) Cortical expression pattern for the cell density of interneuron markers in the DLPFC for the five interneuron markers that showed significant differences. Black lines: control values; blue lines: SZ subjects.

genes showed significant differences only in area 9. The most robust of the density decreases were found for the genes *CALB2* and *SST*, which encode calretinin and somatostatin, respectively. Decreases in densities were also detected in *PVALB*-, *GRIK1*- and *NPY*-expressing cells (Figure 2b). Density was not universally decreased across interneuron markers, however. Density showed no significant alteration for the pan-interneuron markers *GAD1*, *GAD2* or *SLC6A1*, suggesting that the overall density of interneurons remains unchanged in SZ. Additionally, significant differences were not detected for *CALB1*- and *VIP*-expressing interneurons, or for additional markers such as *ERBB4* and the tachykinins (*TAC1* and *TAC3*). Although not significant, the trend for several other interneuron markers such as *TAC1* and *TAC3* was for decreased density in SZ patients, implying a broader decrease in this category of genes.

Staining intensity was also altered in the DLPFC for 7 of the 13 (53.8%) interneuron markers, most notably for *CALB2*, *GAD1*, *GRIK1* and *SST* (Figure 2c). In contrast to the general finding of more significant differences in area 9, *GRIK1*-, *SST*- and *TAC3*-expressing cells were altered only in area 46. These differences, together with the decreased densities for *RG54*-expressing cells noted above and the decreased density of *PCP4* in layer 4 noted below, are the only differences that reached significance between the two groups in area 46; alterations in staining intensity for interneuron markers account for two-thirds of the changes seen in area 46. In all instances, unlike the intensity decreases found for the SZ-associated genes, the direction of change was for an increase in interneuron-staining intensity. A possible explanation for this discrepancy is that unlike the SZ-associated genes, decreased expression in this set of genes resulted in the complete loss of

detectable staining for a subset of cells that express these genes at low levels. While the density of stained cells would therefore decrease, the average staining intensity of remaining cells would increase due to the remaining higher-expressing cells.

Laminar markers

Twenty-two of the genes selected for this study were chosen as markers for specific layers of the cortex. Whereas each of the markers had been chosen because it showed enhanced expression in one or more layers, lighter labeling was also often seen at lower levels or at decreased densities in other layers of the cortex. Of these, 4 (18.2%) showed significant changes in density (Figure 3a), with the changes occurring throughout the depth of the cortex as opposed to only the layer for which the gene was preferentially expressed. One of these genes, *C8orf79* was significantly different in area 9, whereas *PCP4* showed differences only in layer 4 of area 46.

Significant differences in intensity were found in the DLPFC for three genes: *CARTPT*, *NR4A2*, and *SYNPR* (Figure 3b). Changes in intensity of the latter two genes were also seen in area 9, but not in area 46. Layer specific changes in intensity were also found for *CNR1*, *SNCG*, *SCN4B* and *VAMP1* in area 9 only, whereas no changes were found for any gene in area 46.

Remaining cell type markers

Probes directed against oligodendrocytes (*MBP*) or astrocytes (*SLC1A2*) showed no significant differences in either density or intensity. *CLDN5* expression, used as a marker of vascular endothelium, showed significant decreases in intensity in the DLPFC, but these changes were limited to area 9 when the areas were examined independently (data not shown).

DISCUSSION

Reports of differences in cell densities within the prefrontal cortex have varied widely in the SZ literature. Some studies have found loss of specific subgroups of cells^{42,43} or decreases in cell density.⁴⁴ Other studies have reported increased density in specific layers of the cortex^{9,14,15} or alterations in the underlying white matter.^{45,46} In this study, we have found no evidence of decreases in the overall density of cells in SZ as compared to controls. Although specific subgroups of interneurons showed significantly lower cell densities in disease, the densities for *GAD1*, *GAD2* and *SLC6A1* suggest that there is no significant decline in interneurons as a whole. It is also important to note that the findings from this study may be attributable to confounding factors, including increased tobacco use, antipsychotic medication and/or environmental factors such as social isolation or a lack of intellectual stimulation.

In this study, we examined the expression of a variety of genes of several types. Consistent with previous reports, we have shown that a variety of genes expressed in interneurons show alterations in SZ. Disruption of the GABAergic system of the prefrontal cortex is well accepted as being a component of SZ pathology. Akbarian *et al.*⁴⁷ initially reported a decrease in the density of *GAD1*-expressing neurons in the DLPFC of subjects with SZ. Volk *et al.*⁴⁸ reported a similar decrease in *GAD1*-expressing cells in area 9 and in the density of the GABA transporter *SLC6A1*-expressing cells in areas 9 and 10 of the prefrontal cortex,⁴⁹ although the expression per cell for each of these genes did not differ. Both of these studies concluded that the decrease in density was a result of expression levels falling below detectable level, rather than loss of interneurons. In our study, the density of cells identified by pan-interneuron markers (i.e., *GAD1*, *GAD2* and *SLC6A2*) showed no differences, suggesting that the overall population of GABAergic interneurons in the DLPFC is not changed between the two groups.

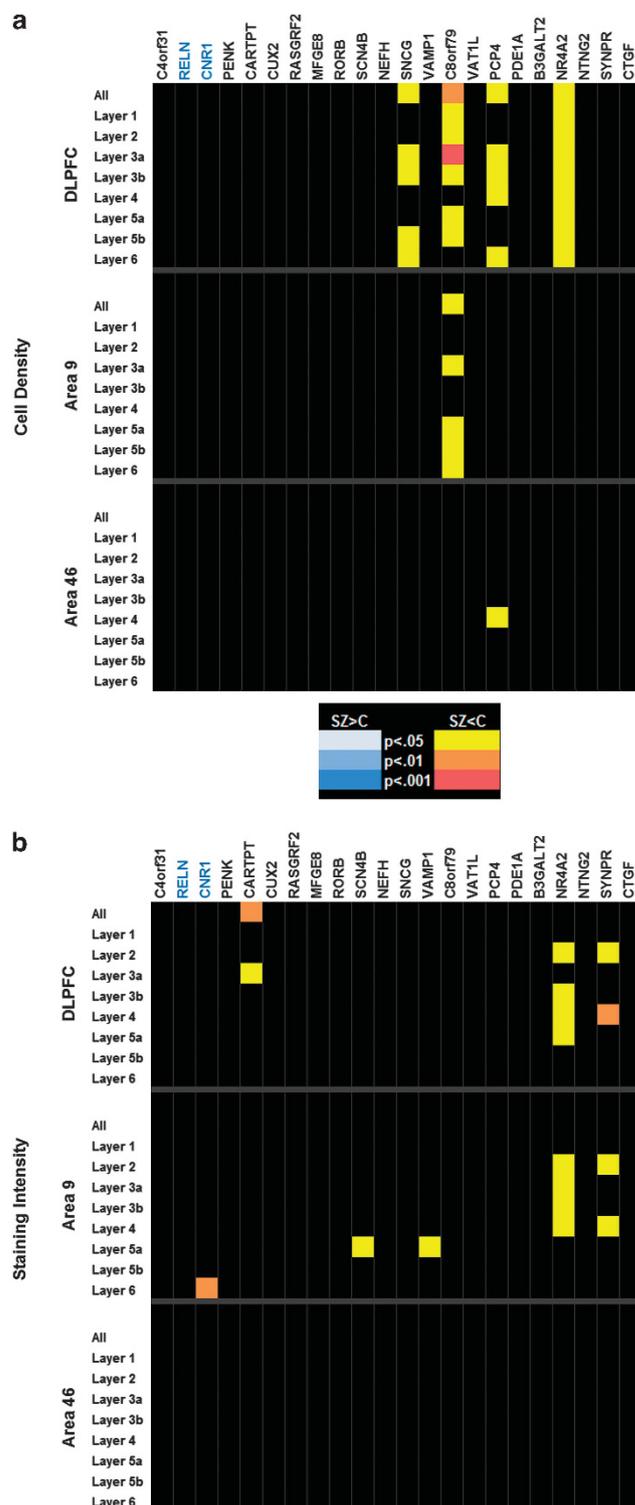


Figure 3. (a–d) *P*-value charts for cell density and staining intensity of laminar marker genes. No significant differences were found in area 46. Genes are listed across the top of the graph (SZ-associated genes are in blue), with genes that are preferentially expressed in layer 1 to the left, and those preferentially expressed in layer 6 to the right. The areas analyzed and their respective layers are on the left. Corrected *P*-values are denoted by color for each layer as shown in the color key between the charts. Black, NS.

However, we did find alterations in the expression patterns of multiple genes that are markers of interneurons, particularly in Brodmann area 9. Specifically, decreased densities were seen in

cells expressing a variety of markers for subsets of interneurons: CALB2, SST and PVALB. Interestingly, these genes do not all highlight the same subset of interneurons, and in many cases, markers for genes in the same subset were unaffected. For example, while SST-expressing cells showed robust changes in both density and intensity, CALB1 showed no detectable changes. Although it is unclear to what degree these two populations may overlap in the DLPFC, it has been shown that in the temporal cortex, over 80% of cells that stained for somatostatin also stain for calbindin.⁵⁰ In that study, somatostatin did not colocalize with calretinin or parvalbumin, both of which show changes in our study. The most plausible explanation is consistent with previous studies showing that the expression of specific genes is decreased below the level of detection in a subset of cells, resulting in what appears to be cell loss. This is further substantiated in light of a failure to identify a loss of interneurons overall with GAD1, GAD2 or SLC1A2. It is possible that the effects we are observing are due to a shift in the proportion of interneurons displaying specific markers; however, we did not find a compensatory increase in any of the markers examined. It should be noted that while the tyramide protocol employed here utilizes amplification, and is therefore inherently less linear of a reaction than what is observed with autoradiography, the relative relationship between expression levels and staining intensity remains applicable. As with all labeling procedures, a threshold for detection exists such that lack of labeling cannot be easily dismissed as a complete absence of expression, but merely failure of the expression levels to reach detection threshold.

Interestingly, our most robust finding involved a decrease in the density of cells expressing CALB2 (Figure 4). Although numerous immunohistochemical studies have shown no decrease in the number of cells that express calretinin,^{51–57} one quantitative PCR study showed a significant decrease in the amount of RNA for CALB2 when examining subjects with SZ,⁵⁸ similar to our results, although an additional study using RT-PCR failed to recapitulate these results.⁵⁹ It is therefore possible that although the CALB2 mRNA expression levels may fall to below the detection levels for ISH, even low-expressing cells are capable of producing enough calretinin to be indistinguishable at the protein level.

It is also worth noting that one of the genes showing the most robust differences was *CHRNA7*, which encodes the $\alpha 7$ nicotinic acetylcholine receptor. Interestingly, *CHRNA7* has been localized to a subset of interneurons, especially in the striatum and the hippocampus,⁶⁰ and studies have suggested that *CHRNA7* expression is decreased in a variety of brain regions in SZ.^{61,62} Additionally, a number of studies have identified various SNPs in *CHRNA7* as possible risk factors for disease development,^{63–65} although earlier studies reported no evidence for linkage.^{66,67} Some of the markers identified, however, are located within or near a region of the gene that is duplicated in a chimeric gene, *CHRFAM7A*. Studies have shown that this chimeric gene may also be implicated in SZ,^{68,69} making it unclear to what extent *CHRNA7* itself is affected. The probe that was used in this study had been specifically designed to have no overlap with the chimeric gene, and our results can therefore be attributed specifically to changes in *CHRNA7* alone.

An intriguing finding from our study is that within the DLPFC, almost all of the significant changes were specific to area 9, extending the results of previous studies^{13–15} that suggest regional specificity in the disease. Similarly, a recent study⁷⁰ using weighted-gene coexpression analysis also found only minimal changes in area 46, whereas additional Brodmann areas (21, 32 and 38) showed significant differences between SZ subjects and controls. Although false negative results due to an insufficient N for area 46 are possible, power analysis on our data set suggests that when significance was reached in area 9, the number of samples required to reach significance in area 46 was significantly greater ($t = -4.07$, $df = 70$, $P = 0.0001$). For genes

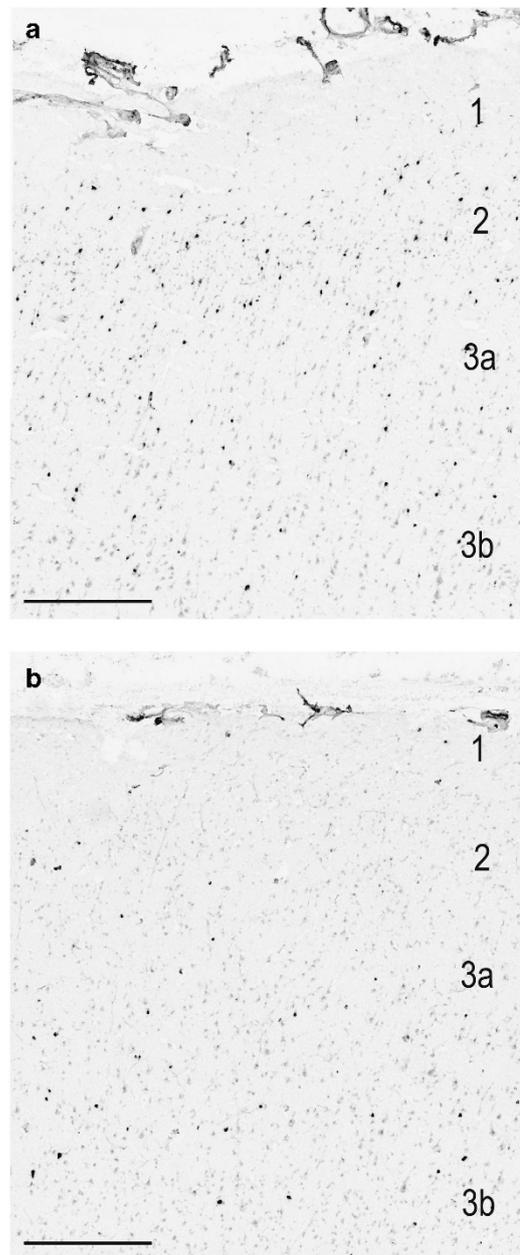


Figure 4. The decrease in the density of cells stained for CALB2 in area 9 is apparent in the supragranular layers of the cortex when control tissue (a), is compared with tissue from an individual with SZ (b). Size bar = 400 μ m.

that failed to reach significance in area 9, there was no such difference. These analyses, together with other studies, suggest that the failure to find significance in area 46 may, at least in part, reflect regional differences in pathology.

The results found with interneuron markers and *CHRNA7* support the use of a high-throughput, cellular resolution platform for identifying changes associated with gene expression level in diseased individuals and the use of image analysis to detect changes that may not be evident with other methodologies. While the laminar markers were not expected to have robust changes between the groups, some showed interesting results that could justify additional studies. Of note is the expression of C8orf79 and NR4A2. C8orf79 encodes a putative methyltransferase, which is interesting in light of the recent focus on epigenetic factors in SZ,

as well as proposed involvement of other methyltransferases such as COMT⁷¹ and DNMT1^{72–74} (DNA methyltransferase-1). NR4A2 is a nuclear receptor protein that can act as a transcription factor. The mouse homolog, *Nurr1*, has been shown to influence the expression of several genes involved in brain development, especially within the dopaminergic system.^{75–78} Haplotype effects of NR4A2 have been proposed in SZ,^{79,80} although multiple studies have failed to find a direct linkage between NR4A2 alleles and SZ.^{81,82} Large-scale ISH studies such as the one presented here can shed light on specifically where and to what degree expression levels of both known and novel genes are altered in human disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank the families of the deceased for the donations of brain tissue, and their time and effort devoted to the consent process and interviews, and the staff of the Offices of the Chief Medical Examiner of District of Columbia and Northern Virginia for their assistance. We would also like to thank the Allen Institute founders, PG Allen and J Allen, for their vision, encouragement and support.

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