Gene expression changes in schizophrenia

PhD Thesis

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Budapest
2010

I. INTRODUCTION

Schizophrenia is a complex and devastating brain disorder that affects 1% of the population and ranks as one of the most costly disorders to afflict humans. This disorder typically has its clinical onset in late adolescence or early adulthood, presenting as a constellation of both positive (delusions, hallucinations, and thought disorganization) and negative (impaired motivation and decreased emotional expression) symptoms. Alterations in cognitive processes, such as attention and working memory, however, may be present prior to the onset of the clinical syndrome and appear to represent core features of the illness. In addition, many individuals with schizophrenia experience difficulties with depression and substance abuse, factors that contributes to the 10-15% lifetime incidence of suicide in this disorder.

The etiology of schizophrenia remains elusive but appears to be multifaceted, with genetic, nutritional, environmental and developmental factors all implicated. In terms of pathophysiology, a convergence of observations from clinical, neuroimaging and postmortem studies have implicated the prefrontal cortex (PFC) and superior temporal gyrus (STG) as major loci of dysfunction in schizophrenia. Abnormal PFC function probably contributes to many of the cognitive disturbances in schizophrenia and appears to be related to altered synaptic structure and/or function in this cortical region. In contrast, changes in STG are likely to be associated with the positive symptoms of this disease.

A convergence of observations from clinical, neuroimaging and postmortem studies have implicated the dorsal prefrontal cortex (PFC) as a major locus of dysfunction in schizophrenia. In subjects with schizophrenia, reductions in gray matter volume in the dorsal PFC have been observed in neuroimaging studies, and these volumetric changes are associated with an increase in cell packing density, but no change in total neuron number in the PFC. Although the size of some PFC neuronal populations appears to be reduced in schizophrenia, these findings are also likely to reflect a decrease in the number of axon terminals, distal dendrites and dendritic spines. Consistent with this interpretation, the levels of synaptophysin, a presynaptic terminal protein, and the density of dendritic spines are decreased in the PFC of subjects with schizophrenia.

Changes in gene expression have also been observed in the PFC of subjects with schizophrenia. In particular, alterations in gene products related to neurotransmission or second messenger systems have been observed. However, each of these studies has focused on only one or a few gene products at a time, without the ability to investigate the simultaneous expression of large numbers of genes. Consequently, complex gene expression patterns in the PFC of subjects with schizophrenia are just beginning to be uncovered.

In the last decades, the scientific community finished the draft of the human genome and we entered the *post-genomic era*, initiating the exploration of *functional genomics* and *proteomics*. DNA microarrays are a crucial part of this technical revolution. In recent years, the advent of a variety of new methods for quantifying messenger RNA (mRNA), the transcription product of genomic DNA, has made it possible to assess tissue levels of virtually every transcript expressed in the brain. Thus far, microarrays have been successfully applied in comparative genomic hybridization, novel gene discovery, polymorphism analysis and gene expression profiling. These studies often are carried out in conjunction with conventional methods for assessment of transcriptome differences, including *in situ* hybridization, suppression subtractive hybridization and representational difference analysis.

II. AIMS / OBJECTIVES

The overall aims of the combined studies were defined as follows:

1) Understanding changes in the gene expression patterns in the postmortem brains of subject with schizophrenia using an unbiased, hypothesis-free, data-driven approach.

2) Verifying the newly discovered gene expression changes.

3) Investigating the relationships between gene expression changes and genetic susceptibility to schizophrenia.

4) Deciphering the role of the gene expression changes in relationship to pathophysiology and clinical presentation of schizophrenia.

III. METHODS

For all our studies fresh-frozen human tissue was obtained from the University of Pittsburgh's Center for the Neuroscience of Mental Disorders Brain Bank. Prefrontal cortex was identified based on histological criteria. In all studies subject pairs were completely matched for sex, race, postmortem interval, and RNA quality. Only samples with exceptional RNA quality were considered for further analysis.

The experiments on synaptic (1), energy metabolism (2), 14-3-3 (3) and RGS4 (4) expression studies were performed on UniGEM-V high-density cDNA microarrays (Incyte Pharmaceuticals, Fremont, CA). The immune-chaperone (5) and GABA gene (6) expression studies were performed on custom-made oligonucleotide arrays manufactured by Nimblegen.

We followed two main strategies in our data analysis: 1) Identifying the differentially expressed transcripts at the level of individual genes; and 2) Assessing the differential expression of transcripts grouped into modules related to functional pathways. In all presented studies, to consider a single gene differentially expressed, it had to fulfill a dual-criteria of magnitude change over baseline (±20%) and statistical significance of $p<0.05$. For gene group analyses, the expression distribution of the whole gene group had to be significantly different between the control and schizophrenic subjects ($p<0.05$), and the false discovery estimates (obtained by random permutation analysis (7)) had to suggest that the chance of obtaining a false-positive finding was $q<0.05$.

For all our *in situ* hybridization studies, we designed custom primers for each of the sequence-verified clones of interest. These primers were used to produce ds cDNA molecules of 750-950 bp using PCR and normal human brain cDNA template. ^{35}S-labeled sense and anti-sense riboprobes were synthesized and purified and hybridized to 20 μm thick cryostat sections from the PFC of each subject. For quantification of the *in situ* hybridization signal, we used Scion Image.

In all our qPCR validation studies cDNA synthesis was performed using two independent reverse transcription, with random hexamer priming. For each sample, amplified product differences were measured with 4 independent replicates using SYBR Green chemistry-based detection. β-actin was used as the endogenous reference

gene. A primer set was considered valid if its efficiency was >80%. The qPCR reactions were quantified using ABI Prism 7300 SDS software and statistically analyzed using a Student's paired t-test in Microsoft Excel.

In all our manuscripts two-way cluster analyses (genes and samples) were performed on normalized log2 transformed signal levels with Euclidian distance analysis in Genes@Work developed by IBM (Armonk, New York).

IV. RESULTS

Presynaptic gene expression changes in the PFC of subjects with schizophrenia

We analyzed global gene expression in PFC area 9 from six matched pairs of schizophrenic and control subjects. Of all genes and expressed sequence tags (ESTs) interrogated by the six microarrays, an average of 3735 (SD = 1345) gene transcripts per array were detectable in the pairwise comparisons. Of these transcripts, 4.8% were judged to be differentially expressed within a subject pair based on the stringent criterion of having $\geq|1.9|$-fold difference (99% confidence level [CL]) between subjects in fluorescent signal intensity. The observed differences for any subject pair, in general, were comparably distributed in both directions.

The changes in schizophrenic subjects were assessed by gene expression profiling for 250 gene groups related to metabolic pathways, enzymes, functional pathways, or brain-specific functions. More than 98% of the gene groups, when compared to the expression pattern of all detectable transcripts, were not significantly different ($p > 0.05$) between the schizophrenic and control subjects. However, several gene groups exhibited significantly changed expression in schizophrenic subjects, both within individual pairs and across pairs (presynaptic secretory machinery, GABA transmission, glutamate transmission, energy metabolism, growth factors, and receptors).

In particular, transcript levels were significantly decreased in the schizophrenic subjects for a group of genes that were functionally related to the presynaptic secretory machinery (*PSYN*). Expression of genes involved in glutamate and GABA neurotransmission were also decreased. Across the six microarray comparisons, of the

62 genes represented in the *PSYN* group, on average 41 *PSYN* genes products were detectable in the subject pairs. Schizophrenic subjects differed in the number of genes in the *PSYN* group that were decreased over the 95% confidence level. This shift in the distribution of the *PSYN* genes was present in *all* subjects, and it was highly significant for both the individual comparisons and at the level of the combined data ($p < 0.0001$).

The hierarchical data analysis revealed an unexpected aspect of altered gene expression. The specific members of the *PSYN* gene group showing decreased expression, and the relative magnitude of individual transcript changes, differed across the pairwise comparisons, revealing different patterns of decreased gene expression in the schizophrenic subjects. We also found that these results were not related to confounding factor such as medication history, sample bias or substance abuse.

Assessment of the expression of genes belonging to various metabolic pathways in the PFC of subjects with schizophrenia

In this study, we wished to determine whether transcript levels in more than 70 different gene groups involved in cellular metabolism, which could impact the quality of neuronal communication, were altered in subjects with schizophrenia and whether the effects on these gene groups were interrelated. Most of the metabolism gene groups that we analyzed did not display significant differences in transcript levels between schizophrenic and control subjects. However, five gene groups did display significant alterations ($p < 0.05$) in transcript levels in five or more of the 10 array comparisons. These included the malate shuttle, transcarboxylic acid (TCA) cycle, ornithine-polyamine, aspartate-alanine, and ubiquitin metabolism groups. Several other gene groups also displayed significantly decreased expression in fewer than five array comparisons. To examine whether there were interactions among the effects on different metabolic gene groups, we next computed a correlation matrix using the $-\log$ of the p value for each gene group and performed a varimax principal component analysis (PCA) on this matrix. Examination of the oblique factor weights in our PCA analysis revealed that the effects on the malate shuttle, TCA cycle, and ubiquitin groups were highly correlated in Factor 2. Factor 3, in contrast, more accurately described the effects on aspartate-alanine and ornithine-polyamine metabolism.

Of the five different metabolic cascades we identified as significantly affected in five or more array comparisons, we were able to establish biological links between four of these groups at the single gene level, with the lone exception being the ubiquitin gene group. The transcripts encoding MAD1, OAT, GOT2, and OAZIN were selected for additional analysis using *in situ* hybridization. I*n situ* hybridization analysis confirmed the microarray finding that expression of each of these four genes was significantly decreased ($p < 0.05$) in the PFC of the subjects with schizophrenia. Moreover, there was no interaction between these decreases and other subject characteristics, such as brain pH, PMI, or tissue storage time.

Altered expression of the 14-3-3 gene family in schizophrenia

The explored microarray data set (1,2) also contained other promising leads, including expression changes in the transcripts encoding the 14-3-3 protein family members. First we examined the expression levels of all seven 14-3-3 genes in the PFC of 10 subjects with schizophrenia and their matched controls using either cDNA microarrays or in situ hybridization (ISH). According to the cDNA arrays, the majority of 14-3-3 genes exhibited moderate to severe decreases in expression in schizophrenia, which were significant at the level of the entire family across all 10 comparisons ($p<0.021$).

Our analysis indicated that the 14-3-3 gene group effect was most correlated with the presynaptic gene group (PSYN) and Aspartate/Alanine gene group (Asp/Ala). Using PCR, we confirmed the robust expression of all seven 14-3-3 genes in area 9 of the human PFC. In a follow-up in situ hybridization for all 14-3-3 isoforms MANCOVA confirmed the significant decreased expression in schizophrenia of two genes: beta -31.9% ($F=5.74$, $p=0.048$) and zeta -18.2% ($F=16.98$, $p=0.005$). Sheffe's post hoc test confirmed the significance of the 14-3-3 beta and zeta findings ($p=0.0094$ and 0.0007, respectively). To examine the potential for antipsychotic medication to influence our results, we examined 14-3-3 gene expression in four PFC areas 9 and 46 from male cynomolgus monkeys treated chronically with haloperidol decanoate and benzotropine and matched control animals. There was no difference in 14-3-3 zeta expression, while 14-3-3 beta increased 28% ($p<0.05$). This increase is in direct

contrast to the decrease seen in the subjects with schizophrenia, and appeared to occur primarily in pyramidal cell layers.

Increased expression of immune and chaperone genes in schizophrenia

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 four independent analytical strategies reduced the dataset to 67 transcripts reporting significant differential expression between PFC-harvested samples from SCZ and CTR (|ALR| > .263; 1.2-fold and $p < .05$). Of these 67 genes, 22 genes (33%) were found upregulated in SCZ samples compared with CTR samples with a mean ALR value of .68 (1.6-fold; Table 2A and Table 2B in manuscript) and 45 (67%) were found downregulated with a mean ALR value of .58 (1.5-fold). 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.

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. 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-like1 (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.

A two-way unsupervised clustering (genes and subjects) of the annotated gene probe signal intensities resulted in the separation of the samples in two different clusters. 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 seemed to be indistinguishable from the control subjects, suggesting a molecular sub-stratification within the SCZ samples.

To validate the microarray findings, we selected 12 genes for real-time qPCR analysis. Six of these transcripts were overexpressed (SERPINA3, CHI3L1, HSPB1, MT2A, IFITM1, IFITM3), whereas six other genes were underexpressed (DIRAS2, CRYM, TF, MOG, MAPK1, RGS4) in the SCZ samples. The agreement between the ΔΔCt of qPCR and DNA microarray ALR datasets was striking: the differential expression for the 12 investigated genes was correlated at r = .93 ($p < .001$) between the two methods

The reported expression findings did not seem to be a result of administration of chronic antipsychotic medication.

GABA-ergic expression changes in schizophrenia

The investigated GABA-related transcripts fell into three categories. The first category involved transcripts encoding presynaptic molecules that regulate GABA neurotransmission, namely GAD_{67} and GAT1. The second category consisted of transcripts encoding neuropeptides, including SST, neuropeptide Y (NPY) and CCK, each of which is expressed and used as a neuromodulator by a subset of GABA neurons. The third category corresponded to different $GABA_A$ receptor subunits, including $\alpha 1$, $\alpha 4$, $\beta 3$, $\gamma 2$ and δ. SST mRNA exhibited the most robust decrease in the schizophrenia group with four probes meeting the criteria and one of them detecting ALR=-0.67, which corresponds to a 1.6-fold decrease in mRNA level. For each of these 10 GABA-related transcripts, expression levels were decreased in the schizophrenia subject in at least 10 of the 14 subject pairs. In order to verify the decreased expression in schizophrenia of six GABA-related transcripts representing the three categories, the expression levels of GAD_{67}, SST, NPY, CCK, $GABA_A$ $\alpha 1$ and $GABA_A$ δ mRNAs were quantified by real-time qPCR in the same 14 subject pairs. We found significant mean decreases in the expression of GAD_{67} (12%, t_{26}=-1.8, P=0.049), SST (44%, t_{26}=-3.3, P=0.003), NPY (28%, t_{26}=-2.9, P=0.007), $GABA_A$ $\alpha 1$ (42%, t_{26}=-8.6, P<0.001) and $GABA_A$ δ (25%, t_{26}=-2.6, P=0.010) mRNAs in the schizophrenia group. For each of these mRNAs, the pattern of pair-wise expression changes in $-ddC_T$ (equivalent to $\log_2$ ratio of schizophrenia to control levels) were highly correlated

across the 14 subject pairs for each of these five transcripts (r=0.71, P=0.005 for GAD_{67} mRNA; r=0.79, P=0.001 for SST mRNA; r=0.71, P=0.005 for NPY mRNA; r=0.53, P=0.053 for $GABA_A$ α1 mRNA and r=0.58, P=0.029 for $GABA_A$ δ mRNA). As a further step of verification, and in order to adjudicate the conflicting findings between the microarray and qPCR analyses for CCK mRNA, we conducted quantitative *in situ* hybridization for SST and CCK mRNAs using 23 subject pairs including 13 pairs (except for pair 7) used in the microarray and qPCR analyses. The expression levels of SST and CCK mRNAs, as measured in the full thickness of the gray matter, were significantly decreased by 36% ($F_{1,20}$=15.4, P=0.001) and 15% ($F_{1,20}$=8.4, P=0.009), respectively, in the schizophrenia group compared to the control group. The percentage expression changes measured by *in situ* hybridization and qPCR were highly correlated for GAD_{67} (r=0.89, P<0.001) and SST (r=0.90, P<0.001) mRNAs across the 13 subject pairs.

As a hierarchical clustering analysis suggested strong co-regulation of transcripts within each of the two groups, we next assessed the relationships among their expression changes in schizophrenia by performing secondary correlation analyses across the 14 subject pairs. In the first group, after Bonferroni's correction, we observed significantly correlated expression changes for GAD_{67} and SST (r=0.71, P<0.005), GAD_{67} and NPY (r=0.72, P<0.004), GAD_{67} and CCK (r=0.84, P<0.001) and SST and NPY (r=0.81, P<0.001). Among $GABA_A$ receptor subunits, there were significant correlations between α1 and γ2 (r=0.84, P<0.001), α1 and δ (r=0.81, P<0.001) and γ2 and δ (r=0.85, P<0.001).

The expression patterns of GAD_{67}, SST and $GABA_A$ α1 mRNAs, as revealed by *in situ* hybridization, did not differ across the groups of monkeys chronically exposed to placebo, haloperidol or olanzapine, suggesting that the observed expression changes were not a result of medication treatment.

RGS4 – a gene critically involved in schizophrenia?

From the UniGem-V cDNA microarray dataset (1-3,8), one strong single gene finding emerged. Of the 4.8% of differentially expressed genes, regulator of G-protein signaling (RGS4) transcript was the only gene decreased at the 99% CL in all schizophrenic subjects. Across the six microarray comparisons these decreases ranged

from 50% to 84%. The magnitude of decrease in RGS4 expression was comparable across the PFC and visual and motor cortices.

In situ hybridization also revealed that the mean level of RGS4 expression was significantly decreased (-34.4%) in the schizophrenic subjects ($F_{1,15}$=6.95; p=0.019). This RGS4 decrease was comparable in schizophrenic subjects with and without any history of alcohol abuse (t-test; p=0.125) and was not present in subjects with major depresive disorders ($F_{1,15}$=0.27; p=0.61) or in monkeys chronically treated with antypsychotic medication (mean=+5.3%; p=0.26).

V. DISCUSSION

In light of the observed presynaptic expression deficits in schizophrenia, we propose a model suggesting that different sequence mutations or polymorphisms in genes related to synaptic communication, combined with other factors, might lead to the shared clinical manifestations of schizophrenia (9). The basic tenets of this model have four components:

1. The etiology of schizophrenia involves a polygenic pattern of inheritance, resulting in altered function of proteins that control the 'mechanics' of synaptic transmission.

2. The heterogeneity in expression defects within the PSYN group reflects distinct adaptive capacities of different populations of neurons. The alterations in RGS4 expression could reflect a primary gene defect or a postsynaptic adaptation in an effort to enhance neurotransmission, which thus compensates for the deficits in PSYN gene function.

3. Deficits in PSYN and *RGS4* gene expression might affect the extended postnatal developmental process of synapse formation and pruning, which ultimately provides a link to the neurodevelopmental timecourse of schizophrenia. We believe that impaired synaptic transmission in subjects with schizophrenia is present from early life (probably in a subset of synapses), but the exuberant synaptic connections that form during the third trimester of gestation and early childhood compensate for a functional

synaptic impairment. These exuberant synapses are pruned by late adolescence, uncovering the existing synaptic impairment. Inadequate synaptic activity might lead to overpruning and manifestations of the symptoms of schizophrenia.

4. Independent of their possible roles in causing the disease, the deficits in PSYN and *RGS4* expression have physiological and behavioral consequences relevant to the pathophysiology of schizophrenia, and could help explain many of the cognitive deficits that arise from prefrontal cortex dysfunction in schizophrenics. Knockout studies in mice, in which specific members of the PSYN group have been deleted, reveal the complexity of physiological deficits that occur because of presynaptic dysfunction, suggesting that the experimental data support the synaptic-neurodevelopmental model of schizophrenia that we propose.

Our analysis has also revealed a consistent and significant decrease in the expression of genes encoding proteins involved in the mitochondrial malate shuttle, the transcarboxylic acid cycle, aspartate and alanine metabolism, ornithine and polyamine metabolism, and ubiquitin metabolism. Furthermore, because of the important relationship that exists between cellular metabolism and synaptic activity in the brain, these findings converge with our studies demonstrating reduced expression of gene groups involved in presynaptic function and the reduced expression of RGS4, a protein involved in postsynaptic signaling. Together, the data suggest that deficits in neuronal communication may contribute to the core pathophysiology of schizophrenia. Whether these transcriptional changes in metabolic gene groups reflect primary or secondary changes, they clearly have the potential to alter neuronal metabolism and activity, thereby contributing to defects in neuronal communication. Our findings indicate that a number of biologically related and mitochondria-dependent processes are affected in schizophrenia, with the altered expression of the malate shuttle genes that is perhaps the most intriguing feature of the dataset. The potential biological consequences of a decrease in malate dehydrogenase activity, and a general decrease in the activity of the malate shuttle, are quite significant. Namely, one of the most important functions of the malate shuttle is to transfer hydrogen ions from the cytoplasm into the mitochondria. Therefore, schizophrenia may be associated with increased [H+]-reducing equivalents in the cytosol. Increases in cytosolic [H+] are known to decrease the activity of the major rate-limiting enzyme of glycolysis, 6-phosphofructokinase. Thus, decreased

malate shuttle activity in the PFC of subjects with schizophrenia could produce secondary effects on the rate of glycolysis, perhaps contributing to the reduced glucose use observed in the PFC of these subjects while they are engaged in cognitive tasks. In addition, the malate shuttle system also acts in concert with a malate-citrate exchange system that is part of the TCA cycle and serves as an entry point for fatty acid synthesis. If the malate shuttle activity is reduced and the activity of the malate-citrate exchange system is reduced as well, one might expect to find a loss in cytosolic citrate and decreased activity of other TCA proteins. Given the role that TCA metabolism plays in fatty acid synthesis, these findings may help explain the reductions in markers of fatty acid metabolism that have been reported in several studies of subjects with schizophrenia. Finally, decreased malate shuttle activity could directly alter cytosolic levels of aspartate and glutamate, given the role that the malate shuttle plays in the exchange of cytosolic malate for mitochondrial α-ketoglutarate and then (after transamination of α-ketoglutarate into glutamate) the exchange of cytosolic glutamate for mictochondrial aspartate. Alterations in cytosolic aspartate and glutamate levels could affect not only the metabolism of these molecules but also ornithine-polyamine metabolism – also present in our dataset.

Our microarray studies of the PFC in subjects with schizophrenia revealed three principal findings regarding the expression of 14-3-3 gene family members: 1) of the six 14-3-3 genes represented on the arrays (beta, eta, epsilon, sigma, theta, and zeta), five were detectable in all 10 pair-wise comparisons, with one (sigma) detectable in 7/10 comparisons; 2) all of the 14-3-3 family members except for sigma were decreased in most comparisons; 3) at the group level, these genes were significantly changed in all 10 arrays ($p<0.021$), representing the single most-changed gene group we have identified to date. The expression changes reported on the microarrays were confirmed by ISH, which revealed decreases for zeta and beta>eta>gamma, supporting the microarray findings. As the 14-3-3 family of proteins plays an integral role in regulating many aspects of cellular function in the brain, including signal transduction, neurotransmitter metabolism, and mitochondrial function (there are at least 100 different binding partners for these proteins that have been identified), it is difficult to precisely define the appropriate context in which to view 14-3-3 gene alterations in schizophrenia. We tentatively suggest that at least in schizophrenia, the most critical

functions, that an effect of the 14-3-3 gene family might exert, are those related to neurotransmission and neurotransmitter metabolism. The decreased synthesis of multiple 14-3-3 transcripts should produce in the cell a decrease in the amount of dopamine available for neurotransmission—an event that could influence both excitatory and inhibitory neurotransmission via D1 and D2 receptor classes. The decreased aspartate metabolism could reduce the amount of excitatory neurotransmitters (including both aspartate and glutamate) available for release, as well as the bioenergetic properties of mitochondria localized to the synapse. The decrease in the presynaptic secretory machinery group genes that also occurs in these subjects suggests that the substrates for neurotransmission as well as the machinery for neurotransmission are collectively and profoundly altered in schizophrenia. Our observations of altered expression of the 14-3-3 gene group may also be of interest in relationship to the disturbances in markers of GABA neurotransmission in the PFC of subjects with schizophrenia. The 14-3-3 proteins (including the zeta and eta forms) have been shown to associate and co-localize with the C-terminus of presynaptic GABA(B)R1 receptors in rat brain preparations and tissue cultured cells. Furthermore, in the presence of 14-3-3, the authors reported a reduction in the dimerization of GABA(B)R1 and R2 subunits. The coupling of GABA(B)R2 to R1 permits surface expression of GABA(B)R1 and the appearance of potassium and calcium currents. Thus, it is possible that the reduction in 14-3-3 gene expression may represent a compensatory mechanism for overcoming a reduction in GABA signaling that is present in the PFC of subjects with schizophrenia.

Detailed analysis of the custom DNA microarray data revealed unexpected and strongly correlated upregulation of a subset of genes involved in immune/chaperone function. Yet, the immune/chaperone signature was primarily present in a subset of subjects with schizophrenia. We believe that the 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, albeit replication across different cohorts of patients has been elusive. 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. 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. In view of the overall 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 INFγ 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? 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. Thus, 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.

Our observation of reduced levels of GAD_{67} and GAT1 mRNAs in the DLPFC of the same subjects with schizophrenia and the correlation between these changes across are consistent with previous studies indicating that both the synthesis and presynaptic reuptake of GABA are reduced in the subset of GABA neurons that express PV. This is likely to be a compensatory response to decreased GABA synthesis in these neurons. The highly significant correlations among the gene expression changes for GAD_{67}, SST and NPY suggest that GAD_{67} mRNA expression is also decreased in another subset of GABA neurons that express both SST and NPY. In the cortex, SST is expressed by the majority of calbindin-containing GABA neurons, a separate population from those that express PV or CR, and a subset of SST-containing neurons largely overlaps with the majority of NPY-containing neurons. The localization of

SST- and NPY-containing neurons predominantly in layers II and V may account for the deficits in GAD_{67} mRNA expression in these layers, which could not be explained by the expression deficits in PV-containing neurons. Because SST- and NPY-containing neurons selectively target distal dendrites of pyramidal neurons, these coordinated gene expression changes suggest that GABA neurotransmission is altered at the dendritic domain of pyramidal neurons in the DLPFC of subjects with schizophrenia. Furthermore, given the functions of SST and NPY as inhibitory neuromodulators, their gene expression deficits indicate the presence of additional mechanisms affecting inhibitory regulation of DLPFC circuitry in schizophrenia. CCK is heavily expressed in GABA neurons that do not contain PV or SST and is expressed at low levels in some pyramidal neurons. The clustering of CCK with GAD_{67} and their highly correlated expression changes suggest a deficit in GABA synthesis in CCK-containing GABA neurons. The axon terminals of CCK-positive basket neurons converge with those from PV-containing neurons on the perisomatic domain of pyramidal neurons. Thus, alterations in GABA regulation on this domain of pyramidal neurons appear to involve at least two subpopulations of GABA neurons in the DLPFC of subjects with schizophrenia. $GABA_A$ receptors containing the $\alpha 1$ and $\gamma 2$ subunits are enriched in postsynaptic sites where they mediate phasic inhibition. In contrast, $GABA_A$ receptors containing the δ subunit, which is often coassembled with the $\alpha 4$ subunit in the forebrain, are selectively localized to extrasynaptic sites. These extrasynaptic receptors, which have a high sensitivity to GABA and thus can be activated by ambient GABA molecules in extracellular space, mediate tonic inhibition which reduces the effects of synaptic inputs over time. Given the predominant localization of the $\alpha 1$, $\gamma 2$ and δ subunits to dendrites, the highly correlated expression deficits for these transcripts suggest coordinated downregulation of $GABA_A$ receptors mediating phasic and tonic inhibition in the dendritic domain of DLPFC pyramidal neurons in schizophrenia. Overall, the findings suggest altered GABA-mediated regulation of both inputs to and outputs from DLPFC pyramidal neurons in subjects with schizophrenia. These alterations are certain to affect information processing in DLPFC circuitry and thus are likely to be major contributors to working memory impairments in schizophrenia.

VI. CONCLUSIONS

We can draw 5 major conclusions from our postmortem gene expression profiling studies:

1) Postmortem human brain tissue can be analyzed by high throughput gene expression profiling, and this analysis gives us unique insight into the pathophysiological disturbances that occur in the PFC of patients with schizophrenia (10,11).

2) Schizophrenia is characterized by complex gene expression changes in the human PFC (10-13). These expression changes appear to be primarily related to the disease process itself, and not a result of medication, sample bias, or quality of postmortem tissue. We uncovered systemic gene expression deficits related to presynaptic markers (1,9), energy metabolism genes (2), 14-3-3 family proteins (3), and GABA-system transcripts (6,14). Furthermore, we reported a strong, sustained and complex induction of immune system and chaperone mRNAs, suggesting that schizophrenia has in important, ongoing and potentially progressive neuroinflammatory component (5). Many of these findings have been also replicated by other investigators, using independent sample cohorts.

3) The molecular variability between subjects with schizophrenia is tremendous (9,15-18), and this is very likely reflected in the vastly different clinical presentations of patients. Yet, the various genetic disturbances are likely to converge and have a sustained effect on well-defined biological functions, resulting in common gene expression patterns – even when the genetic susceptibility to disease is quite different (9-11).

4) It appears that important relationships may exist between altered gene expression and genetic susceptibility to psychiatric disorders (11,13). For example, RGS4 and GABRAB2 show expression alterations in the postmortem brain of subjects with schizophrenia, and these genes have been also implicated as putative, heritable schizophrenia susceptibility genes. Thus, we propose that for some genes, altered expression in the postmortem human brain may have a dual origin: polymorphisms in the candidate genes themselves or upstream genetic-environmental factors that converge to alter their expression level. Based on the currently available data, we

suggest the hypothesis that certain gene products, which function as "molecular hubs", commonly show altered expression in psychiatric disorders and confer genetic susceptibility for one or more diseases (11,13,19).

5) It appears that RGS4 is one of the genes critically involved in the pathophysiology of schizophrenia. First, RGS4 mRNA and protein expression are both reduced in the PFC of subjects with schizophrenia (4). Second, specific genetic variants in the promoter region of RGS4 appear to predispose to developing schizophrenia. Third, genetic variants of RGS4 predict brain structure, PFC function and connectivity, and cognitive performance. Finally, the same RGS4 SNP variant is predictive of certain treatment responses. These combined data suggest that RGS4 is an appealing, knowledge-based therapeutic target for developing novel medications for treatment of schizophrenia.

VII. CANDIDATE'S PUBLICATIONS RELEVANT TO THESIS WORK

1. Mirnics K, Middleton FA, Marquez A, Lewis DA and Levitt P. (2000) Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. Neuron, 28: 53-67.
2. Middleton FA, Mirnics K, Pierri JN, Lewis DA and Levitt P. (2002) Gene expression profiling reveals alterations of specific metabolic pathways in schizophrenia. J Neurosci, 22: 2718-2729.
3. Middleton FA, Peng L, Lewis DA, Levitt P and Mirnics K. (2005) Altered expression of 14-3-3 genes in the prefrontal cortex of subjects with schizophrenia. Neuropsychopharmacology, 30: 974-983.
4. Mirnics K, Middleton FA, Stanwood GD, Lewis DA and Levitt P. (2001) Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia. Mol Psychiatry, 6: 293-301.
5. Arion D, Unger T, Lewis DA, Levitt P and Mirnics K. (2007) Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia. Biol Psychiatry, 62: 711-721.
6. Hashimoto T, Bazmi HH, Mirnics K, Wu Q, Sampson AR and Lewis DA. (2008) Conserved regional patterns of GABA-related transcript expression in the neocortex of subjects with schizophrenia. Am J Psychiatry, 165: 479-489.
7. Unger T, Korade Z, Lazarov O, Terrano D, Sisodia SS and Mirnics K. (2005) True and false discovery in DNA microarray experiments: transcriptome changes in the hippocampus of presenilin 1 mutant mice. Methods, 37: 261-273.
8. Pongrac JL, Middleton FA, Peng L, Lewis DA, Levitt P and Mirnics K. (2004) Heat shock protein 12A shows reduced expression in the prefrontal cortex of subjects with schizophrenia. Biol Psychiatry, 56: 943-950.

9. Mirnics K, Middleton FA, Lewis DA and Levitt P. (2001) Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. Trends Neurosci, 24: 479-486.
10. Mirnics K, Levitt P and Lewis DA. (2004) DNA microarray analysis of postmortem brain tissue. Int Rev Neurobiol, 60: 153-181.
11. Mirnics K, Levitt P and Lewis DA. (2006) Critical appraisal of DNA microarrays in psychiatric genomics. Biol Psychiatry, 60: 163-176.
12. Horvath S and Mirnics K. (2009) Breaking the gene barrier in schizophrenia. Nat Med, 15: 488-490.
13. Mirnics K and Pevsner J. (2004) Progress in the use of microarray technology to study the neurobiology of disease. Nat Neurosci, 7: 434-439.
14. Hashimoto T, Arion D, Unger T, Maldonado-Aviles JG, Morris HM, Volk DW, Mirnics K and Lewis DA. (2008) Alterations in GABA-related transcriptome in the dorsolateral prefrontal cortex of subjects with schizophrenia. Mol Psychiatry, 13: 147-161.
15. Mirnics K, Middleton FA, Lewis DA and Levitt P. (2001) Delineating novel signature patterns of altered gene expression in schizophrenia using gene microarrays. ScientificWorldJournal, 1: 114-116.
16. Mirnics K and Lewis DA. (2001) Genes and subtypes of schizophrenia. Trends Mol Med, 7: 281-283.
17. Mirnics K. (2006) Microarrays in brain research: Data quality and limitations revisited. Current Genomics, 7: 11-19.
18. Mirnics K. (2002) Microarrays in brain research: Data quality and limitations. Current Genomics, 3: 13-19.
19. Winden KD, Oldham MC, Mirnics K, Ebert PJ, Swan CH, Levitt P, Rubenstein JL, Horvath S and Geschwind DH. (2009) The organization of the transcriptional network in specific neuronal classes. Mol Syst Biol, 5: 291.

VIII. ACKNOWLEDGEMENTS

I dedicate this thesis to Željka, Marco and Emma.

First and foremost, I have to thank my wife, Dr. Željka Korade, for everything – her love and support, our wonderful family life, tremendous kids (Emma and Marco), scientific wisdom, patience and self-sacrifice for advancement of my career. Without her at my side, it would not be worth anything.

I also wish to thank my PhD advisor, Prof. Dr. Gábor Faludi from the bottom of my heart – for providing guidance over the years, scientifically challenging me and for being a wonderful friend. He found a way to link me back to the Hungarian scientific community, to rekindle my Hungarian identity, to show me my scientific roots.

I am very grateful for the guidance, help and challenge of my committee members.

Next, I am eternally grateful to SOTE PhD School for giving me a chance to formally become part of the SOTE Alma Mater. I am extremely proud to be a SOTE graduate; it

means a World to me. I also need to thank Tímea Rab, for helping me navigate through the maze of paperwork with kindness and patience.

I also need to acknowledge my parents, who raised me to love, be curious and have an open mind. I wish to thank for the guidance of my teachers, mentors and colleagues who helped me grow and skilled me to believe in myself: Dr. István Görög from Orebić, Croatia, who taught me dedication and unwavering spirit; Kornelija Djaković-Švjacer (University of Novi Sad), who showed me how to love science, Dr. David A. Lewis (U of Pittsburgh), for shaping me into a rigorous scientist and Dr. Miklós Palkovits (Hungarian Academy of Sciences) for continuously amazing me with his scientific and social insights and blazing spirit. However, from all my teachers Dr. Pat Levitt (University of Southern California) had the most influence on my career and scientific thinking. He has been a tremendous mentor, an admired colleague, and a father figure to me over the years, and his friendship and wisdom will stay with me till the rest of my life.

Finally, I have to thank for all the scientists who worked for me and with me over the years, and who are too numerous to name individually. They critically shaped my scientific thinking over the years, and some of the best studies arose from their ideas, thus my success is their success, too.

Most of the research presented here was performed on postmortem brain tissue. I want to thank the donor families for their generosity at their time of sorrow and loss – without their gift none of this research would have been possible.

Over the years our research has received substantial funding from the National Institute of Mental Health (NIMH), National Institute of Aging (NIA), National Institute for Neurological Disorders and Stroke (NINDS), National Institute of Child Health and Human Development (NICHD), National Alliance for Research on Schizophrenia and Depression (NARSAD), American Foundation for Suicide Prevention (AFSP) and Eli Lilly and Company. I am thankful to all of them for funding our research.