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Gene Expression of Metabolic Enzymes and a Protease Inhibitor in the Prefrontal Cortex Are Decreased in Schizophrenia*

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Microarray expression studies have reported decreased mRNA expression of histidine triad nucleotide-binding protein (HINT1) and cytosolic malate dehydrogenase (MDH1) in the dorsolateral prefrontal cortex (DLPFC) of individuals with schizophrenia. Microarray results for neuroserpin (SERPINI1) mRNA in the DLPFC have reported increased and decreased expression in individuals with schizophrenia. The relative abundances of HINT1, MDH1, and SERPINI1 mRNA in the DLPFC in individuals with schizophrenia and controls were measured by real-time quantitative polymerase chain reaction (Q-PCR) and for HINT1 expression by *in situ* hybridization. The Q-PCR results were compared by analysis of covariance between individuals with schizophrenia and controls. Gene expression levels for HINT1, MDH1, and SERPINI1 were significantly different between the groups. The male individuals with schizophrenia compared to male controls showed reductions by 2.8- to 3.7-fold of HINT1, neuroserpin, and MDH1 by Q-PCR. The decreases in mRNA abundance for MDH1 ($P = 0.006$), HINT1 ($P = 0.050$), and neuroserpin ($P = 0.005$) in DLPFC of male individuals with schizophrenia is consistent with prior reports. HINT1 mRNA was reduced significantly by 34% in layer VI. Though there were no significant interactions with gender, gene expression between female patients and the female control group did not differ. These results confirm earlier reports and suggest abnormalities of specific genes related to metabolic and protease activities in the DLPFC might be considered as part of a molecular pathway in male patients with schizophrenia.

KEY WORDS: Cytosolic malate dehydrogenase; gene expression; histidine-nucleotide triad binding protein; *in situ* hybridization histochemistry; microarray; neuroserpin; real-time quantitative PCR.

INTRODUCTION

Schizophrenia is a neuropsychiatric brain disorder affecting an estimated 1% of the US population. Although the precise causes of schizophrenia remain unknown, there is evidence from research implicating neurodevelopmental events, genetic predisposition, neurochemical dysregulations, adverse environmental events, and combinations of these factors. Thus, abnormalities in gene expression in brain might be expected as a result of these factors. However, progress in discovery of molecular pathways related to schizophrenia has been slow because the traditional approach relied on developing a prior hypothesis about which specific molecules might be abnormally expressed. More rapid progress in identifying changes in gene expression has been made by using a microarray screening approach, where thousands of mRNA molecules are

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studied simultaneously. The microarray methodology is a potentially informative methodology for identifying candidate genes for further study in schizophrenia (1).

Numerous postmortem studies have identified gene expression differences between patients with schizophrenia and control using microarray approaches (2). Studies of patients with schizophrenia have used microarray to screen cortical (DLPFC, superior temporal gyrus, and entorhinal cortex) and cerebellar samples from a matched set of subject to determine genes that are differentially expressed (3–11). Broadly, these reports to date have suggested alterations in functional pathways involving myelin, synaptic, metabolic, and proteasome-ubiquitin related processes in schizophrenia, though the specific differences, their magnitude and directionality, have been inconsistent. Before investing valuable resources to understand the relevance of these pathways to the molecular pathology of schizophrenia, further confirmation of expression differences of candidate genes found with the microarray approach is required.

We chose three genes to validate based on the following criteria: 1) these genes were identified as candidate molecules in the neuropathology of schizophrenia via microarray, 2) these genes can show fairly robust changes in expression, and 3) these genes have been identified as abnormally expressed in more than one study. Previous microarray findings reported histidine triad nucleotide binding protein (HINT1) gene was down-regulated across four comparisons of patients with schizophrenia (10,11). These microarray comparisons used pools of RNA representing 20 patients and 20 controls. Another recent microarray study (6) reported that expression of the metabolic enzyme cytosolic malate dehydrogenase (MDH1) was decreased in the DLPFC of patients with schizophrenia, whereas another report showed the opposite (4). Neuroleptic treatment of monkeys increased MDH1 in the DLPFC (6). Cocaine usage also dysregulated MDH1 in the DLPFC (12), raising the possibility that dopamine-related neurotransmission in the DLPFC can disturb MDH1 levels. Microarray studies also had shown neuroserpin increased significantly in the DLPFC of patients with schizophrenia (4), whereas another report showed the opposite direction of change for neuroserpin in the DLPFC (10) (Karoly Mirnics, personal communication, University of Pittsburgh). The candidate gene transcripts (HINT1, MDH1, and neuroserpin) were previously shown to be decreased by microarray in schizophrenia and were therefore measured by real-time quantitative polymerase chain reaction (Q-PCR). Real-time Q-PCR is a powerful tool for measuring transcript levels because it is theoretically sensitive to low starting amounts of mRNA (representing less than 1 copy/10^6 transcripts). Microarray studies based on *in vitro* transcription protocols are sensitive to gene levels representing greater than 1 copy/2.5 to 4.0×10^5 transcripts. Furthermore, real-time Q-PCR has a wider dynamic range of 5–6 log orders compared to a microarray dynamic range of 3–4 log orders. Therefore, Q-PCR is a reliable and sensitive method for confirmation of microarray findings. The microarray studies, whether using a commercial oligonucleotide platform or cDNA array, consistently underestimate the gene expression fold changes when compared to Q-PCR method (13). Only one prior microarray report of schizophrenia has reported real-time Q-PCR gene expression results (14). Thus, it will be important to extend Q-PCR into the validation of findings by microarray in the study of schizophrenia in postmortem brain.

EXPERIMENTAL PROCFEDURE

RNA Tissue Samples for Real-Time Q-PCR. The cohort used in the real-time Q-PCR study included 22 patients with schizophrenia and 21 controls (Table I) group matched for age, gender, race, brain pH, and postmortem interval (PMI). RNA was extracted from the DLPFC of 19 female and 24 male subjects with approximately equal numbers of males and females in the control and schizophrenia groups (Table I). Postmortem brains were collected at the Clinical Brain Disorders Branch (NIMH, St. Elizabeths, Washington, DC, and Bethesda, MD). Briefly, 1.5-cm coronal slabs through the entire cerebrum of each human brain were rapidly frozen in a prechilled dry-ice isopentane slurry bath and stored at −80°C. Tissue was excised from either the right or left side of the middle third of the superior or middle frontal gyrus immediately anterior to the genu of the corpus callosum. All brains (both controls and individuals with schizophrenia) were screened for signs of macroscopic pathology at the time of autopsy; brain sections were examined microscopically with the use of Bielschowsky's silver stain on multiple cerebral areas to exclude the presence of neuritic pathology as seen in Alzheimer's disease. Those cases with an unclear psychiatric diagnosis, evidence of cocaine or PCP abuse (history and/or toxicology), cerebrovascular disease, autolysis, subdural hematoma, neuritic pathology or other pathological features were excluded from the study.

Diagnosis was determined by independent reviews of clinical records by two board certified psychiatrists. Cases that met DSM-IV criteria for schizophrenia were used (15) after two independent reviews. Out of the 22 individuals diagnosed with schizophrenia, 9 patients were classified into the chronic undifferentiated subtype, 10 patients classified as chronic disorganized subtype, and 3 patients classified into the chronic paranoid subtype. There was an overlap of samples from a prior microarray study of schizophrenia (11) and the current real-time PCR samples of 27 of the samples (schizophrenia $n = 15$, controls $n = 12$).

RNA Tissue Samples for In situ *Hybridization Histochemistry.* The DLPFC brain sections obtained for ISH were from individuals with schizophrenia ($n = 14$) and controls ($n = 12$). Out of the 14 individuals diagnosed with schizophrenia, 11 patients were of the chronic undifferentiated subtype, 1 patient of the chronic disorganized

Table I. Demographic Characteristics of the Cohorts for the Real-Time Quantitative PCR and *In situ* Hybridization Studies

Study	Group	Gender	n	Age	Race (Black/ Caucasian)	PMI	pH	Side (right/left)*
Q-PCR	Control	Female	10	56.1	9/1	26.4	6.34	4/2
	Control	Male	11	41.8	7/4	29.1	6.41	5/5
	Schizophrenia	Female	9	58.2	7/2	36.7	6.47	6/2
	Schizophrenia	Male	13	42.3†	11/2	38.1	6.46	7/4
ISH	Control	Female	5	59.2	4/1	29.3	6.57	3/2
	Control	Male	7	38.8	6/1	20.4	6.48	6/1
	Schizophrenia	Female	5	62.0	4/1	32.2	6.52	4/1
	Schizophrenia	Male	9	44.1	7/2	43.5	6.41	3/6

Q-PCR, quantitative polymerase chain reaction; ISH, *in situ* hybridization; PMI, postmortem interval.
* The information for which hemisphere was used for Q-PCR was not available for eight cases.
† The females with schizophrenia were significantly older than male controls ($P = 0.042$) in the Q-PCR study.

subtype, and 2 patients of the chronic paranoid subtype. There was an overlap of subjects between the Q-PCR study and ISH study of 6 controls and 12 individuals with schizophrenia. There was an overlap of samples from a prior microarray study of schizophrenia (11) and the current ISH cohort of 14 of the samples (schizophrenia n = 9, controls n = 5).

pH Determinations. The brain tissue pH was determined by homogenizing cerebellar tissue from the lateral hemisphere; in less than 5% of the cases, frontal cortex was used. Tissue pH has previously been shown to be uniform in different brain regions (16). Tissue (0.4–0.8 g) was thawed and homogenized in 10 times the tissue volume in double-distilled (dd) H_2O adjusted to pH 7.0. The tissue homogenate pH was measured with a Sentron pH meter. The pH electrode was washed thoroughly in ddH_2O (pH 7.0) after each sample and was recalibrated after every 10 samples.

RNA Extraction. Total RNA was extracted from the postmortem DLPFC using a modified version of the Trizol Reagent method (Life Technologies Inc., Grand Island, NY, USA). Frozen tissue specimens were homogenized at 4°C for 45 s using a Polytron tissue homogenizer (Model PT10/35, Brinkmann Instruments, Inc., Westbury, NY, USA) at moderate speed setting (#7), in 10 ml Trizol Reagent per 260–760 mg tissue. The homogenate was maintained on ice for 1 min to allow settling of foam, after which the homogenizing process was repeated. Samples were incubated at room temperature (RT) for 40 min to allow complete dissociation of nucleoprotein complexes. To each sample, 2 ml chloroform was added, shaken vigorously for 15 s, and centrifuged at 12,000 × *g* for 15 min at 4°C. The upper aqueous phase was transferred to fresh tubes, and RNA was precipitated with 5 ml isopropyl alcohol, incubated at RT for 20 min, and centrifuged at 12,000 × *g* for 10 min at 4°C. After removal of the supernatant, the remaining RNA pellet was washed by vortexing in 15 ml of 75% ethanol. The samples underwent a final centrifugation at 12,000 × *g* for 10 min at 4°C. RNA pellets were dried for 20 min, resuspended in 200 μl 0.1% diethylpyrocarbonate-treated distilled water, and stored at −80°C.

RNA Quantity and Quality. The recovery or yield of total RNA/gram tissue was measured with a spectrophotometer. Optical density measurements of total RNA were determined using the relationship of 1 unit at $A_{260} = 40$ μg/ml for RNA. The ratio of A_{260}/A_{280} was used to determine presence of protein contamination. The quality of each total RNA sample was evaluated by one-dimensional 0.75% agarose gel electrophoresis with 0.001% ethidium bromide to allow visualization of the 18S and 28S ribosomal bands. A further check for the integrity or extent of degradation (quality) of the cDNA reactions was monitored by PCR amplification of GAPDH and gel visualization of the amplicons. After running the GAPDH PCR amplicons on a gel, there were five samples that showed no bands for the housekeeping gene GAPDH and were flagged in the Q-PCR analysis. Subsequent Q-PCR analysis revealed that the five samples gave the same amplification patterns as no template controls and were excluded from this study prior to real-time Q-PCR data analysis. Table I shows the number of samples used in the Q-PCR analysis with the five no-amplification cases removed.

cDNA Synthesis. First-strand cDNA from Trizol extracted total RNA samples was synthesized in a 50 μl reverse transcriptase reaction according to the protocol supplied by the manufacturer (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA, USA). Briefly, total RNA (5 μg) and 5 ng/μl of random hexamers were denatured with 1 mM of dNTP at 65°C for 5 min. After addition of reverse transcription buffer, $MgCl_2$ (5 mM final), dithiothreitol (10 mM final), RNAseOUT recombinant ribonuclease inhibitor (100 units), and the reverse transcriptase, SuperScriptII (125 units), the reaction mixture was incubated at 25°C for 10 min, at 42°C for 40 min, and at 70°C for 15 min. RNAse H (5 units) was added to the reaction mixture to destroy the starting RNA by incubating at 37°C for 20 min. This cDNA was diluted and used in the real-time Q-PCR.

Real-Time Quantitative PCR. Real-time quantitative PCR reactions were carried out in an Applied Biosystems 7000 sequence detection system (ABI, Foster City, CA, USA) according to the manufacturer's protocol for SYBR Green PCR using a 25 μl reaction volume. Samples were quantified for HINT1, neuroserpin, and MDH1 by comparison to a standard curve generated with known concentrations of PCR product from amplification in genomic DNA (neuroserpin) or from known cDNA clones (HINT1 and MDH1). The template concentrations for the gDNA standard curve ranged from 100 ng/μl to 0.01 ng/μl in 10-fold dilution series. The cDNA clones for HINT1 (IMAGE Clone: 75415) and MDH1 (IMAGE Clone: 725188) were obtained from ResGen (Huntsville, AL, USA), grown overnight in LBAmp, and the purified mini-prep was used as a standard for HINT1. The standard curves were generated with known concentrations of PCR product from amplification of the purified HINT1 or

MDH1 clones, and the template concentrations ranged from 3.5 ng/μl to 3.5×10^{-7} ng/μl. Standard curves for HINT1 and MDH1 were constructed from purified cDNA grown from the original clones used on the microarray and sequence verified. These standard curves were linear for both the HINT1 and MDH1 clones over a range of 10^6-copy number of the DNA starting template. A cDNA standard was not available for neuroserpin and the primers were designed to amplify within a single exon. The neuroserpin, HINT1, and MDH1 PCR cycle threshold crossings (Ct) for the amplification curves were used for quantitation of each sample. The housekeeping gene cyclophilin was also amplified to determine the integrity and abundance of the starting cDNA. All schizophrenia and control samples were run in duplicate and the average values used in statistical analyses. The standards and housekeeping gene samples were run in triplicate. The averages of triplicate Q-PCR reactions for cyclophilin were used in statistical analyses.

The primer sequences chosen for PCR of the cDNA for HINT1, neuroserpin, and MDH1 were chosen from the 3′ sequence of the gene because the prior microarray results were based on hybridization targets corresponding to 3′ cDNA clones. The HINT1 and MDH1 primers spanned across exon boundaries, eliminating the measurement of any genomic DNA contaminant in HINT1 or MDH1 expression.

HINT1	Forward 5′-AGTCTTCTTGGACACTTAATGATTGTTG-3′
HINT1	Reverse 5′-TCATTCACCACCATTCGATAACC-3′
SERPINI1	Forward 5′-GGATTTTGATGCTGCCACTTATC-3′
SERPINI1	Reverse 5′-TTTGGACTTCACTTTCATCATCTTTAG-3′
MDH1	Forward 5′-CTGTGACCACGTCAGGGACAT-3′
MDH1	Reverse 5′-TCTTGATTACAACAGGGAATGAGTAGAG-3′
Cyclophilin	Forward 5′-AGCCATGGTCAACCCCAC-3′
Cyclophilin	Reverse 5′-CTGCTGTCTTTGGGACCTTGT-3′

The primers chosen for cyclophilin were previously validated by ribonuclease protection assay to generate specific probes. The dissociation curve analysis was used after running the Q-PCR on the ABI 7000 to examine each sample and to determine whether any dimer-primer pairing occurs that would interfere with SYBR green fluorescence measurements. The dissociation curve analysis is run on the ABI 7000 instrument using a program to create a melting profile in small increments of temperature increases and measuring the fluorescence emitted from SYBR green. The melting temperature of pairs of primers will generally be less than the melting temperature characteristics of 75–100 bp amplicons. A plot of temperature and fluorescence will show the peak for SYBR green emission. A single peak at the expected melting temperature of the PCR amplicon indicates that the Q-PCR quantitation is not measuring dimer-primer pairings. The SYBR green master mix (ABI, Foster City, CA, USA) also contains an internal dye used as a reference for determination of the amount of master mix in each sample for normalization of slight variations in pipetting volumes.

In situ *Hybridization and Quantitation*. Fixation, acetylation, delipidation, and dehydration of the slides containing 14-μm-thick sections of frontal cortex were performed as previously described (17). Briefly, 200 μl of hybridization buffer containing the ^{35}S-UTP labeled HINT1 riboprobe (5 ng/ml) was added to each section, and hybridization was allowed to occur at 55°C overnight in humidified chambers. After the *in situ* hybridization procedure, slides, along with ^{14}C standards (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) were exposed to Kodak autoradiographic film (BioMax) for 3 days. Microscopic evaluation of silver grains were conducted on slides dipped in NT-B2 emulsion (Kodak), stored in light-tight boxes in the dark for over 3 months, and then developed in D-19 developer, dehydrated, and lightly stained with a Nissl counterstain. Silver grains were visualized on a Zeiss Axiophot microscope equipped with a video camera.

Statistical Analysis. An ANCOVA analysis of the PCR cycle crossing (Ct) was used to compare controls (n = 21) to patients with schizophrenia (n = 22) using diagnosis and gender as factors. The covariates of age, pH, and cyclophilin were evaluated. Three potential covariates of age, PMI, and pH were first evaluated by Pearson's correlation and there were no significant relationships between gene expression with age or PMI (see Table III) for the three genes measured. The pH showed a significant correlation with the neuroserpin gene measurement, therefore we used pH as a covariate in all three ANCOVAs, as the other two genes showed modest but not significant Pearson correlations (Table III). The ANCOVA procedure (Statistica v 5.1, StatSoft, OK, USA) was applied to each Q-PCR gene measurement (dependent variable) using the factors of diagnosis and gender as independent between group factors. The design is a factorial design and the groups were not hierarchically nested. In the ANCOVA procedure, the gene expression value is adjusted for all subjects for pH covariate regardless of the between-group factors of gender or diagnosis. Optionally, if there were strong reasons to consider that a covariate such as pH plays a differential role on diagnosis or gender, then before making an effect on the gene expression measurement, the means could be adjusted separately between the gender and diagnostic groups. There was no evidence that pH adjustments were needed separately for each of the four groups because the pH was equivalent between groups. Therefore, no further adjustment beyond the overall ANCOVA adjustment was made. We used gender as a between-groups factor in the ANCOVA, as it is not a continuous covariate. The lifetime drug exposure data were not available for each patient with schizophrenia to use for ANCOVA. After conducting the ANCOVA, to evaluate further the effect of diagnosis and gender we examined the group means with a *post hoc* Tukey *t* test that incorporates an adjustment for unequal n and a correction for multiple *t* tests. The Tukey *t* test with both corrections, known as the Tukey HSD (honestly significant difference) *t* test is more conservative than using a noncorrected *t* test. Additionally, the computation of the *post hoc* contrast takes the covariate effect into account.

RESULTS

Real Time Q-PCR

There were no significant group differences for the variables of PMI and pH between the schizophrenia group and control groups nor were there significant differences when stratified by group and gender (Table I). The mean ages of females subjects with schizophrenia was significantly greater compared to the male controls (Table I). The levels of the housekeeping gene cyclophilin were run in triplicate and the average Ct used as an indicator of the concentrations of template cDNA. The cyclophilin gene expression (mean Ct ± SEM) in male

patients with schizophrenia (27.2 ± 0.35) compared to male controls (27.0 ± 0.20) was not significantly different by Tukey's HSD *t* test for unequal n ($P = 0.96$) indicating equivalent estimates of starting cDNA concentrations (Table II). In the female groups, the control level of cyclophilin (26.8 ± 0.31) was not different from the females with schizophrenia cylcophilin level (26.6 ± 0.28; $P = 0.87$). There was a trend for gender differences in cyclophilin gene expression [F (1, 38) = 3.85, $P = 0.056$]. Furthermore, there were no significant differences between groups for the GAPDH housekeeping gene also run on each sample by Q-PCR. The pH was correlated to the gene expression levels (Table III) and used as an additional covariate with cyclophilin in a 2-way ANCOVA with the between-subjects factors of diagnosis and gender. A representative standard curve for HINT1 gene expression (Fig. 1) shows linearity (r = 0.998) spanning 5 log orders of starting cDNA template. The real-time Q-PCR measurements are reliable and have a wide dynamic linear range.

HINT1

HINT1 gene expression was decreased in patients with schizophrenia compared to controls by ANCOVA [F (1, 37) = 5.50, P = 0.024] using cyclophilin and pH as covariates. The mean HINT1 cycle number (Ct ± SEM) for male patients with schizophrenia (26.8 ± 0.71) was significantly different compared to male controls (25.3 ± 0.25) in the Tukey post hoc comparison (P = 0.050). This difference translates to a 2.8-fold decrease in male patients with schizophrenia. The interaction of diagnosis × gender failed to reach significance for the HINT1 gene [F (1, 37) = 0.71, P = 0.40]. The female patients did not show a difference in HINT1 levels compared to female controls (P = 0.99).

Neuroserpin

The expression of neuroserpin was significantly decreased in patients with schizophrenia by 1 Ct (two fold) using cyclophilin and pH as covariates [F (1, 37) = 10.21, P = 0.001]. The interaction of diagnosis × gender did not reach significance for the neuroserpin gene [F (1, 37) = 3.59, P = 0.065]. The male patients showed a significant reduction in the mean neuroserpin cycle number (Ct ± SEM; 30.5 ± 0.59) compared to male controls (28.6 ± 0.33) using Tukey HSD corrected for unequal n (P = 0.005). An increased Ct indicates less neuroserpin in males with schizophrenia by a factor of 3.7-fold compared to male controls. The female patients did not show a difference in neuroserpin levels compared to female controls (P = 0.99).

MDH1

The patients with schizophrenia also showed a significant decreased expression of MDH1 compared to controls

Table II. Mean (SEM) PCR Cycle for Three Genes Measured in DLPFC Samples by Diagnosis and Group

Gender	Diagnosis	n	Mean crossing cycle (SEM) for target genes Neuroserpin	HINT1	MDH1	Mean crossing cycle (SEM) cyclophilin	Mean (SEM) pH
Female	Schizophrenia	**9**	29.3 (0.54)	25.8 (0.58)	30.5 (0.46)	26.6 (0.28)	6.47 (0.07)
	Control	**10**	29.4 (0.53)	25.7 (0.36)	30.5 (0.35)	26.8 (0.31)	6.34 (0.11)
	Fold change		1.1	**−1.1**	1.0	1.1	
	P value		>0.05	>0.05	>0.05	>0.05	>0.05
Male	Schizophrenia	**13**	30.5 (0.59)	26.8 (0.71)	31.9 (0.50)	27.2 (0.35)	6.46 (0.04)
	Control	**11**	28.6 (0.33)	25.3 (0.25)	30.2 (0.28)	27.0 (0.20)	6.41 (0.08)
	Fold change		**−3.7**	**−2.8**	**−3.2**	1.1	
	P value		**0.005**	**0.050**	**0.006**	>0.05	>0.05
Combine	Schizophrenia	**22**	30.0 (0.41)	26.4 (0.46)	31.4 (0.36)	26.9 (0.20)	6.47 (0.03)
	Control	**21**	29.0 (0.30)	25.5 (0.20)	30.4 (0.21)	26.9 (0.16)	6.38 (0.06)
	Fold change		**−2.0**	**−1.9**	**−2.0**	1.0	
	P value		**0.005**	**0.027**	**0.006**	>0.05	>0.05

PCR, polymerase chain reaction; DLPFC, dorsolateral prefrontal cortex; HINT1, histidine triad nucleotide-binding protein; MDH1, cytosolic malate dehydrogenase.

Note: For each gene, the PCR cycle (Ct) and the significance of the group difference (*P* value) are shown, and the differences in PCR cycle between groups are converted to a fold change. The fold change numbers and *P* values shown in **bold** text indicate a significant decrease of gene expression measurements in patients with schizophrenia compared to controls. The housekeeping gene measurements and pH values shown in the far right columns were not significantly different between groups. The combined male and female groups were also analyzed and the results shown in the gender combined rows at the bottom of the table. In the gender combined analysis, the schizophrenia gene expressions were significantly decreased from controls. The differences in PCR cycle threshold for neuroserpin, HINT1, and MDH1 were found in males with schizophrenia compared to male controls.

Table III. The Correlation of Gene Expression PCR Cycle (Ct) with Demographic and Sample Characteristics in the Q-PCR Cohort

	PMI (h)	pH	Neuroserpin Ct	HINT1 Ct	MDH1 Ct	Cyclophilin Ct
Age (years)	−0.19	0.02	0.12	−0.04	0.00	−0.07
PMI (h)		−0.12	0.10	0.00	0.04	−0.17
pH			−0.31*	−0.23	−0.17	−0.30
Neuroserpin (Ct)				0.79*	0.85*	0.70*
HINT1 (Ct)					0.79*	0.66*
MDH1 (Ct)						0.60*

Q-PCR, quantitative polymerase chain reaction; PMI, postmortem interval; HINT1, histidine triad nucleotide-binding protein; MDH1; cytosolic malate dehydrogenase.
Note: A higher cycle number indicates a lower amount of gene transcript.
* Correlations are significant at $P < 0.05$.

by ANCOVA [F (1, 37) = 7.80, P = 0.008] using cyclophilin and pH as covariates. The interaction of diagnosis × gender was not significant for the MDH1 gene [F (1, 37) = 3.89, P = 0.055]. Male patients with schizophrenia showed an increased Ct (31.9 ± 0.50) compared with the male controls (30.2 ± 0.28) representing a 3.2-fold decrease in MDH1 mRNA (Tukey HSD post hoc comparisons P = 0.006). There were no differences in neuroserpin in the female control group and female schizophrenia group (P = 0.99).

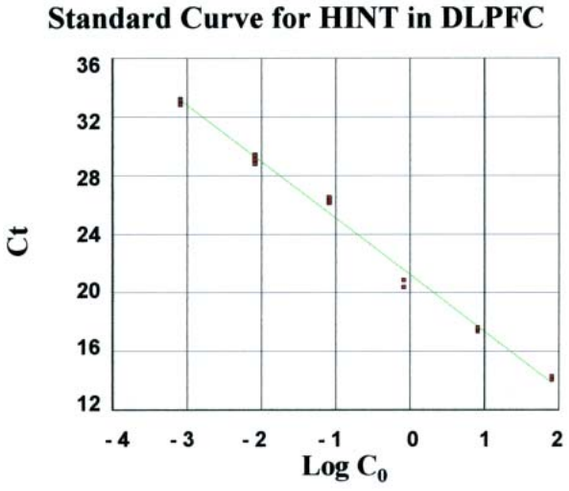

Fig. 1. The standard curve for HINT1 gene expression measured by real-time Q-PCR. The *x* axis (C_o) plots the amount of template HINT1 cDNA added to the real-time Q-PCR reaction on a logarithmic scale in units of ng of DNA. The 0.1 ng standard is labeled −1, the 1 ng standard is labeled 1, and the 10 ng standard is labeled 2 on the *x* axis. The *y* axis represents the cycle for the crossing threshold (C_t) for each standard. A linear response between the the standard concentrations (C_o) and the PCR amplification cycle (Ct) was shown across 5 log orders of starting template concentration (r = 0.998).

Correlation of Gene Expression with Demographic and Sample Characteristics

The Pearson correlations between gene expression (Ct) for HINT1, MDH1, and neuroserpin were evaluated in relation to PMI, age, and pH (Table III). The gene expression Ct values for each of the three genes did not correlate with PMI (correlation range, 0.00–0.10) or age (correlation range, −0.04–0.12). There was a single modest correlation of pH with neuroserpin Ct (r = −0.31), but nonsignificant correlations with HINT1 (r = −0.23) and MDH1 (−0.17).

The Pearson correlations between the expression levels of the three genes (Table III) were significant ($P < 0.05$) indicating consistency in Q-PCR measures. In summary, the mRNA levels of HINT1, MDH1, and neuroserpin measured by Q-PCR were found to be decreased greater than 2.5 fold for all three genes in the DLPFC of male patients with schizophrenia compared to controls, while the reductions were not found in females with schizophrenia.

In situ Hybridization

The distribution of HINT1 by *in situ* hybridization histochemistry was examined as there had been no prior reports in human brain. There are reports of MDH1 and neuroserpin showing neuronal localization (6,18). HINT1 signal intensity on BioMax film was robust in DLPFC sections, indicating high brain expression in adult cortex. Hybridization with the HINT1 plus strand did not give any signal. Representative sections with antisense probes are shown for the DLPFC (Fig. 2). The cellular distribution appears to be strongest in pyramidal neurons and reduced, if at all present, in glial cells (Fig. 2). Figure 2 is overexposed to show the relatively weak distribution of signal intensity in glial cells as compared to the robust neuronal signal.

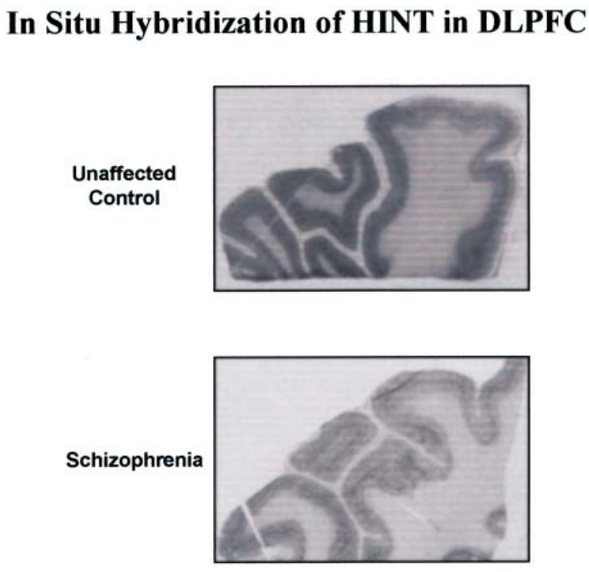

Fig. 2. *In situ* hybridization analysis of HINT1 mRNA in the DLPFC of an unaffected control and individual with schizophrenia. A representative X-ray film autoradiogram image shows HINT1 mRNA from coronal sections of DLPFC for a control and an individual with schizophrenia. There is a strong gray matter labeling with the HINT1 probe in human brain and a relative absence of white matter labeling.

Silver grain analysis from these same sections also confirmed the initial impression made from the film image (Fig. 3). HINT1 is distributed in neurons throughout each cortical layer. Our impression is that HINT1 is expressed in glutamatergic neurons.

Statistical analysis of the HINT1 *in situ* data was conducted with an ANCOVA (diagnosis × gender × hemisphere × layer) with pH as a covariate. There was a significant interaction of diagnosis × layer [$F(5, 90) = 2.38$; $P = 0.044$]. There was a trend for a decrease of HINT1 mRNA in layers II–V of HINT1 mRNA (21.5% ± 2.2% SD) in schizophrenia compared to control tissue sections (Fig. 4). There was a significant decrease in expression in layer VI by 35% in schizophrenia ($P = 0.017$). The other factors in the ANCOVA were not significant for HINT1 ISH measurements.

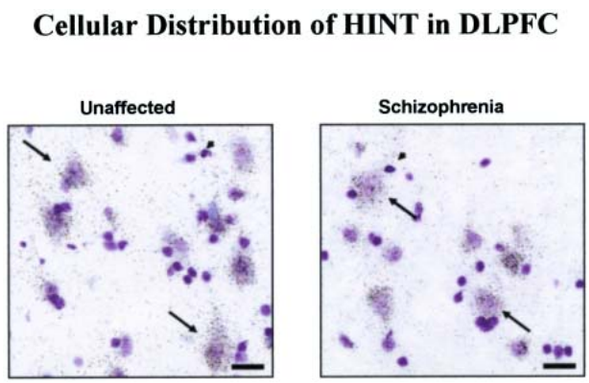

Fig. 3. High-power bright-field images of *in situ* hybridization analysis of HINT1 mRNA in DLPFC of an unaffected control and individual with schizophrenia. Photomicrographs showing HINT1 mRNA expression in layer V of the DLPFC. Note that large pyramidal neurons contain HINT1 silver grains at high levels (arrows). Glial cells identifiable by small dark Nissl-stained nuclei have silver grains at background levels (arrow heads) (scale bar = 20 μm).

DISCUSSION

Alterations in three candidate genes (HINT1, neuroserpin, and MDH1) were originally identified in prior microarray studies of the DLPFC in schizophrenia. We evaluated the mRNA levels of these candidate genes by Q-PCR and found greater than 2.5-fold reductions for all three genes in the DLPFC of male patients with schizophrenia compared to controls, whereas the reductions were not found in females with schizophrenia. Interestingly, these genes appear to be highly expressed in neurons in the DLPFC, HINT1 by our ISH study, and MDH1 and neuroserpin by other ISH studies (6,18). Whether these three genes are coexpressed or co-dysregulated in the same neurons requires further study in specific neuronal populations. In this regard, we did find strong intercorrelations between measures of expression of each of the genes (Table III), supporting the possibility that they are covarying with some common process.

HINT1

HINT1 gene expression was decreased in two prior microarray studies of DLPFC in schizophrenia (10,11) and is confirmed by real-time Q-PCR to be about a 2.8-fold decrease. There is an overlap between cohorts in the microarray study (11) and the current Q-PCR study of 27 of the 43 brains assayed, thus the confirmation is not an independent replication. We further characterize the cellular localization of the HINT1 enzyme by *in situ* hybridization, and this is the first report of HINT1 mRNA expression in human brain tissue. The cellular distribution of HINT1 appears strongest in pyramidal neurons, possibly restricted to glutamatergic, and reduced if at all present in glial cells. HINT1 expression decreases in schizophrenia by 21% in layers II–IV and significantly decreases by 35% in layer VI in comparison to controls by *in situ* hybridization.

The functional significance of decreases in HINT1 expression in neurons is unknown. The human gene,

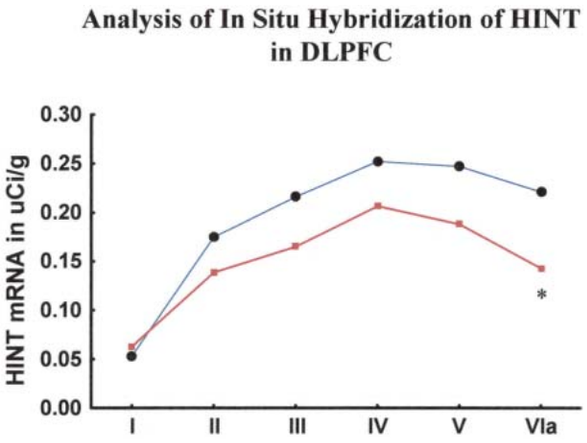

Fig. 4. The results of the HINT1 *in situ* hybridization analysis for individuals with schizophrenia (squares) and controls (circles). There was a 21.5% reduction in layers II–V of HINT1 mRNA in schizophrenia that was not significantly different from controls. There was a significant decrease of HINT1 mRNA expression by 35% in schizophrenia in layer VI ($P = 0.017$).

HINT1, is a member of the histidine triad superfamily of nucleotide hydrolases. Nucleotide transferases consist of three branches: proteins related to Hint and Aprataxin, a branch of Fhit-related hydrolases, and a branch of galactose-1-phosphate uridylytransferase (GalT)-related transferases (19). A mutation of a gene in these branches has been associated with functional consequences such as ataxia-oculomotor apraxia syndrome, epithelially derived tumors, and galactosemia (19). HINT1 appears to be structurally conserved from yeast through mammals suggesting a highly conserved function. However, no grossly observable deficiencies were noted in hint −/− mice, suggesting that other redundant genes can functionally compensate (20). HINT enzyme homologues hydrolyze adenosine-5′-monophosphoramidate ($AMPNH_2$) to AMP plus the leaving NH_2 group (19,21). HINT enzyme activity is a positive regulator of Kin28, the human homologue is CDK7 (21). Human CDK7 interacts with the transcriptional machinery in the nucleus (22). There are likely a variety of substrates/target proteins for Hint and Aprataxin that regulate a variety of cellular processes. One possibility is the HINT enzymes have adenylated proteins as substrates, although the potential ligands of HINT and subsequent reactants remain unknown. A loss of HINT activity restricted growth rate in yeast (19,21,23,24). Thus, a 35% reduction in HINT1 enzyme mRNA in neurons in cortical layer VI (21% decrease in layers II–V) might be the result of abnormal cell transcription, although results in yeast leading to alterations in cell growth rate do not translate into alterations in the growth of neurons that are terminally differentiated cells.

MDH1

The current findings are consistent with a reported decrease of MDH1 in schizophrenia in an earlier study (6). Others have suggested that decreased MDH1 does not appear to be reflective of treatment with typical neuroleptics as the administration of haloperidol actually increased MDH1 in the PFC of monkeys (6). Malate dehydrogenase catalyzes the reversible oxidation of malate to oxaloacetate and is part of the citric acid cycle. The protein encoded by this gene is localized to the cytoplasm and may play pivotal roles in the malate-aspartate shuttle that operates in the metabolic coordination between cytosol and mitochondria. A decrease of MDH1 in schizophrenia is hypothesized to result in an increased cytosolic [H+] limiting the enzyme activity of 6-phosphofructokinase (6) resulting in decreased energy production pathways via glycolysis. MDH1 is located at chromosome 2p13-14. Suggestive linkages in the region of 2p13-14 to schizophrenia (25,26) and to bipolar disorders (27) have been reported. A large meta-analysis of schizophrenia linkage studies showed one region to be highly significant (2p11.1-q21.1), which met the criterion for genome-wide significance (28).

Neuroserpin

The current Q-PCR analysis of neuroserpin gene expression in the DLPFC agrees with prior data from a cohort of Stanley Foundation Brain Bank schizophrenia patients (10). An oligonucleotide microarray analysis, which showed an increased expression of neuroserpin in the DLPFC (4) used a cohort that is older than the cohort in the current study. Thus, age and duration of illness might account for these opposite findings, though it is not obvious how advanced age would account for this change in direction. Interestingly, another cDNA microarray study also found a reduction in neuroserpin in the DLPFC of patients with schizophrenia (Karoly Mirnics, personal communication, University of Pittsburgh). One difference between two microarray studies involved using a cDNA microarray platform which showed a decrease in neuroserpin (10) whereas the oligonucleotide microarray result showed an increased neuroserpin expression in schizophrenia (4). The platform might contribute to this difference, as it has been suggested that cDNA and oligonucleotide arrays do not agree, but show weak correlation (29). Again, however, it is difficult to understand how this inconsistency would

change the direction of the difference in expression between groups.

Neuroserpin is an endogenous protease inhibitor that is activity dependent, expressed in neurons in the late stages of neurogenesis during the process of synapse formation, and continues to be expressed in adult brain (30,31). Point mutations in neuroserpin lead to a neurodegenerative disease characterized by adult-onset dementia and progressive cognitive decline with neuroserpin inclusion bodies (32,33). Neuroserpin polymer fibril accumulates in the brain in familial dementia cases in degenerating neurons. A decrease in neuroserpin protein could ultimately increase proteolytic events in the synaptic cleft. Serine proteases break down cell adhesion molecules (34) and neuroserpin inhibits serine proteases. The reduction in neuroserpin, possibly due to a decreased neuronal activity, could lead to a breakdown of synaptic proteins such as N-CAM creating an increased N-CAM degradation found in schizophrenia and eventual spilling into the CSF (35).

Gender appears to be an important factor in gene expression in brain tissue. Gene expression differences between genders have been shown in previous microarray studies in brain (36). Gender dissociation in gene expression might be part of the course of illness and perhaps restricted environmental inputs to males with chronic schizophrenia. It has been argued that an early-onset psychosis in males is associated with the presence of structural cerebral pathology specifically involving the dominant hemisphere and that a later onset form of schizophrenia in some females is characterized by less pronounced structural cerebral involvement (37). Sexual dimorphisms in cortical regions (38) and ventricular brain ratio in schizophrenia have been reported (39). There are well-known examples of dimorphic gene expression in regions of the human brain such as the hypothalamus that are age-related (for a review, see Ref. 40). One example is found in the suprachiasmatic nucleus of which there is a vasoactive intestinal peptide positive subnucleus. This VIP-positive subnucleus is twice as large in young men compared to young women, but actually shows a sex difference reverse above the age of 40 (40). Thus, some of the differences in gene expression between male patients and male controls might result from alterations in brain structure observed in males with schizophrenia but not in females. There is the possibility that age-related deficits are more pronounced in male patients with schizophrenia. To define whether an age-related gender deficit is found in the frontal cortex in gene expression for male patients will require a larger cohort of patients and controls.

One report of schizophrenia and microarray reported Q-PCR results to validate microarray candidate gene expression levels for apo L gene family members (14). There were no differences found due to gender, although the gender results were not specifically reported for the apo L gene family (14). Other microarray study cohorts of predominantly male individuals with schizophrenia did not report gender differences in gene expression (4,8,10,11). However, each of these studies employed different methods to address the gender issue. In one study (8), the female patients were paired to female controls, thus differences between male patients could predominate because the pairs were largely male. In the Hakak et al. study,[4] the authors used ANOVA to test for an interaction of gender and diagnostic category and did not find any significant interactions, thus, specific comparisons were not further reported. Vawter et al.[10,11] used pools of predominantly male individuals with schizophrenia and compared these to matched control pools of samples, thereby possibly generating microarray results that select for male differences in patients with schizophrenia. The inclusion of genes in the current Q-PCR study might be biased toward a gender difference as previous microarray cohorts for schizophrenia have about 70% male and 30% female composition across 5 studies (4,8,10,11,14). Vawter et al., 2001a; Vawter et al., 2002). Further Q-PCR studies will be needed to determine whether these gender differences in gene expression can be replicated for male patients with schizophrenia. The current results suggest an interaction between diagnosis and gender in the metabolic and protease processing pathways, but the limits of a small sample caution against further interpretation of our results.

Regulation of mRNA expression by neuroleptic treatment in patients with schizophrenia might partially explain the current results, although MDH1 has previously been shown to increase with neuroleptic treatment in cortex (6). Perhaps these genes would actually be overexpressed in nonmedicated patients, and medication would reduce the expression levels. This hypothesis was preliminarily examined in never-medicated patients compared to matched controls (10). MDH1 and neuroserpin gene expression levels in the DLPFC were overexpressed in untreated patients compared to controls. The HINT1 gene expression levels in never-medicated patients were similar to the control group (10). Although the interpretation of these results is limited due to a small subject pool, further study of never-medicated patients is required to determine whether differences in candidate genes exist at onset prior to medication. Unfortunately, few cases are usually available. Thus, treatment might ameliorate genes expressed

in the DLPFC of patients. Overexpression of these candidate genes and subsequent neuroleptic treatment to study further these candidate gene levels in the brain would help clarify the association of schizophrenia and expression in postmortem brain. A parallel question is whether antisense knock-down strategies in adult animals might lead to behavioral consequences such as impaired cognitive functioning. These candidate genes could also be tested for SNPs that might have some regulatory role in expression levels. The HINT1 gene is located on chromosome 5q within a broad linkage region. Genome-wide scans are suggestive of linkage regions to schizophrenia on chromosome 5q exceeding LOD scores of 2 (41–43).

Schizophrenia appears to be a heterogeneous disorder, perhaps manifesting in the brain of affected individuals differently. Multiple genes conferring moderate effects have been proposed to provide susceptibility to schizophrenia (44). Further complicating this picture, all three candidate genes (MDH1, HINT1, and neuroserpin) are localized in neurons in the DLPFC and thus might be down-regulated because of lowered neuron size, neuronal inputs, or neuronal number. This neuronal hypofunction may represent a generalized decrease in neuronal markers associated with frontal dysfunction in patients with schizophrenia (45). However, a generalized decrease in mRNA did not occur in the male patients as the levels were equivalent to male controls for the cyclophilin housekeeping gene. Alternatively, these candidate genes might be dysregulated in different sets of neurons that are important regulators of information processing, which could lead to impairment in cognitive function. The expression of HINT1 is primarily localized to neurons and was decreased in the DLPFC of schizophrenia. The findings of a decrease in enzyme activities relating to cell growth and energy metabolism accompanying an increased proteolytic activity (as a result of decrease inhibition of protease activity) suggests a neuronal phenotype in schizophrenia. Finding the functional consequences of this neuronal phenotype will be important in determining if these genes are directly involved in the pathophysiology of schizophrenia.

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