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Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia

Dominique Arion¹, Travis Unger¹, David A. Lewis^{1,2}, Pat Levitt^{3,5}, and Károly Mirnics^{1,4,5,*}

¹Department of Psychiatry, U of Pittsburgh, Pittsburgh, PA 15261

²Department of Neuroscience, U of Pittsburgh, Pittsburgh, PA 15261

³Department of Pharmacology, Vanderbilt University, Nashville, TN 37232

⁴Department of Psychiatry, Vanderbilt University, Nashville, TN 37232

⁵Department of Vanderbilt, Kennedy Center for Human Development, Vanderbilt University, Nashville, TN 37232

Abstract

Background—Schizophrenia is characterized by complex gene expression changes. The transcriptome alterations in the prefrontal cortex have been the subject of several recent postmortem studies which yielded both convergent and divergent findings.

Methods—To increase measurement precision, we used a custom-designed DNA microarray platform with long oligonucleotides and multiple probes with replicates. The platform was designed to assess the expression of >1,800 genes specifically chosen because of their hypothesized roles in the pathophysiology of schizophrenia. The gene expression differences in dorsolateral prefrontal cortex samples from 14 matched pairs of schizophrenia and control subjects were analyzed using two technical replicates and four data mining approaches.

Results—In addition to replicating many expression changes in synaptic, oligodendrocyte and signal transduction genes, we uncovered and validated a robust immune/chaperone transcript upregulation in the schizophrenia samples.

Conclusions—We speculate that the overexpression of SERPINA3, IFITM1, IFITM2, IFITM3, CHI3L1, MT2A, CD14, HSPB1, HSPA1B and HSPA1A in schizophrenia subjects represents a long-lasting and correlated signature of an early environmental insult during development that actively contributes to the pathophysiology of prefrontal dysfunction.

Keywords

Immune system; DNA microarray; heat-shock protein; schizophrenia; qPCR; prefrontal cortex; postmortem brain

*Correspondence should be addressed to: Károly Mirnics, Department of Psychiatry, Vanderbilt University, 8130 MRB 3, 465 21st Ave South, Nashville, TN 37232, USA. e-mail: karoly.mirnics@vanderbilt.edu

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INTRODUCTION

Schizophrenia is a severe mental disorder that affects ~1% of the population (Jablensky 2000). Although both genetic and environmental factors play critical roles in the etiology of schizophrenia, the molecular substrate for the pathophysiology of the disease remains elusive and is recognized as complex (Lewis and Levitt 2002). To address this issue, a number of DNA microarray studies have been conducted (for review see (Iwamoto and Kato 2006; Mirnics et al 2006)). These high throughput transcriptome profiling experiments have identified several altered molecular processes in schizophrenia, including downregulation of genes in the GABA and glutamate systems (Mirnics et al 2000; Mirnics et al 2001; Vawter et al 2002), gene expression changes related to synaptic and mitochondrial functions (Altar et al 2005; Iwamoto et al 2005; Middleton et al 2005; Mirnics et al 2000; Prabakaran et al 2004) and a repression of oligodendrocyte mRNAs in the prefrontal cortex (Aston et al 2004; Hakak et al 2001; Pongrac et al 2002; Tkachev et al 2003).

There have been both convergent and divergent results from the transcriptome profiling studies (Mirnics et al 2006). Differences could be attributed to 1) systematic cohort biases arising from brain collection procedures (e.g., hospitalized subjects vs. subjects who died suddenly, divergent inclusion/exclusion criteria, etc.); 2) sample processing differences in RNA isolation procedures, cDNA synthesis, in vitro transcription or fluorescent labeling; 3) microarray platform differences; or 4) differences in segmentation, data extraction, normalization and statistical analyses (Hollingshead et al 2005). Thus, the combined experimental variation and noise in every microarray experiment is considerable, and the microarray data can be less precise and accurate than desired.

Given these limitations, the present study was aimed at analyzing putative schizophrenia-associated molecular pathways at increased measurement precision. We postulated that the ideal microarray would: 1) be built with longer oligonucleotides in order to achieve a higher level of probe specificity; 2) contain multiples probes against the same gene; 3) have a high probe replicate redundancy; and 4) incorporate microarray probes based on published schizophrenia datasets and hypotheses. In addition, we established that the comprehensive microarray experiments would analyze only samples with the highest RNA integrity using two technical replicates. Finally, we utilized several analytical approaches to maximize the identification of convergent data.

The present study reports on a cohort composed of 14 pairs of control and schizophrenic brain samples matched for age, sex and postmortem interval (PMI). We identified significant changes in expression for 67 genes and ESTs between schizophrenia and control samples. In addition to confirming some of the previously reported gene expression changes, we found a highly correlated alteration in the expression of genes involved in immune/chaperone function as part of the transcriptome disturbances in schizophrenia. These results are discussed in the context of the environmental influences that may predispose to the disease.

MATERIALS AND METHODS

1-Human brain samples and tissue preparation

Fresh-frozen human tissue was obtained from the University of Pittsburgh's Center for the Neuroscience of Mental Disorders Brain Bank. In this study, 14 pairs of schizophrenia and control post-mortem brains were matched for gender, and as closely as possible for age, race and postmortem interval (PMI). The two groups of normal control (CTR) and schizophrenia (SCZ) subjects (Table 1) did not differ in mean ($\pm$ SD) age at the time of death (42.21 ± 14.8 and 42.57 ± 12.31 years, respectively), PMI (17.34 ± 5.39 and 17.43 ± 8 hours, respectively), tissue storage time (67.4 ± 24.8 and 73.7 ± 18.2 months, respectively), brain pH (6.88 ± 0.21

and 6.88 ± 0.20 , respectively), and RNA integrity number (RIN) number (8.6 ± 0.40 and 8.4 ± 0.67 , respectively). Consensus DSM-IV diagnoses for all subjects were made using data from clinical records, toxicology studies and structured interviews with surviving relatives, as described previously (Volk et al 2000). The details of the demographic and clinical features of this subject cohort have been previously described in (Hashimoto et al 2006; Hashimoto et al 2003).

2-Nimblegen Array design and processing

Our custom-designed Nimblegen DNA microarrays were composed of 60-mer single-stranded oligonucleotides synthesized by maskless in situ photolithographic synthesis. Each microarray contained probes against ~1,800 genes. The expression of each gene was assessed by 5 independent probe sequences and each of the probes was printed at 4 technical replicates on a sub-array (Figure 1). Each microarray consisted of 5 sub-arrays, resulting in a total of 100 measurements/gene/array (5 probes $\times$ 4 replicate spots $\times$ 5 identical sub-arrays). In addition, the experiment was performed in two technical replicates using independent cDNA and cRNA synthesis steps. As a result, our dataset consisted of 56 microarrays.

The ~1,800 gene expression probes were chosen based on 1) previous microarray studies conducted in our laboratory, 2) previously published gene expression and genetic data in schizophrenia, and 3) genes participating in cellular processes implicated in the pathophysiology of PFC dysfunction in schizophrenia. As a result, the list of microarray probes was enriched in GABA, glutamate, synaptic, glial, dopamine, serotonin system and other transcripts. Furthermore, based on previous microarray studies of subjects with schizophrenia and matched controls, an additional ~400 genes with unaltered expression were chosen to ensure proper normalization across the microarrays.

Coronal blocks containing area 9 of the prefrontal cortex (PFC) were cut on a cryostat at 20 μ m thickness. The sections were collected into tubes containing Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was isolated according to the manufacturer's instructions. RNA quality was assessed using an Agilent BioAnalyzer 2100 system (Agilent, Palo Alto, CA). After cDNA synthesis (cDNA SuperScript Custom Kit by Invitrogen Corporation, Carlsbad) and in vitro transcription (MEGAscript T7 Kit, Ambion Inc., Austin), the resulting cRNA was labeled according to Nimblegen recommendations. Hybridization was performed at the Nimblegen Systems facility using established protocols (for further information, see <http://www.nimblegen.com/technology/hybe.html>).

3- Data analysis

The microarray images were segmented and analyzed using a customized system named NimbleScan (<http://www.nimblegen.com/products/software/nimblescan.html>). Data were then normalized and analyzed using Robust Multi-array Analysis (RMA) (Irizarry et al 2003). For analysis purposes and for each gene, the 5 probes were treated as independent measurements and for each probe the result of the 20 measurements/ array were averaged into a single value. In order to define differentially expressed genes between SCZ and CTR brain samples and across the two technical replicates (R1 and R2), data were analyzed using four different strategies (Figure 2):

A: Group wise strategy with R1 and R2 analyzed independently In this strategy, the normalized expression levels for all 14 SCZ samples and all 14 CTR samples were first averaged and then compared. This analysis was performed for both R1 and R2 independently. A gene was then considered differentially expressed if: 1) it showed an absolute average Log₂ ratio (ALR) between SCZ and CTR samples of more than 20 % ($|ALR| > 0.263$; 1.2-fold); 2) at

least one gene probe showed a groupwise Student's t-test significance of $p < 0.05$ between SCZ and CTR samples; and 3) the differential expression was observed in both R1 and R2.

B: Group wise strategy with R1 and R2 averaged In B, the normalized expression levels for R1 and R2 were averaged for each SCZ and CTR samples. Then, the resulting expression levels for all 14 SCZ and 14 CTR samples were averaged and compared. A gene was then considered differentially expressed if: 1) at least one gene probe showed an absolute average Log2 expression difference between SCZ and CTR of more than 20% ($|ALR| > 0.263$; 1.2-fold); and 2) it showed a groupwise Student's t-test significance of $p < 0.05$ between SCZ and CTR samples.

C: Pair wise strategy with R1 and R2 analyzed independently In C, the normalized expression levels were compared in a pair wise fashion for each of the 14 SCZ and CTR samples. Then, the results for all 14 pairs were averaged. The same comparison was performed for each R1 and R2 independently. A gene was then considered differentially expressed if: 1) at least one gene probe showed an absolute average Log2 expression difference of more than 20% ($|ALR| > 0.263$; 1.2-fold) between SCZ and CTR samples; 2) it showed a pairwise Student's t-test significance of $p < 0.05$ between SCZ and CTR samples; and 3) the differential expression was observed in both R1 and R2.

D: Pair wise strategy with R1 and R2 averaged In D, the normalized expression levels for all CTR and SCZ pairs in R1 and R2 were averaged. Then, each matched SCZ and CTR brain samples were compared in a pair wise fashion to determine gene expression differences. A gene was then considered differentially expressed if: 1) at least one probe showed an absolute average Log2 expression difference of more than 20% ($|ALR| > 0.263$; 1.2-fold); and 2) it showed a pairwise Student's t-test significance of $p < 0.05$ between SCZ and CTR samples.

A gene was considered differentially expressed if it reported significant and unidirectional differential expression in all 4 analysis strategies.

4- Clustering

Two-way cluster analyses (genes and samples) were performed on normalized log2 transformed signal levels across the 28 subjects using Euclidian distance analysis in Genes@Work developed by IBM (Lepre et al 2004).

5- Testing for confounds

The two potential confounding factors in our study are antipsychotic medication and ethanol abuse. For *post hoc* testing for antipsychotic medication effects, we divided the subjects into two groups: A) pairs of subjects where the schizophrenic subjects were off medication at the time of death (Pairs 2, 9 and 13) and B) pairs of subjects where the schizophrenic subjects were on antipsychotic medication at the time of death. For *post hoc* testing of effects of ethanol dependence on the observed gene expression differences, we also separated the subject pairs into two groups (EtOH group: pairs 5, 9, 11, 13 and 14), this time by history of ethanol dependence and compared the expression ALRs of the two cohorts. For testing of both confounds, we assessed the gene expression of the 20 critically changed genes in across the two newly created pair groups. Data were not available about smoking history of the subjects.

6- Correlation testing

Pearson's correlation testing was performed on the normalized log2 values for 8 selected genes (SERPINA3, IFITM1, IFITM3, CHI3L1, CD14, HSPA1B, HSPB1 and HSPA1A). The correlation was calculated on the averaged R1R2 data and across all 28 human subjects. We

obtained 5 correlation values for each gene (one for each probe). The highest across-gene and within gene correlation is reported.

7- False discovery assessment

The false discovery rate (FDR) assessment, a built-in feature of this experimental design, was assessed by establishing how often the independent probes from the same gene reported opposite expression changes. In all of our analyses, two or more different probes against the same gene never reported a statistically significant expression change across the SCZ-CTR comparison in opposite directions (e.g. increase vs. decrease). This suggests a very high measurement precision and a negligible FDR for the differentially expressed genes. However, we acknowledge that our converging data mining strategy may carry an increased amount of type II error (false negative observations), which are not possible to address in current microarray experiments.

8- RT-qPCR

For selected genes showing differential expression between CTR and SCZ subjects, cDNA synthesis was performed using two independent reverse transcriptions for each sample with the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA). For each reaction, we used 50 ng of total RNA from each subject. Priming was performed with random hexamers according to the manufacturer's recommendations. For each sample, amplified product differences were measured with 4 replicates using SYBR Green chemistry-based detection (Mimmack et al 2004). Mitochondrial ATP synthase 6 (MTATP6) and GAPDH were used as the endogenous reference genes, because they did not display significant variation in gene expression between CTR and SCZ samples. The efficiency for each primer set was assessed prior to qPCR measurements, and a primer set was considered valid if its efficiency was between 92 and 100 %. The qPCR reactions were carried out in an ABI Prism 7000 thermal cycler (Applied Biosystems Inc.) using the ABI Prism 7000 SDS software with the automatic baseline and threshold detection options selected. These quantified data were exported to Microsoft Excel for establishing SCZ and CTR ΔC_t , determining $SCZ_{\Delta C_t} - CTR_{\Delta C_t}$ ($\Delta \Delta C_t$) and significance testing. A one-tailed Student's t-test was used to determine the significance of the qPCR-reported SCZ-CTR expression differences.

9 - Data sharing

All microarray data will be made publicly available at the authors' Web site at the time of publication.

RESULTS

In this study, a Nimblegen custom microarray analysis allowed us to compare the expression levels of >1800 different genes, including >400 control genes between SCZ and CTR samples from area 9 of human postmortem PFC. The combination of 4 independent analytical strategies reduced the dataset to 67 transcripts reporting significant differential expression between PFC-harvested samples from SCZ and CTR ($|ALR| > 0.263$; 1.2-fold and $pVal < 0.05$; Table 2). Of these 67 genes, 45 (67%) were found downregulated in SCZ samples compared to CTR samples with a mean ALR value of -0.58 (-1.5-fold) and 22 genes (33%) were found upregulated with a mean ALR value of 0.68 (1.6-fold). These results are in agreement with previous studies reporting a global downregulation of the expression of a variety of functional gene groups in schizophrenia (for review see (Iwamoto and Kato 2006)). The 67 differentially expressed genes between SCZ and CTR samples represented a heterogenic group of transcripts, some of which have been previously associated with the pathophysiological processes in schizophrenia. Downregulation of transferrin (TF), synapsin 2 (SYN2), synaptotagmin 1 (SYNJ1), regulator of G-protein signaling 4 (RGS4), mitogen-activated protein kinase 1 (MAPK1), glutamic-

oxaloacetic transaminase 1 (GOT1), potassium channel, subfamily K, member 1 (KCNK1) and mu-crystallin (CRYM) have all been previously reported to be downregulated in subjects with schizophrenia (Aston et al 2004; Hakak et al 2001; Iwamoto et al 2005; Middleton et al 2002; Mirnics et al 2000; Pongrac et al 2002; Vawter et al 2002). Finally, this experimental series also confirmed and extended previous findings of altered GABAergic system transcriptome in schizophrenia (Lewis et al 2005). Due to the volume and complexity of these findings, the GABA system related expression changes are the subject of a separate manuscript (Hashimoto et al 2006).

The analyses revealed an unexpected upregulation of genes related to immune function and chaperone system. Of the 19 fully annotated transcripts that reported increased expression in subjects with schizophrenia, 10 (>50%) have been linked to functions in immune/chaperone systems (Table 2). For example, in subjects with schizophrenia >50% expression upregulation was observed for the transcripts encoding 28kDa heat shock protein (HSPB1), 70kDa heat shock protein 1B (HSPA1B), 70kDa heat shock protein 1A (HSPA1A), metallothionein 2A (MT2A), interferon induced transmembrane protein 1 (IFITM1), interferon induced transmembrane protein 3 (IFITM3), CD14 antigen (CD14), chitinase 3-like 1 (CHI3L1), serine-cysteine protease inhibitor A3 (SERPINA3) and paired immunoglobulin-like receptor beta (PILRB). These transcripts and their protein products are known to exhibit increased expression in response to cellular stress and/or immune stimulation (Chung et al 2004; Chung et al 2003b; Gosslau and Rensing 2000; Kohler et al 2003; Lewin et al 1991; Mosser et al 2000; Mousseau et al 2000; Penkowa and Hidalgo 2001; Potter et al 2001; Seidberg et al 2003; Shiratori et al 2004; Smith et al 2006; Takekawa and Saito 1998; van Bilsen et al 2004; Xia et al 2006).

A two-way unsupervised clustering (genes and subjects) of the annotated gene probe signal intensities resulted in the separation of the samples in 2 different clusters (Figure 3). Of the 14 SCZ subjects, 9 clustered together and these subjects clearly were major contributors to the statistical significance across the dataset. In contrast, the remaining 5 SCZ subjects appeared to be indistinguishable from the controls, suggesting a molecular sub-stratification within the SCZ samples. The clustering also yielded an interesting separation along the dimension of expressed genes; here, the immune/chaperone-related transcripts (MT2A, IFITM1, IFITM3, CHI3L1, SERPINA3, CD14, TNC, GADD45G, HSPB1, HSPA1B and HSPA1A) strongly clustered together as a distinct sub-group, suggesting a potential co-regulation of these molecules.

The pre-established criterion of the differentially expressed transcripts needing to fulfill a combination of 4 distinct data analysis strategies resulted in the reporting of only the most reliable expression changes (Table 2). Due to the conservative nature of this data mining approach, however, a number of true differential expressions may have been eliminated from our dataset. In **Supplemental Material 1** we report all other non-GABA system related transcripts that were differentially expressed by at least one of the four analysis strategies. Of these additional 170 putatively changed transcripts, ~15% were found changed in previous schizophrenia studies, suggesting that these data may also contain findings for further exploration.

Testing for effects of medication and ethanol abuse

The reported expression findings did not appear to be a result of administration of chronic antipsychotic medication: subjects with schizophrenia that were off medication at time of death showed similar expression changes to subjects on medication at the time of death. For subset of the 21 most changed genes, subjects with schizophrenia on medication showed an average ALR = -0.53 for decreased, and 0.60 for the increased expression (**Supplemental Material 2A**). Similarly, subjects off medication at the time of death showed for the same genes an

average ALR of -0.80 and 1.11, respectively, with an overall correlation of $r=0.94$ ($p<0.01$). Furthermore, in an unsupervised clustering of the gene expression changes in Figure 3, two of the three subjects with schizophrenia that were off medication (829s,622s,537s) clustered together with the subjects that were on medication at the time of death. Similarly, sample pairs where the schizophrenic subject had current alcohol abuse/dependence at the time of death showed the similar gene expression changes as the rest of the pairs where the schizophrenic subject had no active EtOH abuse/dependence. The expression differences for the critical genes were highly correlated ($r=0.90$, $p<0.01$) between these two groups, with a trend of more robust expression changes in pairs without EtOH abuse/dependence (average ALRs 0.59 vs 0.77 and -0.26 vs -0.77 - **Supplemental Material 2B**). Thus, based on these data, it appears that antipsychotic medication and EtOH consumption were not confounding factors in our dataset.

qPCR findings—In order to validate the microarray findings we selected 12 genes for real-time quantitative PCR (qPCR) analysis. Six of these transcripts were overexpressed (SERPINA3, CHI3L1, HSPB1, MT2A, IFITM1, IFITM3) while six other genes were underexpressed (DIRAS2, CRYM, TF, MOG, MAPK1, RGS4) in the SCZ samples. The agreement between the $\Delta\Delta Ct$ of qPCR and DNA microarray ALR datasets was striking: the differential expression for the 12 investigated genes was correlated at $r=0.93$ ($p<0.001$) between the two methods (Figure 4).

In addition, as there were no probes against IFITM2 on the microarray, in the context of IFITM1 and IFITM3 transcript increases, we quantified the expression of the third family member, IFITM2. By qPCR measurement the IFITM2 overexpression in the SCZ samples was comparable to that seen to the other two IFITM family members ($-\Delta\Delta Ct = 0.54$ or 1.45 fold increase, $p<0.05$).

The successful verification of the immune/chaperone system transcript findings raised the question of the origin of these robust transcriptome changes. To determine whether the observed transcriptome changes were the result of an active immune process in the brains of subjects with schizophrenia, we investigated the levels of 2-prime,5-prime oligoadenylate synthetase 1 (OAS1), a critical marker of acute viral infection and interferon gamma ($IFN\gamma$), a critical first activator of multiple downstream immune cascades, including the OAS system. Subjects with schizophrenia, when compared to matched controls, did not show altered transcript levels of OAS1 or $IFN\gamma$ (data not shown).

Immune/chaperone transcript co-regulation

The clustered data strongly suggested that the upregulated immune- and HSP-related transcripts may represent a robust, selectively regulated gene expression network. To test this, we performed an expression level cross-correlation of 5 immune genes with 3 HSP family members. This correlational analysis was performed across the whole dataset (28 human PFC samples) for the 5 probes/gene (Figure 5). The data revealed that the expression levels for the 5 immune markers tested (SERPINA3, IFITM1, IFITM3, CHI3L1 and CD14) were highly correlated within the human PFC (max $r=0.73-0.93$, all $p<0.01$). Similarly, the 3 HSP family members (HSPA1B, HSPB1 and HSPA1A) showed a high within-group correlation (max $r=0.85-0.99$, all $p<0.01$). In contrast, only a weak to moderate correlation was observed between the members of the chaperone and immune genes, suggesting that the modulation of the changed immune-related genes and altered chaperone-related transcripts may occur independently, and not as a direct adaptive relationship between these two systems.

DISCUSSION

Detailed analysis of the custom DNA microarray data from a cohort of 14 matched pairs of CTR and SCZ brain samples from area 9 of human PFC revealed a number of important

observations related to changes in gene expression in schizophrenia. First, using rigorous and convergent statistical approaches, we identified 67 genes that were differentially regulated across CTR and SCZ samples. Second, the number of downregulated transcripts outnumbered the upregulated ones by ~2:1 margin, confirming previous reports that gene expression changes in the PFC of subjects with schizophrenia are predominantly characterized by transcript reductions, rather than increases (Iwamoto and Kato 2006; Mirnics et al 2000). Third, we confirmed many of gene expression changes reported in previous postmortem studies, including downregulation of TF (Hakak et al 2001), RGS4 (Mirnics et al 2001), SYN2, SYNJ1, MAPK1, GOT1, KCNK1 and CRYM (Mirnics et al 2000) and upregulation of CHI3L1 (Chung et al 2003a), HSPB1 (Kuromitsu et al 2001), HSPA1B (Hakak et al 2001) and MT2A (Aston et al 2004). Fourth, we identified an unexpected and strongly correlated upregulation of a subset of genes involved in immune/chaperone function. Fifth, we found that the immune/chaperone signature was primarily present in a subset of subjects with schizophrenia. Although we recognize that chaperones can function in a wide range of roles that are independent of immune processes (e.g. response to cellular stress and signal transduction) (Garcia-Osta et al 2003; Kim et al 2001; Pae et al 2005), in our dataset we favor the interpretation that the observed chaperone and immune changes are of common origin and causally interrelated, and they will be discussed in this context.

The neuroimmune hypothesis of schizophrenia has been debated for decades (Giovannoni and Baker 2003; Hanson and Gottesman 2005; Jones et al 2005; Muller et al 2000; Muller and Schwarz 2006; Patterson 2002; Rothermundt et al 2001; Strous and Shoenfeld 2006). Relative risk for developing schizophrenia is increased more than 2-fold compared to the general population with 2nd trimester infection (reviewed by (Cannon and Clarke 2005)). Additionally, epidemiological, serological, gene expression and pathological findings all suggest an infective-immune component of the disease, albeit replication across different cohorts of patients has been elusive (Rothermundt et al 2001). Nevertheless, the combined evidence suggests an infective-immune predisposition to schizophrenia, and that this predisposition is likely to interact with genetic susceptibility for developing the disease. In this context, the changes related to immune/chaperone functions can represent either a response to an ongoing infective-immune challenge or a long-lasting signature of an immune system challenge that may have acted during brain development, which in the human extends in a lengthy fashion from 1st trimester through puberty. Most of the studies of schizophrenia to date suggest that the observed neuroimmune changes are a long-lasting consequence of a previous infective-immune challenge (for review see (Nawa and Takei 2006; Sperner-Unterweger 2005)). Here, we addressed this more directly by determining that there is no change in the transcript levels of OAS1 and INF γ , two critical markers of acute immune response (Rothwell and Hopkins 1995; Rothwell et al 1996). Thus, we also favor the interpretation of a developmentally-based, long-term alteration in the transcriptome of genes related to immune/chaperone function, although one must be aware that certain pre-mortem life stressors and adverse socio-economic conditions, which are highly prevalent in patients with schizophrenia, may also contribute to some of the observed expression changes.

Long-term consequences of early immune challenge are not unprecedented. Studies in rodents that undergo prenatal or perinatal exposure to immune challenges (such as maternal exposure to polyriboinosinic-polyribocytidilic acid - poly(I:C), a synthetic cytokine inducer), high levels of pro-inflammatory cytokines or viral infections develop post-adolescent behavioral deficits that are similar in nature to clinical manifestations in schizophrenia (Ashdown et al 2006; Meyer et al 2005; Tohmi et al 2004; Zuckerman and Weiner 2005). In the mouse, prenatal exposure in mid-pregnancy to poly(I:C) reduces the number of reelin positive cells in hippocampus (Meyer et al 2006). Furthermore, poly(I:C) administration also causes increased dopamine turnover, prepulse inhibition deficits and cognitive impairments in the adult offspring, and the latter is improved by administration of clozapine (Ozawa et al 2006). Finally,

in a rat model, poly(I:C) administration during pregnancy also produced long-lasting pathophysiological changes that are also observed in schizophrenia, including dopaminergic hyperfunction and loss of latent inhibition (Zuckerman et al 2003).

In view of these data, we propose that the transcriptome signature of altered genes related to immune/chaperone function may be a consequence of early life TNF- α , IL-1, IL-6 and/or INF γ brain activation. In this proposed mechanism, the elevated pro-inflammatory cytokine levels during late embryonic development or perinatal period could not only impair normal differentiation and/or refinement of neural connectivity, but also leave behind a specific immune/chaperone signature primarily consisting of altered IFITM, SERPINA3 and HSP transcript increases.

How does elevation of these immune/chaperone system molecules contribute to the symptoms of schizophrenia? The present study does not address this directly, but we speculate that this immune/chaperone signature extends beyond a correlation with an early environmental insult and may actively contribute to the clinical features of the illness. Many immune/chaperone genes are known to be essential for the normal functioning of the CNS, and immune function genes are capable of altering cognitive performance (Blalock et al 2003; Heyser et al 1997; Hoffman et al 1998; Wilson et al 2002; Ziv et al 2006). The causality between the immune/chaperone gene expression changes and altered cognitive performance in schizophrenic patients needs to be addressed in comprehensive clinical studies and in animal models.

In conclusion, our data suggest that a subgroup of subjects with schizophrenia carries an immune/chaperone transcriptome signature of an early environmental insult in the PFC. The identification of this putative immune/chaperone molecular sub-phenotype of schizophrenia, if it is validated across other cohorts of subjects, will allow a separate, mechanistic follow-up in the context of behavioral changes associated with the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. DNA microarray design

For each of the >1,800 genes 5 independent, non-overlapping 60-mer DNA probes were designed. Each array contained five identical probe-containing, photolithographically synthesized blocks (sub-arrays). Each block contained 4 probe replicates for each of the 5 probes. Fluorescent intensity values were first averaged within the block, and then across the blocks for each of the 5 probes. This gave rise to 5 intensity values, one for each probe (P1-P5). As the experiment was performed with two replicates, we obtained 10 intensity values for each of the investigated genes (5 for R1 + 5 for R2). These RMA normalized intensities entered the data mining process described in Figure 2.

Figure 2. Data analysis design

A total of four data analysis methods were employed. All 5 probes were treated independently: data across the 5 probes were never combined or averaged. **A.** In the first approach (*groupwise analysis with independent replicates*) the data from the first replicate (R1) was compared across schizophrenic (S1-S14) and control subjects (C1-C14). A similar comparison was performed across the second replicate (R2). To be considered a differentially expressed gene, a corresponding microarray probe had to show $|ALR| > 0.263$ and $p > 0.05$ in both replicates.

B. In the second approach (*groupwise analysis with averaged replicates*), log2-normalized intensity values were averaged across the two technical replicated originating from a same subjects, and the averaged values were compared in the groupwise fashion between the controls and schizophrenic subjects ($|ALR| > 0.263$ and $p < 0.05$). **C.** In the third approach (*pairwise analysis with independent replicates*) the 14 matched pairs were compared in a pairwise fashion, generating two ALRs for each of the pairs (one for each technical replicate). Gene probes showing similar expression changes across the R1 and R2 analysis were considered differentially expressed. **D.** In the fourth approach (*pairwise analysis with averaged replicates*), the normalized log2 probe intensities were averaged across R1 and R2 for each of

the five probes. Then, these average values were compared across the 14 S-C subject pairs in a matched, pairwise fashion.

Figure 3. Hierarchical clustering of gene expression changes

Normalized log₂ intensities were clustered by Genes@Work in two dimensions (horizontal-genes; vertical-samples) based on Euclidian distance. Each colored pixel represents a single gene expression value in a single subject. The color intensity is proportional to its relative expression level (green-underexpressed; red-overexpressed). Note that a subset of subjects with schizophrenia (9/14, vertical clustering on the left) shows a commonly altered expression pattern that is distinct from the rest of the schizophrenic and control subjects. ESTs and unannotated probesets were removed from the dataset, while GABA-ergic transcripts are denoted with ***.

Figure 4. qPCR and microarray data are highly correlated

We performed qPCR validation for 12 genes showing robust gene expression differences in the microarray experiments. Validation was performed on 13 of the 14 pairs of matched subjects. Differential S-C microarray expression ALRs are plotted on X axis, while Y axis denotes $\Delta\Delta C_t$ in the qPCR experiment. Each symbol corresponds to a single gene. Note that the microarray data are highly correlated with the qPCR data ($r=0.93$, $p < 0.001$).

r=	SERPINA3	IFITM3	IFITM1	CHI3L1	CD14	HSPA1B	HSPB1	HSPA1A
SERPINA3	0.97							
IFITM3	0.86	0.91						
IFITM1	0.79	0.93	0.86					
CHI3L1	0.90	0.76	0.73	0.97				
CD14	0.83	0.74	0.82	0.76	0.89			
HSPA1B	0.31	0.34	0.50	0.72	0.32	0.95		
HSPB1	0.63	0.57	0.64	0.74	0.61	0.88	0.97	
HSPA1A	0.24	0.22	0.44	0.44	0.30	0.99	0.85	0.97

Figure 5. Immune and chaperone transcripts are highly correlated

Microarray data from 5 immune and 3 chaperone genes were examined for correlation across all schizophrenic and control samples. Numbers in black boxes report maximal self-correlation between the 5 different probes for the same gene. Brown boxes denote high correlation ($r > 0.70$), tan boxes suggest moderate ($r = 0.40-0.60$) while grey boxes correspond to low correlation ($r < 0.39$). Note that IFITM1, IFITM3, SERPINA3, CHI3L1 and CD14 show a very high correlation, and so do the HSP family molecules. However, the correlation between the immune and chaperone molecules was only moderate to weak.

Table 1

Subjects studied

Demographic data of these 14 matched pairs of schizophrenic and control subjects. RNA RIN denotes RNA integrity number measured by BioAnalyzer 2100 (Agilent Technologies).

Pairs	Control	Sex/ Race	Age	PMI [*]	Cause of Death ^{***}	pH	RNA RIN	Schizophrenia	Sex/ Race	Age	PMI [*]	DSM IV Diagnosis	Cause of Death ^{***}	pH	RNA RIN
1	592c	M/B	41	22.1	ASCVD	6.72	9	533s	M/W	40	29.1	Chronic undifferentiated schizophrenia	Accidental asphyxiation	6.82	8.4
2	567c	F/W	46	15	Mitral valve prolapse	6.72	8.9	537s	F/W	37	14.5	Schizoaffective disorder ⁺	Suicide by hanging	6.68	8.6
3	516c	M/B	20	14	Gun shot wound to chest	6.86	8.4	547s	M/B	27	16.5	Schizoaffective disorder	Heat stroke	6.95	7.4
4	630c	M/W	65	21.2	ASCVD	6.95	9	566s	M/W	63	18.3	Chronic undifferentiated schizophrenia ^c	ASCVD	6.8	8
5	604c	M/W	39	19.3	Hypoplastic coronary artery	7.08	8.6	581s	M/W	46	28.1	Chronic paranoid schizophrenia ^{a,e}	Accidental combined overdose	7.22	7.9
6	546c	F/W	37	23.5	ASCVD	6.74	8.6	587s	F/B	38	17.8	Chronic undifferentiated schizophrenia ^c	Myocardial hypertrophy	7.02	9
7	1047c	M/W	43	12.4	ASCVD	6.63	9	722s	M/B	45	9.1	Undifferentiated schizophrenia ^f	Gastrointest hemorrhage	6.71	9.2
8	551c	M/W	61	16.4	Cardiac tamponade	6.63	8.3	625s	M/B	49	23.5	Chronic disorganized schizophrenia ^b	ASCVD	7.32	7.6
9	685c	M/W	56	14.5	Hypoplastic coronary artery	6.57	8.1	622s	M/W	58	18.9	Chronic undifferentiated schizophrenia ⁺	Right MCA infarction	6.78	7.4
10	681c	M/W	51	11.6	Hypertrophic cardiomyopathy	7.15	8.9	640s	M/W	49	5.2	Chronic paranoid schizophrenia	Pulmonary embolism	6.86	8.4
11	806c	M/W	57	24	Pulmonary thromboembolism	6.94	7.8	665s	M/B	59	28.1	Chronic paranoid schizophrenia ^a	Intestinal hemorrhage	6.92	9.2
12	822c	M/B	28	25.3	ASCVD	7.04	8.5	787s	M/B	27	19.2	Schizoaffective disorder ^d	Suicide by gun shot	6.67	8.4
13	727c	M/B	19	7	Trauma	7.15	9.2	829s	M/W	25	5	Schizoaffective disorder ^{d,f,+}	Suicide by drug overdose	6.8	9.3
14	871c	M/W	28	16.5	Trauma	7.14	8.5	878s	M/W	33	10.8	Disorganized schizophrenia ^a	Myocardial fibrosis	6.72	8.9
mean			42.2	17.3		6.88	8.63			42.5	17.4			6.88	8.41
StDev			14.8	5.39		0.21	0.40			12.3	8.01			0.20	0.67

* PMI indicates postmortem interval in hours

*** ASCVD indicates atherosclerotic coronary vascular disease

^a Alcohol dependence, current at time of death

^b Alcohol abuse, current at time of death

^c Alcohol abuse, in remission at time of death

^d Other substance dependence, current at time of death

^e Other substance abuse, current at time of death
^f Other substance abuse, in remission at time of death
⁺ Schizophrenic subjects off medications at time of death.

Table 2 Differentially expressed genes in the PFC: A) increased and B) decreased expression in schizophrenia

UniGene	Gene name	Chr	SYMBOL	mALR	pval
Hs.534293	Serine (or cysteine) proteinase inhibitor A3 (alpha-1 antitrypsin)	14q32.1	SERPINA3	1.29	0.0017
Hs.382202	Chitinase 3-like 1 (cartilage glycoprotein-39)	1q32.1	CHI3L1	1.12	0.0039
Hs.274402	Heat shock 70kDa protein 1B	6p21.3	HSPA1B	1.06	0.0004
Hs.520973	28 kDa heat shock protein	7q11.23	HSPB1	0.89	0.0001
Hs.374650	Interferon induced transmembrane protein 3 (1-8U protein)	11p15.5	IFITM3	0.78	0.0002
Hs.458414	Interferon induced transmembrane protein 1 (9-27 protein)	11p15.5	IFITM1	0.77	0.0013
Hs.9701	Growth arrest and DNA-damage-inducible, gamma	9q22.1-2	GADD45G	0.76	0.0204
Hs.520028	Heat shock 70kD protein 1A	6p21.3	HSPA1A	0.75	0.0044
Hs.78788	Leucine-zipper-like transcriptional regulator 1	22q11.21	LZTR1	0.60	0.0005
Hs.163867	CD14 antigen	5q31.1	CD14	0.58	0.0021
Hs.534330	Metallothionein 2A	16q13	MT2A	0.58	0.0073
Hs.632314	Paired immunoglobulin-like receptor beta	7q22.1	PILRB	0.56	0.0004
Hs.298280	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha 1	18q12-21	ATP5A1	0.52	0.0014
Hs.632099	Transferrin	11q23.2	TAGLN	0.49	0.0065
Hs.143250	Tenascin C (hexabrachion)	9q33	TNC	0.48	0.0014
Hs.113275	Orphan purinergic receptor P2X-like 1	22q11.21	P2RX1L1	0.45	0.0005
Hs.166204	PHD finger protein 1 variant 2	6p21.3	PHF1	0.44	0.0002
Hs.593722	Transmembrane protein 63C	14q24.3	TMEM63C	0.43	0.0016
Hs.474935	Semaphorin 4B	15q25	SEMA4B	0.41	0.0004
UniGene	Gene name	Chr	SYMBOL	mALR	pval
Hs.386726	Regulator of G-protein signaling 4	1q23.3	RG54	-0.99	0.0016
Hs.591198	Ecotropic viral integration site 2A (EV12A)	17q11.2	EV12A	-0.99	0.0062
Hs.518267	Transferrin (TF)	3q21	TF	-0.86	0.0253
Hs.4865	Sodium channel, voltage-gated, type III, beta	11q24.1	SCN3B	-0.75	0.0183
Hs.431850	Mitogen-activated protein kinase 1, transcript variant 2	22q11.21	MAPK1	-0.73	0.0070
Hs.165636	GTP-binding RAS-like 2	9q22.31	DIRAS2	-0.72	0.0074
Hs.482587	Secretory carrier membrane protein 1, variant 2	5q13.3-q14.1	SCAMP1	-0.71	0.0073
Hs.516656	Ephrin-A3 precursor (EHK-1 tyrosine kinase ligand EFL-2)	1q21-q22	EFNA3	-0.70	0.0037
Hs.924	Mu-crystallin	16p13.1-12.3	CRYM	-0.64	0.0068
Hs.481133	Sparc/osteonectin (testican) 3	4q32.3	SPOCK3	-0.63	0.0129
Hs.500015	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	17q11.2	CDK5R1	-0.62	0.0087
Hs.208544	Potassium channel, subfamily K, member 1	1q42-q43	KCNK1	-0.60	0.0045
Hs.473632	Synaptotagmin 1	21q22.2	SYNJ1	-0.60	0.0038
Hs.253844	DnaI (Hsp40) homolog, subfamily C, member 14	12q13.2	DNAJC14	-0.59	0.0179
Hs.371218	Ephrin type-A receptor 4 precursor	2q36.1	EPHA4	-0.57	0.0192
Hs.559412	HMP19 protein	5q35.2	HMP19	-0.56	0.0015
Hs.433845	Keratin 5	12q12-q13	KRT5	-0.56	0.0038
Hs.213264	Tyrosyl-tRNA synthetase	1p34.3	YARS	-0.56	0.0044
Hs.132902	CAP, adenylate cyclase-associated protein, 2	6p22.3	CAP2	-0.55	0.0357
Hs.590868	Zinc finger, CCH-type with G patch domain	20q13.3	ZGPAT	-0.53	0.0048
Hs.180414	Heat shock 70kDa protein 8, transcript variant 1	11q24.1	HSPA8	-0.52	0.0135
Hs.75249	ADP-ribosylation factor-like 6 interacting protein	16p12-p11.2	ARL6IP	-0.50	0.0060
Hs.500756	Glutamic-oxaloacetic transaminase 1, soluble	10q24.1-25.1	GOT1	-0.50	0.0029
Hs.77318	Platelet-activating factor acetylhydrolase Ib, alpha 45kDa	17p13.3	PAFAH1B1	-0.50	0.0021
Hs.237825	Signal recognition particle 72kDa	4q11	SRP72	-0.47	0.0130
Hs.433442	Kinesin-associated protein 3	1q23.3	KIFAP3	-0.47	0.0162
Hs.34560	LIM domain only 2 (rhombotin-like 1)	11p13	LMO2	-0.47	0.0147
Hs.528222	NADH dehydrogenase Fe-S protein 4 (NADH Q reductase)	5q11.1	NDUFS4	-0.47	0.0259
Hs.482301	Small glutamine rich protein with tetrapeptide repeats 2	5q12.3	SGTB	-0.47	0.0077
Hs.792	ADP-ribosylation factor domain protein 1, 64kDa	5q12.2	ARFD1	-0.44	0.0175

Hs.445503	Synapsin II (SYN2), transcript variant 1a	3p25	SYN2	-0.43	0.0023
Hs.463456	Non-metastatic cells 1, protein (NM23A)	17q21.3	NME1	-0.40	0.0135
Hs.29665	Calsynenin 1	1p36.22	CLSTN1	-0.38	0.0133
Hs.503453	Discs, large homolog 2, chapsyn-110	11q21	DLC2	-0.37	0.0061

UniGene - Cluster identifier; Chr - cytogenetic location on chromosome; Symbol - NCBI gene symbol; *mALR* - average log2 ratio for the probe showing the greatest gene expression difference between S-C; *pval* - p value for the probe showing the greatest gene expression difference between S-C. The table has been culled of ESTs, unannotated DNA fragments and GABA system transcripts.