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Molecular Characterization of Clinical Study Schizophrenia Viewed by Microarray Analysis of Gene Expression in Prefrontal Cortex

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Summary

Microarray expression profiling of prefrontal cortex from matched pairs of schizophrenic and control subjects and hierarchical data analysis revealed that transcripts encoding proteins involved in the regulation of presynaptic function (*PSYN*) were decreased in all subjects with schizophrenia. Genes of the *PSYN* group showed a different combination of decreased expression across subjects. Over 250 other gene groups did not show altered expression. Selected *PSYN* microarray observations were verified by in situ hybridization. Two of the most consistently changed transcripts in the *PSYN* functional gene group, N-ethylmaleimide sensitive factor and synapsin II, were decreased in ten of ten and nine of ten subjects with schizophrenia, respectively. The combined data suggest that subjects with schizophrenia share a common abnormality in presynaptic function. We set forth a predictive, testable model.

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 (Carpenter and Buchanan, 1994; Hyman, 2000b). 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 (Lewis, 2000). 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 contribute 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 (Weinberger, 1995; Andreasen, 1996; Hyman, 2000a; Pulver, 2000; Tsuang, 2000). In terms of pathophysiology, a number of brain regions, including the hippocampus, superior temporal gyrus, and thalamus, appear to be disturbed in this disorder (Harrison, 1999; McCarley et al., 1999). In particular, 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 (Weinberger et al., 1986; Selemon et al., 1995; Andreasen et al., 1997; Bertolino et al., 2000). 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. For example, in subjects with schizophrenia, reductions in gray matter volume in the dorsal PFC have been observed in neuroimaging studies (Goldstein et al., 1999; Sanfilipo et al., 2000), and these volumetric changes are associated with an increase in cell packing density (Selemon et al., 1995, 1998; Lewis and Lieberman, 2000) but no change in total neuron number in the PFC (Pakkenberg, 1993). Although the size of some PFC neuronal populations appears to be reduced in schizophrenia (Rajkowska et al., 1998; Pierri et al., 1999), these findings are also likely to reflect a decrease in the number of axon terminals and in the distal dendrites and dendritic spines that represent their principal synaptic targets (Selemon and Goldman-Rakic, 1999). Consistent with this interpretation, both the levels of synaptophysin, a presynaptic terminal protein (Karson et al., 1996; Perrone-Bizzozero et al., 1996; Glantz and Lewis, 1997), and the density of dendritic spines (Garey et al., 1998; Glantz et al., 2000) are decreased in the PFC of subjects with schizophrenia. Interestingly, the typical age of onset of schizophrenia coincides with the termination of the adolescence-related reduction in the densities of synapses and dendritic spines in the primate PFC (Huttenlocher, 1979; Bourgeois et al., 1994; Anderson et al., 1995).

Changes in gene expression also have been observed in the PFC of subjects with schizophrenia. In particular, alterations in gene products related to neurotransmission (Akbarian et al., 1995a, 1996; Meador-Woodruff et al., 1997; Volk et al., 2000) or second messenger systems (Dean et al., 1997; Shimon et al., 1998; Hudson et al., 1999) have been observed. However, each of these studies have 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 currently unknown.

Microarray technology provides an opportunity for application of gene expression analysis to complex clinical diseases (Eisen et al., 1998; Brown and Botstein, 1999; Duggan et al., 1999; Alizadeh et al., 2000), and these approaches have been successful in addressing fundamental biological questions in human cancer (Alizadeh et al., 2000). However, due to the inherent complexity of nervous tissue and the need to utilize postmortem material, few microarray studies of the human central nervous system have been conducted (Whitney et al., 1999). Furthermore, in contrast to cancer, where many biological measures of the disease reveal differences of

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several orders of magnitude, alterations in brain structure or chemistry in schizophrenia appear to be much more moderate, often not exceeding a 2-fold change (Collinge and Curtis, 1991; Akbarian et al., 1995a, 1996; Woo et al., 1998; Glantz and Lewis, 2000; Volk et al., 2000). In addition, the clinical heterogeneity of schizophrenia probably reflects a complex biological substrate, but with key elements that are common to the clinical phenotype. Consequently, in order to determine whether there are modest, but consistent, changes in gene expression in the PFC across schizophrenic subjects, we designed a three-step experimental approach. First, we used high-density cDNA microarrays (Brown and Botstein, 1999; Duggan et al., 1999; Whitney et al., 1999; Alizadeh et al., 2000) to compare the relative levels of over 7000 gene transcripts in the PFC of matched pairs of schizophrenic and control subjects. These data were analyzed by gene expression profiling of over 250 gene groups, including custom-designed cascades of gene products related to specific nervous system functions. Following hierarchical analysis of these data, we verified selected microarray findings using in situ hybridization. Finally, we determined whether the most significant differences in gene expression also were present in a second cohort of schizophrenic and control subjects.

Results

Microarrays Reveal Defects in Expression of Synaptic Function–Related Gene Groups

We performed several control experiments to assess the feasibility of microarray analysis on postmortem human brain tissue. First, we hybridized two aliquots of the same cortical Cy3- and Cy5-labeled mRNA from a control subject onto one UniGEM-V cDNA array (Figure 1A). The obtained data showed a normal distribution with variability that was in agreement with Incyte's extensive UniGEM-V reproducibility studies (http://www.incyte.com/gem/technology/GEM-reproducibility.pdf). Second, to assess interexperimental variability, pair 685c/622s was hybridized to three different UniGEM-V microarrays. The results obtained in the three experiments were consistent and reported the same changes in gene expression between the schizophrenic and control subject (data not shown). These control data indicated that microarray analysis is a reliable tool for analyzing gene expression changes in human postmortem brain tissue.

Next, using high-density cDNA microarrays, 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: 2.6% of the genes were expressed at higher levels in schizophrenic subjects than in the matched controls, whereas 2.2% were expressed at lower levels in the schizophrenic subjects.

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 (Figures 1E–1H), establishing that other changes that we did detect are not due simply to human subject variability. This observation also is in agreement with previous findings that total mRNA levels in schizophrenic subjects are comparable to those in the unaffected human population (Harrison et al., 1997). 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*) (Figure 1B). Expression of genes involved in glutamate and GABA neurotransmission (Figures 1C and 1D) were also decreased, supporting previous studies that have documented changes of individual genes within these latter groups in the PFC of schizophrenic subjects (Collinge and Curtis, 1991; Harrison et al., 1991; Akbarian et al., 1995b, 1995c, 1996; Chen et al., 1998; Volk et al., 2000). For example, the microarrays reported decreased glutamic acid decarboxylase 1–67 kDa (*GAD67*) expression, essentially reproducing our previous study of in situ hybridization (Volk et al., 2000) on the same subjects with schizophrenia. Furthermore, less consistently reported changes in glutamate receptor 1 and 2 (*AMPA1–2*, *GluR1–2*) expression in other cortical regions (Eastwood et al., 1995, 1997) were also reproduced. It is possible that the deficits in these systems observed in the present study may be related to the changes in the presynaptic secretory machinery transcripts. However, the limited number of microarray targets related to these specific neurotransmitter systems hindered our ability to further interpret these observations. Furthermore, we were not able to fully assess changes in a number of other neurotransmitter systems (e.g., dopamine, norepinephrine, serotonin) due to the low number of transcripts related to these systems on the microarrays.

The Expression of the *PSYN* Gene Group Is Decreased in the PFCs of All Schizophrenic Subjects

We focused our attention on what appeared to be the most robustly affected functional group, those genes related to the *PSYN* cascade. Although this group primarily includes transcripts encoding presynaptic secretory machinery proteins, a substantial number of these gene products are also involved in other cellular functions, mostly related to membrane trafficking. 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% confi-

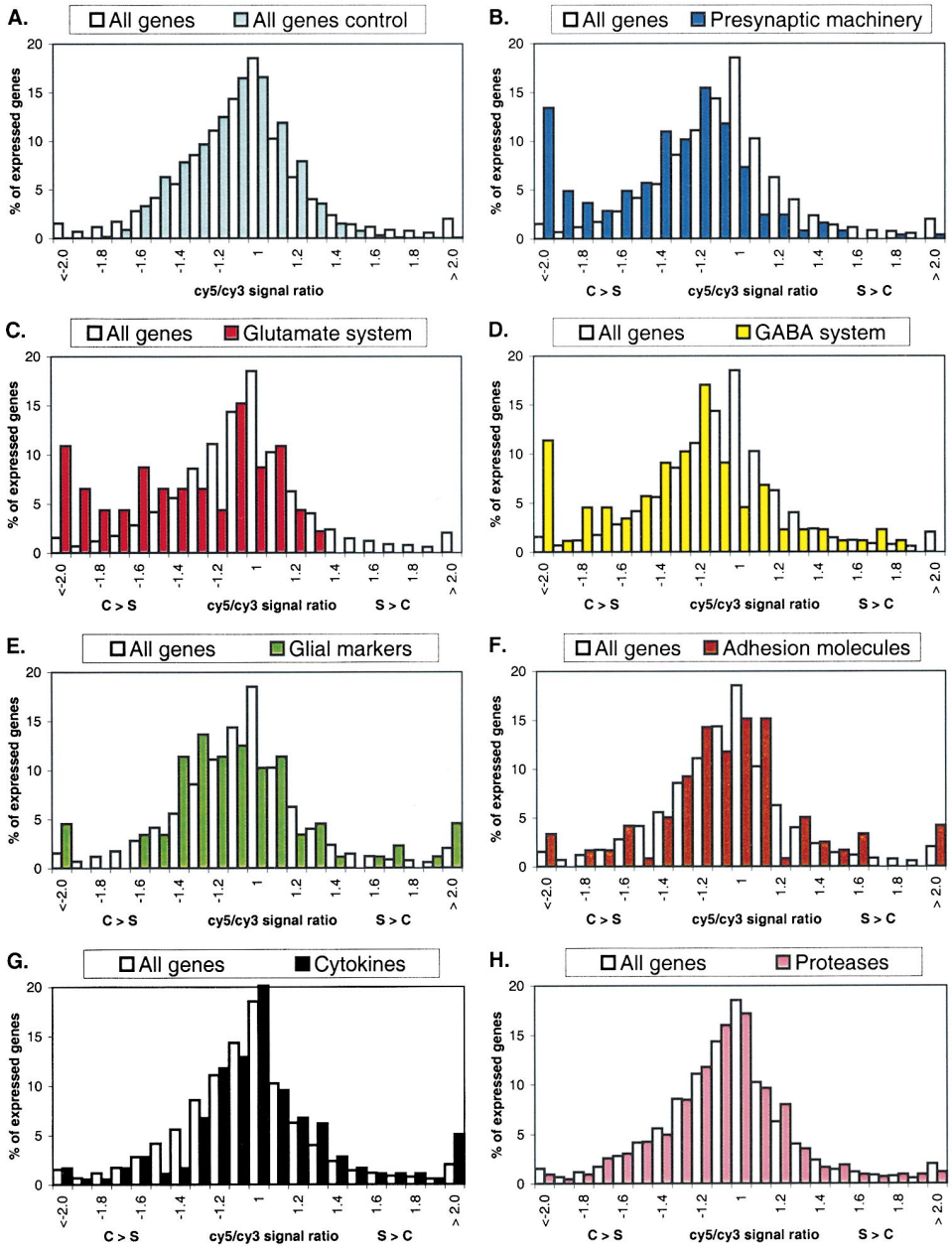

Figure 1. Gene Expression Differences in PFC Area 9 for Six Pairs of Schizophrenic and Control Subjects as Revealed by Microarray Analysis

For each gene group, all expressed genes were classified into signal intensity difference intervals (0.1 bins) according to their Cy5/Cy3 signal ratio. Transcripts in a "1" bin had identical Cy5 versus Cy3 signal intensities. Positive values (to the right) on the x axis denote higher Cy5 signal in schizophrenic subjects (S > C), negative values (to the left) correspond to higher Cy3 signal intensity in the control subjects (C > S). The y axis reports the percentage of expressed genes across the six subject pairs per bin for each gene group. In all panels, the white bars (all genes) denote distribution of all expressed genes across the six PFC pairwise comparisons (n = 22,408).

(A) Hybridization of two aliquots of the same cortical mRNA from one control subject (all genes control). One aliquot of control cortical mRNA was labeled with Cy3, the other with Cy5, and the combined sample was hybridized onto a single UniGEM-V cDNA microarray. At the 99% confidence level ($\geq|1.9|$), only four of the 4844 expressed genes (0.08%) reported a false positive Cy5 bias, and two genes (0.04%) reported a false positive Cy3 bias. In contrast with this control experiment, 4.8% of all expressed genes in the six combined PFC comparisons showed a differential expression over the 99% confidence level. Of all expressed genes in the six combined PFC comparisons, 95% of the expressed genes reported Cy3/Cy5 signal intensity ratios $\leq|1.6|$.

(B–H) Distribution of expressed genes for transcripts related to *PSYN* group (B), glutamate system (C), GABA system (D), glial markers (E), adhesion molecules (F), cytokines (G), and proteases (H). Note that genes classified in the *PSYN* group, glutamate system, and GABA system groups exhibit a pronounced decrease (shift to the left) in transcript expression in the schizophrenic subjects. Statistical tests of this leftward shift are noted in Table 1.

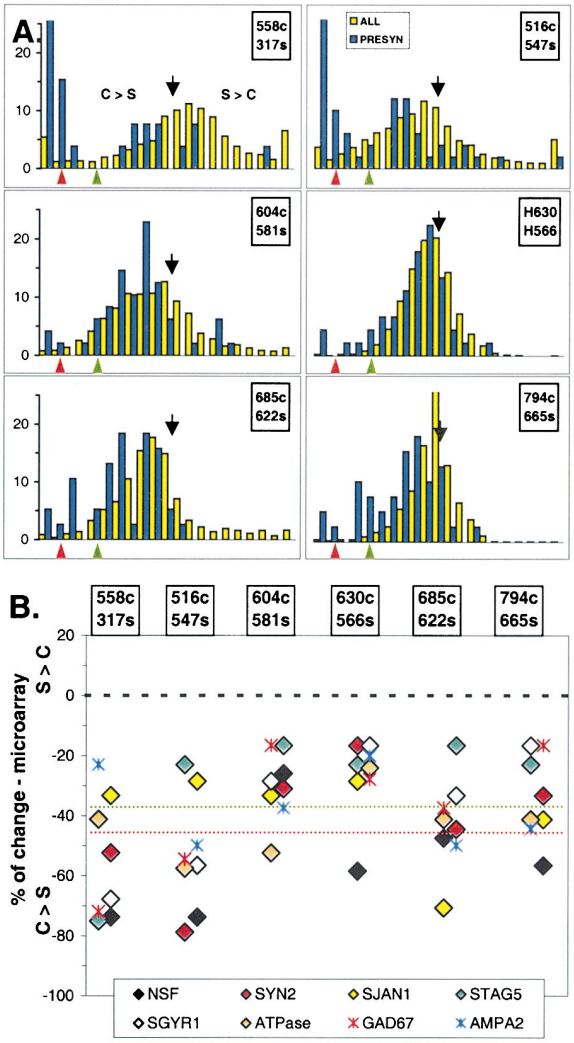

Figure 2. The *PSYN* Group Changes Consistently in Each Schizophrenic Subject

Pairs of control and schizophrenic subjects ("c" and "s" following subject number, respectively) are denoted in all figures in closed boxes.

(A) Each graph represents a single microarray experiment, with the layout the same as in Figure 1B. Histogram bars represent distribution of expressed genes as a function of Cy5/Cy3 signal ratio. Blue bars denote distribution of *PSYN* group. Yellow bars denote distribution of all expressed genes within the same microarray comparison. Black arrows denote "1" bin (identical Cy3/Cy5 signal intensity). Green arrowheads depict 95% and red arrowheads denote 99% confidence interval (CL) of transcript decrease, respectively. The shift to the left in the distribution of the *PSYN* group was highly significant ($p < 0.001$) in each individual microarray comparison.

(B) Expression differences for six genes related to synaptic machinery and one gene each for the glutamate and GABA systems in six microarray experiments. The six *PSYN* genes were chosen to represent three consistently changed genes (*NSF*, *SYN2*, and *ATPase*) and three occasionally changed genes (*STAG5*, *SJAN1*, and *SGYR1*). Y axis denotes expression differences between control and schizophrenic subjects measured by percent of Cy5/Cy3 intensity. Red dashed line represents a transcript decrease of 47.5% (99% CL), green dashed line corresponds to 37.5% decrease trend (95% CL), black dashed line denotes equal Cy5/Cy3 signal (0% change). Note that the pattern of decrease in *PSYN* transcripts differs across schizophrenic subjects. Abbreviations: *NSF*, N-ethylmaleimide sensitive factor; *SYN2*, synapsin II; *SJAN1*, synaptojanin 1; *STAG5*, synaptotagmin 5; *SGYR1*, synaptogyrin 1; *ATPase*, vacuolar proton pump (42 kDa); *GAD67*, glutamic acid decarboxylase 1 (67 kDa); and *AMPA2*, glutamate receptor 2 (*AMPA2*, *GLUR2*, *GLURB*, *GRIA2*).

dence level (Figure 2A). For each of the six comparisons, between 6 and 26 genes were changed (13.3%–56.9% of expressed *PSYN* genes). Furthermore, when combined, out of the total of 246 *PSYN* group observations, 17.9% of genes showed a decrease at the 99% CL (≤ -1.9), and 29.3% showed a significant decrease at the 95% CL (≤ -1.6) in the schizophrenic subjects across the six pairwise comparisons (Table 1A). 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 ($p < 0.0001$). This highly skewed distribution of the *PSYN* gene group was not due to individual variability between subjects, because individual random variability must occur in both directions. At the 99% CL, 2.2% of all genes were underexpressed in the schizophrenic subjects, suggesting that less than one *PSYN* gene per subject would be decreased due to the to individual variability (41 expressed genes $\times$ 0.022 = 1 gene for a single comparison or 246 $\times$ 0.022 = 5.5 genes across all six pairs). Furthermore, in a random distribution, at least six *PSYN* genes would be expected to show *increased* expression across the six schizophrenic subjects (246 $\times$ 0.028). However, this was not the case for the genes in the *PSYN* group. Over the 99% CL across the six schizophrenic subjects, only one (0.4%) *PSYN* gene reported an increase, and 44 (17.9%) *PSYN* transcripts were decreased. In addition, at the 95% CL we observed only 2 (0.8%) increased and 72 (29.3%) decreased *PSYN* genes across all the schizophrenic subjects.

In addition to the frequency distribution data, central tendency measurements also revealed a 38% decrease (-1.48-fold) ($p < 0.005$) of all expressed genes in the *PSYN* group compared to the average expression of all genes (-1.10) (Table 1B). Removing either the two most consistently changed *PSYN* genes (N-ethylmaleimide sensitive factor [*NSF*] and synapsin II [*SYN2*]), or the two most affected schizophrenic subjects, did not eliminate the significance of the findings (Table 2). The *PSYN* group average, without *NSF* and *SYN2*, still showed a 33% decrease (-1.43-fold) ($p = 0.004$) in comparison to the mean of all expressed genes (-1.10). These data indicate that many different genes contributed to the consistent transcript decrease of the *PSYN* group, including those genes whose expression did not reach the expression level difference of ≤ -1.6.

PSYN Gene Expression Deficits Are Different between Schizophrenic Subjects

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 (Figure 2B), revealing different patterns of decreased gene expression in the schizophrenic subjects. However, while individual differences across subject pairs can be inter-

Table 1. Summary Statistics of Gene Group Data Observed by Microarrays

	Distribution of Expressed Genes across Six PFC Comparisons							
A. Percent of Change	(22408) ALL	(246) *PSYN*	(46) *GLU*	(88) *GABA*	(88) *GLIAL*	(119) *ADH*	(178) *CYT*	(425) *PROT*
>1.9	2.6	0.4	0.0	0.0	5.7	4.2	5.6	2.1
1.6 to 1.89	2.8	0.4	0.0	4.5	3.4	3.4	3.4	2.6
−1.59 to +1.59	86.7	69.9	65.2	70.5	83.0	81.5	83.7	87.3
−1.6 to −1.89	5.7	11.4	17.4	12.5	3.4	7.6	5.1	6.6
<−1.9	2.2	17.9	17.4	12.5	4.5	3.4	2.2	1.4
Chi-square, p <	NA	0.0001*	0.0001*	0.0001*	0.12	0.63	0.43	0.97
	Average of Expressed Genes across Six PFC Comparisons							
B.	(>7,000) ALL	(62) *PSYN*	(11) *GLU*	(18) *GABA*	(22) *GLIAL*	(37) *ADH*	(82) *CYT*	(240) *PROT*
Mean number of expressed genes	3735	41.0	7.7	14.7	14.7	19.8	29.7	70.8
± SD	1344	8.76	1.97	3.72	4.32	6.79	7.66	25.37
Mean expression ratio	−1.10	−1.48	−1.42	−1.36	1.09	−1.06	1.01	−1.04
±SD	0.05	0.27	0.10	0.14	0.52	0.06	0.10	0.12
t test (df = 5), p <	NA	0.005*	0.001*	0.002*	0.222	0.216	0.065	0.221

Data analysis was performed across all six pairs of schizophrenic and control subjects. ALL, all expressed genes; *PSYN*, presynaptic secretory machinery; *GLU*, glutamate system; *GABA*, GABA system; *GLIAL*, glial markers; *ADH*, adhesion molecules; *CYT*, cytokines; *PROT*, proteases.
(A) Chi-square was calculated against all expressed genes in the six PFC comparisons. The total number of expressed gene observations across the six microarrays is given in parenthesis above each gene group. Note that transcript decreases in the *PSYN*, *GLU*, and *GABA* groups in schizophrenic subjects were highly significant ($p \leq 0.0001$) by comparing distribution of expressed ratios.
(B) Mean expression ratios (average of six mean expressions) for each gene group were calculated across the six microarray comparisons. The mean expression ratio for each gene group was compared to the mean expression ratio of all expressed genes using a t test (df = 5). Numbers in parentheses denote number of genes that comprise each gene group. Note that transcript decreases in the *PSYN*, *GLU*, and *GABA* groups in schizophrenic subjects were significant ($p \leq 0.005$) by comparing distribution of expressed ratios.

preted as a result of different patterns of decreased *PSYN* gene expression across the schizophrenic subjects, some of the observed individual pattern changes also could be due to the pairwise experimental design and the comparison of each schizophrenic subject to a different, matched control. Consequently, we tested the later interpretation in a post hoc manner using the in situ hybridization data (see below).

Within the *PSYN* group, the two most consistently altered gene products, *NSF* and *SYN2*, were decreased by 26%–74% and 17%–79%, respectively, in the six schizophrenic subjects (Figure 2B). To verify the microarray findings for these genes, and for two others that exhibited modest changes and only in a few subjects (synaptojanin 1 [*SJAN1*] and synaptotagmin 5 [*STAG5*]), we performed in situ hybridization for five of the same subject pairs used for the microarray experiments. For pair 794c/665s, no sections were available from the same block of tissue used in the microarray experiment. Radiolabeled cRNA probes specific for *NSF*, *SYN2*,

Table 2. *PSYN* Central Tendency Measures with and without Selected Genes and Distribution Classes

Pairs/MEAN Expression	All Expressed Genes	Expressed *PSYN* Genes			
		All	w/o *NSF* & *SYN2*	w/o <−1.9 & >1.9	w/o <−1.6 & >1.6
H558c/H317s	−1.19	−1.90	−1.82	−1.25	−1.20
H516c/H547s	−1.10	−1.71	−1.60	−1.21	−1.08
H604c/H581s	−1.09	−1.27	−1.26	−1.22	−1.18
H630c/H566s	−1.07	−1.25	−1.22	−1.20	−1.15
H685c/H622s	−1.09	−1.41	−1.38	−1.31	−1.28
H794c/H665s	−1.04	−1.34	−1.31	−1.28	−1.19
Average of means	−1.10	−1.48	−1.43	−1.24	−1.18
SD	0.05	0.27	0.23	0.04	0.07
t test (df = 5) p <	NA	0.005	0.004	0.001	0.025

PSYN Frequency Distribution with and without *NSF* and *SYN2*

	All Expressed Genes		Expressed Genes—*PSYN*		*PSYN* w/o *NSF* and *SYN2*	
Category	Number of Genes	Percent	Number of Genes	Percent	Number of Genes	Percent
>1.9	576	2.6	1	0.4	1	0.4
1.6 to 1.89	629	2.8	1	0.4	1	0.4
−1.59 to +1.59	19426	86.7	172	69.9	169	72.2
−1.6 to −1.89	1278	5.7	28	11.4	26	11.1
<−1.9	499	2.2	44	17.9	37	15.8
TOTAL	22408	100	246	100	234	100
Chi-square p <		NA		0.0001		0.0001

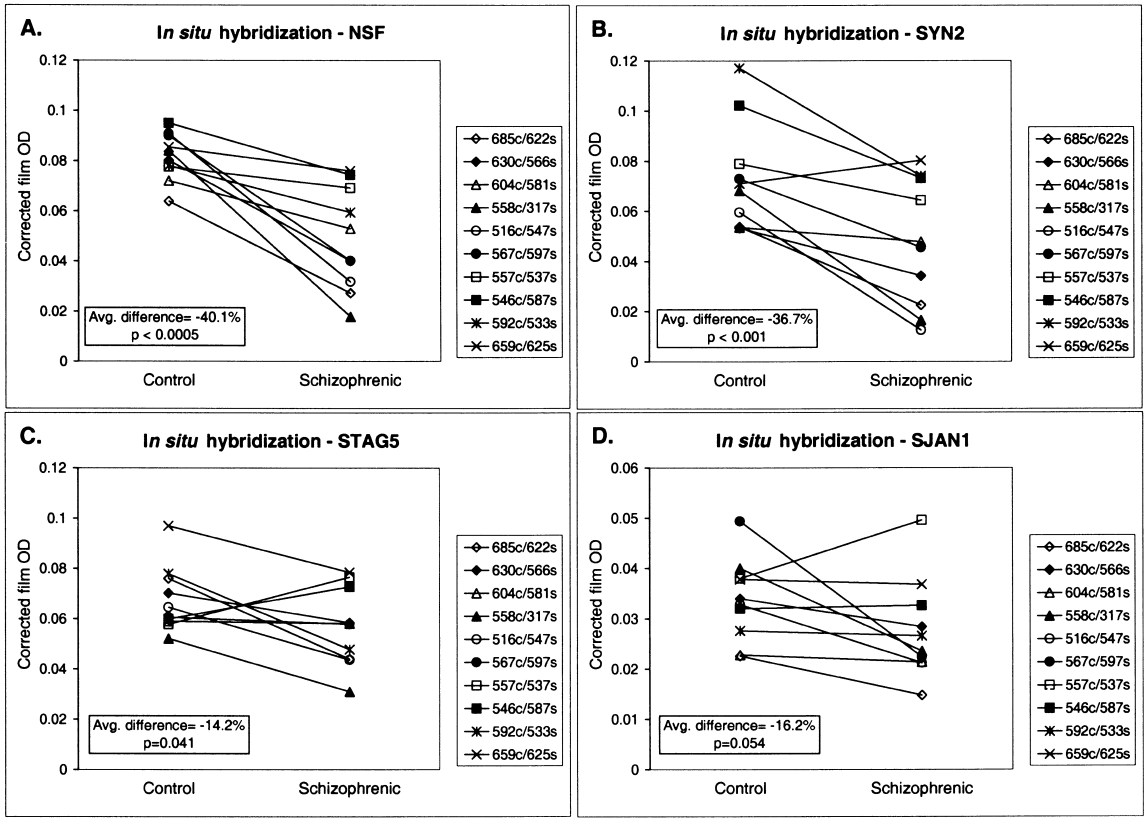

Figure 3. PFC Expression of Two Consistently Changed *PSYN* Genes (*NSF* and *SYN2*) and Two Occasionally Changed Genes (*SJAN1* and *STAG5*)

The in situ hybridization data from ten PFC pairwise comparisons were quantified using film densitometry. X axis represents ten pairwise comparisons, Y axis reports corrected film optical density measured across all layers for each control and schizophrenic subject. Lines connecting symbols indicate a matched subject pair. Note that *NSF* (A) and *SYN2* (B), both consistently decreased across the initial six microarray comparisons were also significantly decreased across the ten in situ pairwise comparisons. However, as expected, the two occasionally changed genes in the microarray comparisons, *STAG5* (C) and *SJAN1* (D), showed inconsistent changes that were marginally significant (indicated in boxes) between the control and schizophrenic subjects.

SJAN1, and *STAG5* were used to localize and quantify relative transcript levels. The in situ hybridization experiments confirmed the microarray findings for the schizophrenic subjects (Figures 2B, 3, and 4). At the group level, the decreases determined by microarray and in situ hybridization experiments were highly correlated ($r = 0.70$; $p < 0.001$).

Our findings also suggested that the most consistently observed decreases in transcripts across the microarray experiments (e.g., *NSF* and *SYN2*) would reproduce by in situ hybridization in a different cohort of schizophrenic subjects. To test this prediction, we examined whether *NSF* and *SYN2*, the most affected *PSYN* transcripts in the six original subject pairs, were also altered in a different cohort of five matched pairs of schizophrenic and control subjects. Indeed, in these additional comparisons *NSF* transcript levels were decreased in all five schizophrenic subjects, and *SYN2* levels were decreased in four of five schizophrenic subjects. Across all ten subject pairs, the mean levels of *NSF* and *SYN2* expression were significantly decreased in the schizophrenic subjects by 40% ($p < 0.001$) and 37% ($p < 0.005$), respectively (Figures 3A and 3B). In contrast, we predicted that the occasional and more modest *SJAN1* and *STAG5* changes reported by the microarrays would be altered to a lesser degree only in a subset of schizophrenic subjects. Indeed, from the second set of pairs, for both *STAG5* and *SJAN1*, only one of five comparisons exhibited a >20% decrease (Figures 3C and 3D). Across the ten pairs, due to less consistent decreases in expression, overall *STAG5* and *SJAN1* expression levels were only marginally changed (-16.2%, $p = 0.05$, and -14.2%, $p = 0.04$, respectively).

To test if the apparently distinct patterns of decrease of the *PSYN* group in individual schizophrenic subjects are present for the *NSF*, *SYN2*, *STAG5*, and *SJAN1* genes, we analyzed the in situ hybridization data post hoc in an unpaired experimental design. The corrected OD values, obtained for each schizophrenic subject, were compared to the mean OD values for each of the ten control subjects, as well as to the overall average OD values of the ten control subjects (Figure 5). Across the ten schizophrenic subjects, at least four different patterns of decreased expression of *NSF*, *SYN2*, *STAG5*, and *SJAN1* genes emerged, regardless of control subject used for comparison. The patterns of decreased expression also were present when single schizophrenic subjects were compared to the pooled, "reference" con-

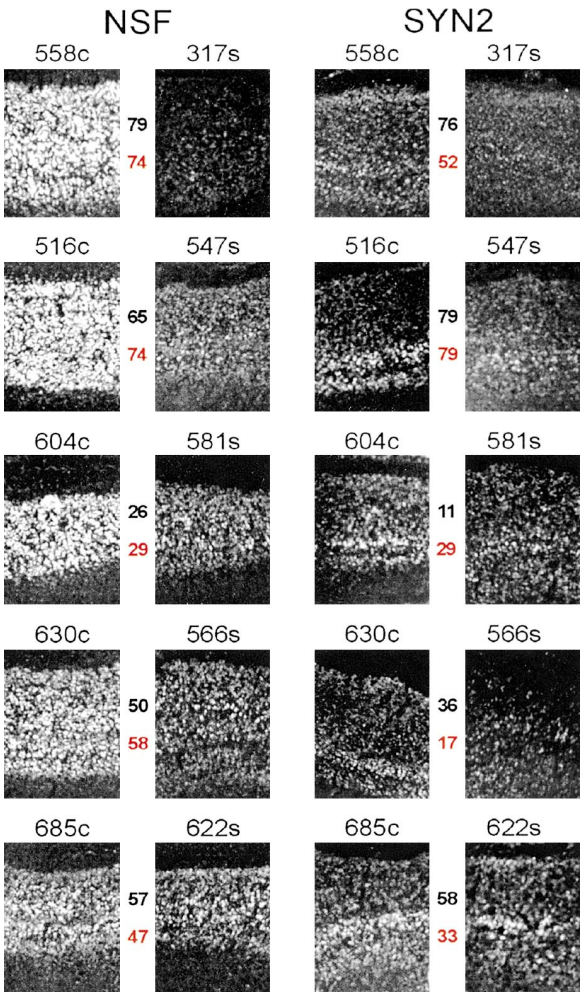

Figure 4. In Situ Verification of UniGEM-V Data for *NSF* and *SYN2*

Dark-field photomicrographs of PFC tissue sections from matched pairs of control and schizophrenic subjects (denoted by "c" and "s" following subject number, respectively). Complete set of micrographs for each gene was taken under identical illumination conditions at 25× magnification. Pial surface is towards the top of each image. Numbers denote percentage of transcript decrease in schizophrenic subjects as reported by the OD measurements of X-ray film (black numbers) and by microarray analysis of same pairs (red numbers).

trol. The variation patterns observed by in situ hybridization for these four genes are highly suggestive that distinct patterns of *PSYN* gene decreases seen on the microarrays are characteristic for subjects with schizophrenia.

Decreased *PSYN* Gene Expression Is Not a Result of Confounds

For each gene examined by in situ hybridization, group differences in OD measurements were examined using analysis of covariance (ANCOVA) with diagnosis as the main effect and sex, age, postmortem interval, brain pH, and tissue storage time as covariates. These potential confounds did not account for the observed changes in the schizophrenic subjects, except for *STAG5*, where at group level, in an unpaired design, the effect of diagnosis was no longer significant. In addition, for each of the four genes, no differences in gene expression were observed between the schizophrenic subjects with ($n = 4$) or without ($n = 6$) a history of substance abuse, or between those with a diagnosis of schizoaffective disorder ($n = 3$) or "pure" schizophrenia ($n = 7$).

Decreased *PSYN* Expression Is Not Induced by Antipsychotic Treatment

Two subjects with schizophrenia were not receiving antipsychotic medications at the time of death (622s and 547s), and both showed decreased expression of the *PSYN* group. As an additional strategy to address the influence of chronic exposure to antipsychotic medications (Nakahara et al., 1998; Johnson et al., 1999; Selemon et al., 1999), we compared gene expression in the frontal cortex of haloperidol-treated and control monkeys by microarray and in situ hybridization analyses. In one such pairwise comparison on a cDNA microarray, chronic haloperidol treatment produced changes in gene expression, but none of these were related to the *PSYN* group (Figures 6A and 6B), or to the glutamate and GABA systems (data not shown). In situ hybridization analysis of *NSF*, *SYN2*, *STAG5*, and *SJAN1* in two matched pairs of haloperidol-treated and control animals also failed to detect any decreases in expression (Figures 6B and 6C). Although the effects of chronic antipsychotic medication on *PSYN* gene expression cannot be completely excluded, evidence from both human and monkey studies suggests that the observed alterations in the expression of *PSYN* genes reflect the pathophysiology of schizophrenia and not treatment with antipsychotic medications. Indeed, our previous investigations have confirmed the utility of this combined approach by demonstrating that certain gene products in the PFC were increased, rather than decreased, both in schizophrenic subjects receiving antipsychotic medication relative to schizophrenic subjects not receiving antipsychotic medication at the time of death and in haloperidol-treated monkeys relative to control monkeys (Lewis, 2000).

Discussion

This study of a complex psychiatric disease combines the power of initial data acquisition from high-density cDNA microarrays, function-related data mining, and conventional molecular biology techniques to confirm findings for individual genes. Our finding of a deficit in the expression of genes encoding functionally defined cascades of proteins underscores the utility of microarrays as screening tools for complex changes in gene expression patterns in psychiatric diseases.

Reduced Expression of Genes Related to the Presynaptic Secretory Machinery

The number of PFC neurons appears to be normal in schizophrenia, and thus loss of neurons cannot account for our findings (Pakkenberg, 1993). Furthermore, through our statistical analysis, we demonstrate that the reduced expression of *PSYN* genes is related to the disease process of schizophrenia and not a consequence of its treatment or a confound of subject characteristics. At

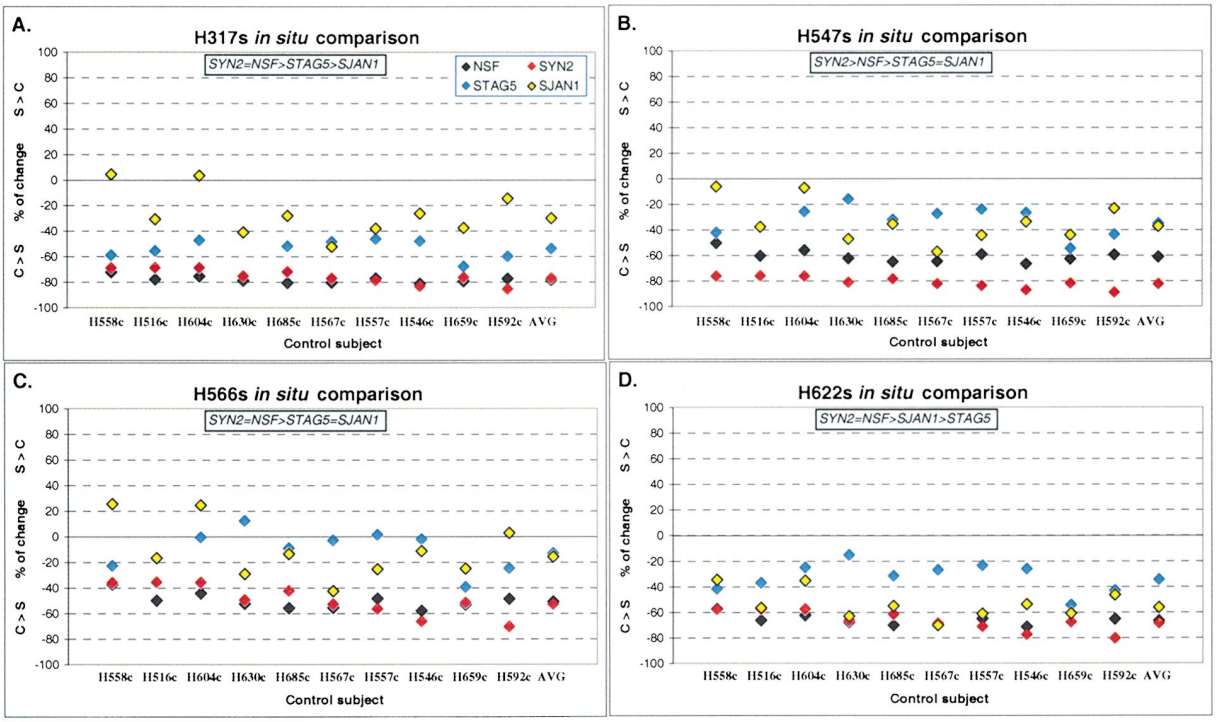

Figure 5. Individual and Distinct *NSF*, *SYN2*, *SJAN1*, and *STAG5* Gene Expression Patterns in Schizophrenic Subjects

OD comparison of in situ hybridization data between schizophrenic subjects 317s (A), 547s (B), 566s (C), 622s (D) and individual control subjects. Y axis reports *NSF*, *SYN2*, *STAG5*, and *SJAN1* expression changes compared to each control subject and the average of the control subjects on the x axis (AVG). Note the distinct gene expression patters in these four subjects (boxes).

the 99% CL, 4.5%–54% of the expressed genes in the *PSYN* group were decreased in each schizophrenic subject, but the specific genes affected differed across subjects. Although some of the less consistent and moderate changes cannot be ruled out as contributing to the differences in individual expression profiles, the data suggest that, at least in part, our findings are not due to a generic loss of neuropil. Such a loss would be expected to affect gene expression similarly in all schizophrenic subjects without marked individual differences. Furthermore, general synapse loss should produce comparable decreases across all synaptic machinery transcripts within a single brain comparison. This did not occur in any of the microarray pairwise comparisons: some transcripts encoding presynaptic markers were always unchanged (e.g., syntaxin 1), some were occasionally changed (e.g., synaptogyrin 1, *SJAN1*, *STAG5*), and others were consistently changed (e.g., *NSF*, *SYN2*, and

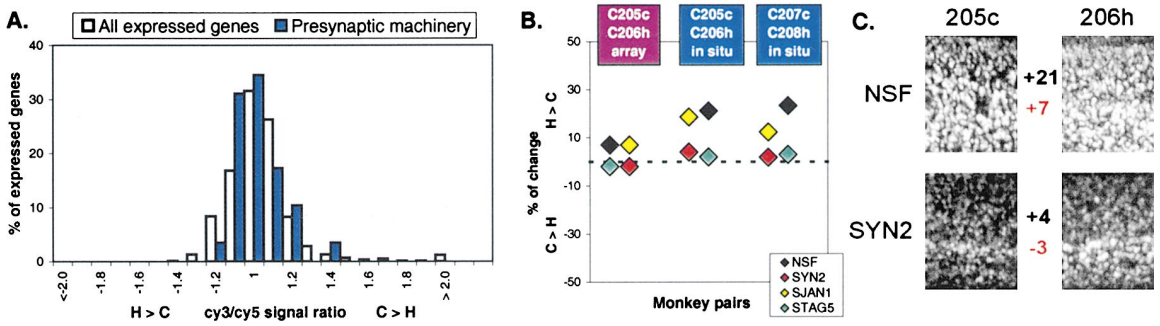

Figure 6. Chronic Haloperidol Treatment Does Not Decrease Expression of *PSYN*-Related Genes in Cynomolgus Monkeys

(A) Microarray hybridization data from the PFC of a haloperidol treated (206h) and matched control (205c) monkey. Figure layout is the same as in Figure 1B. h, haloperidol treated; c, matched control.

(B) Expression changes for one microarray (206h/205c) and two in situ pairwise comparisons (206h/205c and 208h/207c) for *NSF*, *SYN2*, *SJAN1*, and *STAG5*. Figure layout is the same as in Figures 2B and 2C.

(C) In situ hybridization micrographs from the dorsal PFC of a haloperidol-treated (206h) and matched control (205c) monkey viewed under dark-field microscopy at 25× magnification. Pial surface is toward the top of each image. Numbers denote percent differences reported by in situ (black) or microarray (red) analysis.

vacuolar proton pump-42 kDa [*ATPase*]). In addition, a previous analysis in the same pairs of subjects used in the present study, showed that the number of neurons expressing synaptophysin 1 mRNA, and the level of expression per neuron, did not differ between schizophrenic and control subjects (Glantz et al., 2000). Thus, we propose that subjects with schizophrenia share a common abnormality in the control of presynaptic function in the PFC but that they may differ in terms of the specific combination of genes that exhibit reduced levels of expression. This latter possibility is supported by the in situ hybridization results, in which variations in the decrease patterns for *NSF*, *SYN2*, *STAG5*, and *SJAN1* were evident in some schizophrenic subjects, regardless of the control subject used for comparison.

As part of the overall deficit in expression of the *PSYN* gene group in schizophrenic subjects, we also report a significant and consistent decrease in expression of *NSF*, *SYN2*, and *ATPase* in subjects with schizophrenia. For *NSF* and *SYN2*, in which in situ analysis also was done, these deficits were evident using both paired and unpaired comparisons. Alterations in transcripts for these genes have not been reported previously in schizophrenia and could be central to the pathogenesis of the disorder. Alternatively, *NSF*, *SYN2*, and *ATPase* transcripts may simply be particularly sensitive to deficits in other genes related to overall synaptic function. In addition, we wish to emphasize that other synapse-related genes, not yet identified, also may be consistently decreased in schizophrenia.

Many synaptic proteins also play a role in membrane trafficking. Consequently, *PSYN* group gene changes, in addition to reflecting potential alterations in synaptic release, also may affect overall protein trafficking. Presynaptic secretory function, however, is likely to be a principal target of altered *PSYN* gene expression. For example, the *Drosophila* mutant *comatose*, deficient in the widely expressed and multifunctional *NSF* gene, exhibits a primary defect in maintaining a readily releasable pool of synaptic vesicles (Tolar and Pallanck, 1998).

Multigene *PSYN* Deficits Link to Schizophrenic Loci

Our hypothesis that schizophrenia is a disorder of a functionally related group of genes is consistent with a multiloci pattern of linkage. Indeed, loci *22q11-13*, *17q21*, *1q21.3*, *3p24-26*, and *5q12-q13* have each been implicated in schizophrenia (Bird et al., 1997; Karayiorgou and Gogos, 1997; Pulver, 2000; Tsuang, 2000) (http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?181500#MAPPING) and contain genes encoding proteins related to *PSYN* genes or synaptic function. In fact, there are clear indications that alterations at some of these loci may be involved in the primary pathophysiology of schizophrenia. For example, deletions in the *22q11-12* region, encoding numerous synapse-related genes, have been associated with a prevalence of schizophrenia that is 25-fold greater than the general population (Murphy et al., 1999). Furthermore, a linkage study with a logarithm of odds score of 5.0 on chromosome *17q21* (containing, among others, the *NSF* and *ATPase* genes) has been linked (Bird et al., 1997) to the development of schizophrenia-like symptoms and classic neurofibrillary tangle pathology. However, just as decreased expression of all genes in the *PSYN* group was not present in each schizophrenic subject, alterations in one particular chromosomal locus are not likely to be present in all schizophrenic subjects.

Schizophrenia as a Deficit of a Final Common Path of Conserved Functional Abnormalities

Our results suggest that schizophrenia may be caused by multigene defects that lead to a final common path of conserved functional abnormalities. For disorders other than schizophrenia, single or multigene defects in different members of a functional cascade have been shown to produce the same disease with virtually indistinguishable symptoms. For example, over 70 gene loci or genes have been implicated in the pathogenesis of retinitis pigmentosa (Inglehearn, 1998; van Soest et al., 1999). In addition, first episode, never-medicated, and chronically ill schizophrenic subjects exhibit similar changes in functional and structural imaging studies of the PFC, and thus the deficits in *PSYN* gene expression are likely to contribute to PFC dysfunction at all stages of the illness (Pettegrew et al., 1993; Stanley et al., 1996; Harrison, 1999).

A Testable Model of Schizophrenia

The preponderance of data in the literature still reflects a lack of credible evidence of a specific biological mechanism being responsible for schizophrenia and its expressed symptoms (Weinberger, 1999). We know that the essence of the disease has a complex neurobiological basis, influenced by both polygenic, non-Medelian inherited genetic variation and epigenetic factors (Risch and Baron, 1984).

Toward which directions of future investigations can the present microarray analysis point us regarding falsifiable models of the disease? We put forth a model with four important components that address issues of genetic susceptibility, developmental time course, and synaptic dysfunction that underlie the pathophysiology of schizophrenia.

The Etiology of Schizophrenia Involves a Polygenic Pattern of Inheritance that Results in Altered Function of Proteins Controlling the "Mechanics" of Synaptic Transmission

Several *PSYN* genes and/or gene products already had been shown by conventional methods to be decreased in schizophrenia (Browning et al., 1993; Tcherepanov and Sokolov, 1997; Harrison and Eastwood, 1998; Davidsson et al., 1999; Karson et al., 1999). The present results, however, highlight what we believe to be a key feature of gene expression that only could have been ascertained by a high throughput genomics approach, in which dozens of genes in multiple molecular cascades are profiled simultaneously, without a constraining hypothesis or a need for robust linkage to a particular locus. In the present report, we show that the particular altered transcripts in the *PSYN* cascade, and the extent to which they change, varies among subjects, but with consistency in a small number of "most affected" genes. This fits with polygenic, non-Mendelian inherited disorders, in which independent studies produce maximal evidence for linkage at different loci, but with examples

of common alleles that have sufficiently large effects to be detected (Risch, 2000). Our model encompasses the scenario that mutations/polymorphisms in one or several of these genes, which may result in dysregulated expression of normal gene products, are sufficient to lead to a final common path of conserved functional abnormalities. Risch (2000) suggests that this polygenic view of the inherited component of schizophrenia can be tackled more effectively using a forward genetics approach. Using leads from our and future microarray studies that would probe the *PSYN* group more completely, a forward genetics approach would embrace a prioritized search for single nucleotide polymorphisms (SNPs) that, depending upon their location in coding or regulatory regions, could effect PSYN gene expression and function. The ability to narrow the SNP search from the hundreds of thousands of possibilities is clearly a major advantage for defining disease associations. Using the growing human genome and SNP databases, we can design novel "sequencing," protein and expression arrays to more fully characterize the underlying molecular mechanism of schizophrenia. We suggest that across schizophrenic subjects altered gene expression patterns will sort into a continuum related to the variations across individuals in clinical features. Thus, SNPs and gene expression patterns could be predictive of the age of onset, severity of illness, and, ultimately, of the most effective treatments.

The Heterogeneity in Expression Defects within the PSYN Group Is a Reflection of Distinct Adaptive Capacities of Different Populations of Neurons

In our model, a modest number of susceptibility genes, including *PSYN* or postsynaptic components (such as G protein–coupled signaling), would be primarily dysfunctional and would lead to phenotypic adaptations, which in some individuals would present clinically. This aspect of the model requires heterogeneous expression of *PSYN* genes across different neuronal types and brain areas, otherwise widespread peripheral and central neural dysfunction, incompatible with life would occur. Although surprisingly understudied, a few reports in the literature document that such heterogeneity exists in rodents (Melloni et al., 1993; Matus-Leibovitch et al., 1997; Porton et al., 1999), but detailed analysis is needed in primate brain. The added feature of adaptive capacities of different neurons leads directly to the hypothesis that some circuits naturally will be more susceptible to functional disruption than others. This is readily testable in forward genetic studies in which mutations are created conditionally, both spatially and temporally, providing a substrate for analysis of predictable molecular adaptations and physiological consequences. Few of these experiments have been performed, but recent reports provide evidence for complex and not necessarily predictable changes in synaptic plasticity and molecular adaptations. Indeed, studies of mice with deletions of individual synapse-related genes indicate that single genetic abnormalities can affect the transcription levels of a functionally defined gene group. For example, deletion of synapsin I results in major decreases in the expression of synaptic vesicle proteins and acceleration of synaptic depression, but no change in LTP (Rosahl et al., 1995). Furthermore, in mice deficient in the synapsin II gene, expression levels for many (but not all) of the other genes in the *PSYN* group are decreased (Rosahl et al., 1995), though to varying degrees. In addition, mice lacking synapsin I (Rosahl et al., 1995), synapsin II (Rosahl et al., 1995), *SCAMP1* (Fernandez-Chacon et al., 1999), synaptophysin I, synaptogyrin 1 (Janz et al., 1999), or Rab3a (Jahn and Sudhof, 1999) are viable and lack gross functional deficits, but display impairments in repetitive synaptic release and synaptic plasticity, while synaptotagmin IV–deficient mice show impaired learning and memory (Ferguson et al., 2000).

Deficits in PSYN Gene Expression Are Linked to the Neurodevelopmental Time Course of Schizophrenia

How might this occur? The extended period of synaptogenesis and pruning, from just before birth through adolescence in the primate cerebral cortex, is perhaps the most susceptible component of development to epigenetic factors that can influence both the molecular adaptation of *PSYN* gene expression and subsequent neuronal function. Thus, alterations in synaptic efficacy will have an impact on the organization and refinement of circuits during the experience-expectant and -dependent phases of synaptic development. For example, phase 4 of synaptic development corresponds to the period when net synapse number is stable, from early youth to prepuberty (Bourgeois, 2001). During this time, however, normal synapse reorganization occurs (e.g., the balance of synapses on spines versus shafts) without a change in synapse number. In the primate, altered visual experience has a pronounced negative impact on the balance of spine and shaft synapses that are formed in visual cortex (Bourgeois, 2001). Phase 5 of synaptic development, initiated around puberty in monkey and human (Bourgeois et al., 1994; Huttenlocher and Dabholkar, 1997), includes the period of synaptic pruning, in which a normal elimination of 30%–40% of synapses leads to a stable adult complement. Experimentally, altered synaptic function in the cortex, tested by eye removal in the embryonic monkey to alter visual experience (Bourgeois, 2001), leads to dramatic changes in the rate and type of synapses that are lost, but still results in a near normal number of total synapses in the neuropil. Though somewhat far-reaching, we suggest in our model that *PSYN* dysregulation gives rise to a "catch-22 scenario" for specific neural circuits, in which subclinical synaptic dysfunction during adolescence and puberty, due in part to defects in *PSYN* gene expression, leads to maladaptive mechanisms for synaptic stabilization and circuit function. Given our hypothesis that neuronal dysfunction is dependent on the expression of the altered *PSYN* susceptibility genes, certain developing circuits will be affected in a more pronounced fashion than others. A falsifiable experiment would incorporate the use of additional microarray data to define adaptive *PSYN* gene expression patterns, which will help investigators create appropriate genetic hypomorphs or conditional mutants, with subsequent analysis of the formation and pruning of synapses. The prediction includes, of course, a postpubertal onset of behavioral dysfunction that can be tested in standard assays. The expression of pronounced behavioral disturbances, appearing postpubertally and with a neurodevelopmental

etiology, have been described recently in genetic (Burrows et al., 2000) and lesion models (Wood et al., 1997).

Deficits in PSYN Expression Have Physiological and Behavioral Consequences Relevant to the Pathophysiology of Schizophrenia

In the context of reduced expression or mutation of multiple *PSYN* genes, neurons will have an impaired release of synaptic vesicles at nerve terminals. Such deficiencies may not be particularly meaningful under conditions of low synaptic activity but would have dire functional consequences when either high rates of sustained or synchronous firing are required. Indeed, mice deficient in different *PSYN* genes do display deficits in sustained release and in long-term potentiation (Rosahl et al., 1995; Fernandez-Chacon et al., 1999; Jahn and Sudhof, 1999; Janz et al., 1999). The situation in schizophrenia is obviously more complex, but multiple defects in *PSYN* genes could cause deficiencies in the ability to maintain sustained firing or synchronize firing within certain cortical and subcortical networks. Such a mechanism could underlie the consistent finding that subjects with schizophrenia show less activation of PFC during tasks that require the sustained neural activity involved in working memory. Indeed, considerable evidence from human and monkey studies indicate that disturbances or interruptions in sustained neural activity of the PFC during working memory tasks directly affect the performance of the task (Stamm, 1969; Brandt et al., 1998; Chafee and Goldman-Rakic, 2000). Thus, our model holds that deficits in expressing multiple *PSYN* genes in the PFC of subjects with schizophrenia are partly responsible for some of the cognitive symptoms associated with this disorder. This suggestion is testable in animals, using antisense RNA or conditional knockouts to create hypomorphs, coupled with evaluation of working memory.

The essence of our model incorporates the now widely accepted premise of polygenic complexity of schizophrenia (with a spotlight on *PSYN* genes), which in turn produces maladaptive responses of developing neural circuitry in an experience-dependent fashion, ultimately resulting in clinical manifestations with a common set of phenotypes. In addition, we believe that it may be possible eventually to identify different subtypes or etiologies of schizophrenia produced by different fundamental molecular defects in a *PSYN* functional cascade.

Experimental Procedures

Characteristics of Subjects

Two groups of subjects, consisting of six and five pairs of schizophrenic and control subjects, were used in these studies. Subject pairs were completely matched for sex (18 males and 4 females). Eight subjects were diagnosed with "pure" schizophrenia, while in three subjects the diagnosis was schizoaffective disorder. All but two of the schizophrenic subjects were receiving antipsychotic medications at the time of death, and five had a history of alcohol abuse or dependence.

The mean (± SD) difference within pairs was 4.6 ± 3.5 years for age and 4.4 ± 2.7 hr for postmortem interval (PMI). The entire group of schizophrenic and control subjects did not differ in mean (± SD) age at time of death (46.5 ± 10.7 and 45.1 ± 11.5 years, respectively), PMI (19.4 ± 7.1 and 17.7 ± 5.0 hr, respectively), brain pH (6.85 ± 0.29 and 6.81 ± 0.15, respectively), or tissue storage time at −80°C (45.4 ± 12.3 and 37.7 ± 13.1 months, respectively). Consensus DSM-IIIR diagnoses were made for all 22 subjects using data from clinical records, toxicology studies and structured interviews with surviving relatives as previously described (Pierri et al., 1999). Ten of the schizophrenic subjects had been used in a previous study of GAD67 mRNA expression in PFC area 9 (Volk et al., 2000).

Human Postmortem Tissue Preparation

The cause of death and the pH of the brain tissue suggested a short agonal state in all subjects, indicating that the brain tissue was appropriate for use in microarray experiments (Barton et al., 1993). Fresh-frozen human tissue was obtained from the University of Pittsburgh's Center for the Neuroscience of Mental Disorders Brain Bank and area 9 from the right hemisphere was identified as previously described (Glantz and Lewis, 2000; Volk et al., 2000). For microarray analysis, cryostat sections (30–50 μm) were collected into 50 ml tubes at −24°C under nuclease-free conditions, providing 300–900 mg of wet weight per specimen. Total RNA was extracted using Promega kit #Z5110, with modification. Total RNA yields were 0.2%–0.8% of the wet tissue weight with an $OD_{260/280}$ over 1.70. The mRNA was extracted by Qiagen kit #70022. Typical yields for mRNA were about 0.1%–0.5% of the total RNA with an $OD_{260/280}$ of over 1.80. The volume was adjusted using Microcon columns YM-30 #42,409 to 50 ng/μl. The integrity of the isolated material was comparable to those seen in rat brains with a 2–3 min PMI (our unpublished data).

Experimental Design

We used a matched sample design, in which each of the schizophrenic samples was compared to a matched control on each of the microarrays. There were two principal reasons for using this design.

First, aging is known to affect gene expression levels in the brain (Long et al., 1999). Furthermore, age, sex, substance abuse, and other factors also influence the course of the disease (Frederikse et al., 2000; Gearon and Bellack, 2000; Seeman, 2000). Consequently, baseline expression levels of many genes may significantly depend on race, sex, age, PMI, and brain pH differences between the subjects. Hence, combining in a reference sample material from multiple control brains would create an artificial baseline and may misrepresent the physiological levels of transcripts for any given subject.

Second, if the combined racial, PMI, brain pH, and sex differences between samples result in an expression variability for a gene product that is greater than the disease-specific difference, the reference pool comparison becomes unreliable. However, a pairwise design will still show a consistent and significant change between the schizophrenic and matched control brain samples.

As there are virtually no consistent gene expression changes reported over 2-fold in schizophrenia, the pairwise design in this system seemed advantageous over a more commonly used reference pool comparison strategy for microarray studies (Alizadeh et al., 2000). In retrospect, however, the ANCOVA and unpaired t test data for *NSF*, *SYN2*, *SJAN1*, and *STAG5* are suggestive that in the microarray comparisons, at least for some gene expression changes, comparison of schizophrenic subjects to a pooled control sample standard is a viable alternative to a matched experimental design.

Microarrays

The experiments were performed on UniGEM-V high-density cDNA microarrays (Incyte Pharmaceuticals, Fremont, CA). These cDNA microarrays test human gene expression using genes and ESTs from the public domain UniGene database. Each array compares a sample to a control by Cy3/Cy5 dual fluorescent hybridization. Each UniGEM-V array contains over 7000 unique and sequence verified cDNA elements mapped to 6794 UniGene Homo sapiens annotated clusters. The gene names are updated to build 110.

Probe Preparation and Array Hybridization

From each subject within a pair, 200 ng of mRNA was labeled with a fluorescent dye (either Cy3 or Cy5) during reverse transcription and then combined to use as probes for gene expression on the same microarray. The labeling and hybridization was performed according to protocols established by Incyte Pharmaceuticals, Inc. If a gene or EST was differentially expressed, the cDNA feature on the array bound more of the labeled probe from one sample than

the other, producing either a greater Cy3 or Cy5 signal intensity. The arrays were scanned at Incyte, the signal intensities were color coded and downloaded into a local database (SQL Server 7.0). Note that the operators were blind to the specific category to which each sample belonged. Data analysis was performed using GemTools (Incyte's proprietary software). Internal controls were excluded from the analysis. Gene group data was exported to MS Excel 2000 and further analyzed.

Microarray Data Analysis

A gene was called present in the sample if all of the following criteria were met: (1) the arrayed element was successfully amplified by PCR; (2) the arrayed element produced signal from at least 40% of the spot surface; (3) the signal/background ratio was over 5-fold for either the Cy3 or Cy5 probe; and (4) the arrayed element was not a spiked-in control.

Incyte's control hybridization studies (http://www.incyte.com/gem/GEM-reproducibility.pdf) suggested array data reliability and reproducibility cutoffs as listed below. In addition to this, we performed a single control brain hybridization experiment (see Figure 1 legend). To assess interexperiment variability, pair 685c/622s was hybridized to three different UniGEM-V microarrays, producing essentially the same overall results. In summary, our own control data were consistent with Incyte's control experiments. Based on these control data, all expressed gene observations were sorted into one of the following three categories.

Balanced Cy3/Cy5 or Cy5/Cy3 Signal Intensity Ratio $<$ 1.59

Observations in this category suggested comparable expression between the schizophrenic and control sample. In our Cy3/Cy5 control experiment (Figure 1A) and in Incyte's verification study, $>$95% of all observations fell into this category. These observations may contain real differences between samples that are indistinguishable from experimental noise.

Balanced Cy3/Cy5 or Cy5/Cy3 Signal Intensity Ratio between 1.6 and 1.89

Observations in this category report a real expression difference between the schizophrenic and control sample at the 95% confidence level. In the control experiments, $<$5% of the observations fell into this category.

Balanced Cy3/Cy5 or Cy5/Cy3 Signal Intensity Ratio $\geq$ 1.9

Observations in this category report a real expression difference between the schizophrenic and control sample at the 99% confidence level. In the control experiments, $<$0.5% of the observations fell into this category.

Gene Groups

The gene groups related to metabolic pathways, enzymes, and functional pathways are part of GemTools' gene hierarchy analysis capability. To obtain the list of gene groups, visit http://pittarray.neurobio.pitt.edu/neuron/. In addition, gene groups related to brain-specific functions were designed using recent review articles. UniGEM-V microarrays did not contain probes for all known genes related to a single gene group. Unless it was certain that only one member of a given gene family performed a certain synaptic function, all gene family members represented on the microarrays were included in the gene groups (e.g., synaptotagmin I, II, IV, V, VII, IX, XI). To maximize the potential of novel discovery, isoforms with an uncertain brain expression were also occasionally included in the custom gene groups (e.g., syntaxin family genes). However, as only expressed genes were included in the analysis, inclusion of family members with absent brain expression did not affect the analysis and interpretation of the data.

The custom-designed gene groups related to brain-specific functions included transcripts for structural proteins, glial markers, neuronal markers, transcription factors, protein kinases, apoptosis markers, growth factors, growth factor receptors, cytokines, adhesion molecules, G proteins, cAMP pathway, DNA binding proteins, metalloproteinases, heat shock proteins, presynaptic secretory machinery, GABA system, catecholamine system, glutamate system, neuropeptides, voltage-gated channels, ligand-gated channels, and Alzheimer disease-related genes.

Genes in the PSYN group represented on the arrays (n = 62) were as follows: clathrin (both light and heavy polypeptides), clathrin coat assembly protein, clathrin-associated adaptor protein (large, β-1), dynamin (1 and 2), dynein (axonemal and cytoplasmic), endobrevin, kinesin (1, 2, 4 heavy chains), neurexin 1, NSF, P/Q α-1 Ca channel, rab3a, rabaptin 5, rabphillin 3a, SAP90A, SCAMP 3, SNAP (α and γ), synaptotagmin (I, IV, V, VII, IX, XI), syntaxin (1, 3, 4, 5, 7, 8, 16), syntaxin binding protein (1 and 2), synaptogyrin (1, 2, 3), synapsin (I and II), synaptoporin, calcineurin (β and γ), synaptojanin (1 and 2), synaptophysin 1, synaptopodin, vacuolar proton pumps (21 kDa, 42 kDa, 56/58 kDa, 110/116 kDa), vesicle-associated membrane protein (1, 2, 5, 8), vesicle docking protein p115, voltage-sensitive Ca channel (β-1, β-3, and γ-1 subunits), zinc transporter 4 (Rosahl et al., 1995; Sudhof, 1995; Linial, 1997; Bajjalieh, 1999; Fernandez-Chacon et al., 1999; Jahn and Sudhof, 1999; Janz et al., 1999).

Glutamate system group represented on the microarrays (n = 11) were as follows: AMPA receptors (1, 2, 3), high-affinity aspartate/glutamate transporter, kainate receptor 2, metabotropic receptors (1, 2, 3, 8), NMDA receptor 2c, N-methyl-D-aspartate receptor–associated protein.

GABA system group represented on the microarrays (n = 18) were as follows: Betaine/GABA transporter, calbindin 1, calretinin, calmodulin, CAM2 kinase, $GABA_A$ receptor (β-3, γ-2, γ-3, π), $GABA_B$ receptor 1, glutamic acid decarboxylase 1 and 2, parvalbumin, phosphodiesterase 1b and 1c (calmodulin-dependent), solute carrier family 6, SRP72, vesicular GABA/glycine transporter.

In Situ Hybridization

For in situ hybridization, 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. The products of these reactions were ligated into a plasmid vector and transformed into competent *E. coli* cells. ^{35}S-labeled sense and antisense riboprobes were synthesized and purified using Riboprobe In Vitro Transcription System (Promega) and RNeasy kit (Qiagen).

Sections (20 μm) from area 9 of each subject in this study were cut on a cryostat and stored on slides at −80°C until processing. The methods used for in situ hybridization were those of D. B. Campbell, J. B. North, and E. Hess (Campbell et al., 1999), with subject pairs always processed together.

For quantification of the in situ hybridization signal, we used Scion Image (version 3). The investigators were blind to the sample category. Uncalibrated optical density (OD) values were measured from high resolution scans of X-ray film (X-Omat AR, Kodak) that had been exposed to the processed slides for 2–3 days prior to being developed. The mean relative OD was calculated for each section in five nonoverlapping rectangular regions with a width of ~500 μm and adjusted in length to span cortical layers 2–6. The long axis of each rectangular region was positioned in parallel with the orientation of cortical cells. The OD of the white matter in the section was subtracted from each of these measurements to give five relative OD values for each subject. For group comparisons, the mean of these five values was used for each subject.

Monkey Experiments

Two pairs of male cynomolgus (*Macaca fascicularis*) monkeys, matched for age and weight, were studied. One pair was compared on the cDNA microarrays, and both pairs were analyzed by in situ hybridization. In each pair, one animal was treated for 10–12 months with the antipsychotic medication haloperidol decanoate at doses that produced trough serum levels (4–5 ng/ml), known to be in the therapeutic range for the treatment of schizophrenia. Consistent with our attempt to mimic the clinical model of neuroleptic threshold dosing, all haloperidol-treated animals developed extrapyramidal symptoms that were effectively controlled with maintenance administration of benztropine mesylate (Akil et al., 1999; Pierri et al., 1999). The brain tissue was processed identically to the human samples. For cross-species hybridizations, Incyte required three times the amount of mRNA for array hybridization. Based on the detection of expressed genes, the hybridizations were as successful as the human sample hybridizations. The in situ analysis of monkey tissue was performed on areas 9 in sections matched for rostrocaudal location.

Statistical Analysis

Microarray Gene Group Analysis

To compare relative levels of expression for genes in the different groups, we employed two types of statistical measures. First, for individual pairs, chi-square analysis was performed on the distribution of genes in a group versus the distribution of all genes called present on that microarray. For this analysis, the distribution of gene expression ratios was divided into five different bins representative of confidence intervals for individual gene comparisons: <-1.9; -1.89 to -1.6; -1.59 to 1.59; 1.6 to 1.89; and >1.9. Then, a paired t test (df = 5) was used to compare mean expression ratios for a given gene group to the mean expression ratios for all expressed genes across all six subject pairs.

To distinguish between single gene changes and gene group changes, we established a list of criteria. In our experiments, we judged gene group expression to be changed if it met the following conditions. (1) More than five genes were expressed in the gene group per each subject. (2) By independent statistical measures, the gene group across the experiment was significantly changed ($p < 0.05$) both by frequency distribution (chi-square test) and central tendency measures (t test) compared to the expression of all genes across the experiments. (3) In each of the individual array comparisons, the gene group was significantly changed ($p < 0.05$) both by frequency distribution (chi-square test) and central tendency statistics (t test) compared to the expression of all genes in the same experiment. (4) Analyzing the group effect without 5% of the most changed individual genes still showed an overall chi-square and t test significance. (5) No single gene accounted for more than 20% of the group effect. More than 30% of the genes in the group contributed to the group effect.

In Situ Hybridization Data Analysis–Paired Design

For each gene, averages are reported as a mean of expression changes between schizophrenic-control subject pairs. Because microarray data indicated a decrease in gene expression, probabilities for in situ hybridization changes were calculated by a one-tailed t test in a pairwise design. Unless indicated differently, these pairwise comparison statistics are reported throughout the manuscript.

In Situ Hybridization Data Analysis–Group Design

To evaluate the effect of confounds, for each gene examined by in situ hybridization, group differences were examined using analysis of covariance (ANCOVA) with diagnosis as the main effect and sex, age, postmortem interval, brain pH, and tissue storage time as covariates.

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