Evidence for Linkage Between Regulatory Enzymes in Glycolysis and Schizophrenia in a Multiplex Sample

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Observations of impaired glucose regulation in schizophrenia are long-standing, although their pathological and etiological significance is uncertain. One approach to the issue that minimizes environmental variables (e.g., medication and diet) is to determine whether genes related to glucose regulation show genetic linkage to schizophrenia. We examined the potential role of glucose metabolism in schizophrenia through a genome scan of affection status in schizophrenia and an empirical method for deriving $P$-values. Data were utilized from the NIMH Genetics Initiative for Schizophrenia dataset, which comprises a total sample consisting of 71 pedigrees containing 218 nuclear families and 987 individuals. A genome scan with 459 markers spaced at an average of 10 cM intervals was conducted using the linkage analysis program Genehunter separately for European- and African-American groups. Enzymes that regulate glycolysis were identified and the

INTRODUCTION

Abnormal glucose regulation in schizophrenia has been reported over much of the last century [e.g., Kooy, 1919; Braceland et al., 1945; Franzen and Nilsson, 1968]. Between 1930s and 1950s (when neuroleptics

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were introduced), insulin coma therapy was a treatment of choice for schizophrenia [Cramond, 1987]. While it fell into disfavor for several reasons, its effectiveness in some patients has never been understood fully. It is significant, however, that recent animal studies suggest the mechanisms of insulin therapy action may include modulation of dopamine neurotransmission in a manner similar to that produced by typical neuroleptic medications [Lozovsky et al., 1985]. Moreover, numerous studies report either impaired glucose tolerance or increased resistance to insulin in schizophrenia [e.g., Braceland et al., 1945; Franzen and Nilsson, 1968; Schimmelbusch et al., 1971; Brambilla et al., 1976], or relationships between fasting levels of glucose or insulin, and abnormal movements or tardive dyskinesia [Ganzini et al., 1991; Schultz et al., 1999].

The nature of the relationship between glucose regulation and schizophrenia is significant for several reasons. First, glucose administration attenuates deficits in verbal declarative memory in schizophrenia through mechanisms that require clarification [Fucetola et al., 1999; Newcomer et al., 1999; Stone et al., 2003]. Moreover, glucose regulation itself is related to memory performance in rodents and humans [e.g., Hall et al., 1989; Stone et al., 1990; Messier et al., 1999]. Second, current interest in glucose regulation in schizophrenia stems from hyperglycemic effects associated with some of the newer, atypical antipsychotic medications [Popli et al., 1997; Haggl et al., 1998]. Interestingly, impaired glucose regulation is not limited to an association with the newer pharmacological treatments. Some of the earlier observations in this area were made before neuroleptic treatments were introduced [e.g., Braceland et al., 1945], or later, in un-medicated patients [Schimmelbusch et al., 1971] and in patients treated with typical neuroleptic medications [e.g., Mukherjee et al., 1989].

Although there are methodological problems with some of the early studies (e.g., those that did not report relationships between body weight/adiposity and glycemic control), studies with appropriate methodological controls confirm relationships between antipsychotic medications and impaired glucose regulation [Baptista et al., 2001b; Newcomer et al., 2002]. These findings raise the possibility that impaired glucose regulation in schizophrenia is related to the underlying disorder, as well as to the medication used to treat it. Consistent with this view, Mukherjee et al. [1989] reported elevated rates of non-insulin dependent diabetes (NIDDM) in the relatives of patients with schizophrenia. If these conditions are related, however, the nature of the relationship remains uncertain. In this study, we explored one facet of the hypothesis that glucose regulation/availability is abnormal in schizophrenia, by determining whether genes that are related to glucose regulation show linkage to schizophrenia.

MATERIALS AND METHODS
Subjects

Data for these analyses were derived from subjects who participated in the NIMH Genetics Initiative for Schizophrenia, which is a multi-site, collaborative family study of schizophrenia [Cloninger et al., 1998; Faraone et al., 1998]. Probands who met DSM-III-R diagnostic criteria for schizophrenia were identified through systematic screening of patients in psychiatric hospitals and clinics at Washington University, Harvard University, and Columbia University. Families with at least one additional first-degree relative with schizophrenia or schizoaffective disorder were recruited to participate in the study. All first-degree relatives of each proband were included. Families were excluded if both parents met criteria for schizophrenia. The total sample consisted of 71 pedigrees containing 218 nuclear families and 987 individuals. Subjects were mainly either European-Americans or African-Americans. The diagnostic distribution of the sample is described in Cloninger et al. [1998].

Due to allelic heterogeneity between the two ethnic groups [Faraone et al., 1998], data from European-Americans and African-Americans were analyzed separately in this study. The European-American sample consisted of 39 pedigrees that contained 137 nuclear families and 644 individuals. Of these, 137 subjects (64 female, 73 male) were genotyped and utilized for the current analyses. The African-American sample consisted of 29 pedigrees that contained 81 nuclear families and 343 individuals. Of these, 103 subjects (57 female, 46 male) were genotyped and utilized for the current analyses. Additional details are provided in Table I.

Genotyping Procedures

The Millennium Schizophrenia Consortium completed a genome-wide scan of 459 short-tandem repeat (STR) markers with an average spacing of 10 cM in all subjects [Cloninger et al., 1998]. The markers were selected from the CHLC-6 set and supplemental markers were added from the Genethon map. The markers were di-, tri-, and tetra-nucleotide repeats that can be reliably scored using automated methods. The polymerase chain reactions (PCR) were set up with 5.0 µl genomic DNA (4 ng/µl), 0.50 µl primer cocktail, and 4.95 µl Taq cocktail. The PCR cycling consisted of 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, and concluded with 72°C for 10 min. The gels were run on Applied Biosystems (ABI)

<table>
<thead>
<tr>
<th>TABLE I. Description of Families</th>
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<tr>
<td></td>
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<tr>
<td>European-American</td>
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<tr>
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</tr>
<tr>
<td>No. of pedigrees</td>
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<tr>
<td>Total no. of genotyped</td>
</tr>
<tr>
<td>No. of males</td>
</tr>
<tr>
<td>No. of females</td>
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</tbody>
</table>
Gene Identification and Location

Five enzymes critical to the regulation of glycolysis were selected for the analysis. These were selected because all of these serve regulatory functions in glycolysis. Initially, they were also selected to determine whether the genes that encode them fell within the 95% confidence intervals of chromosomal regions that are implicated in schizophrenia (see Table II). However, while our initial analyses showed whether the genes fell within the confidence intervals, they did not yield $P$ values for these genes. To correct this, we instead adopted a permutation testing approach [Faraone et al., submitted] that could assess the statistical significance of the locations of these enzymes with regard to the NIMH schizophrenia linkage findings. The results of the permutation testing (see additional details, below) analyses, rather than the confidence interval analyses, are reported here.

The enzymes selected included two forms of hexokinase, including hexokinase 1 (HK1) and hexokinase 3 (HK3); phosphofructokinase, platelet type (PFKP), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2); and a form of pyruvate kinase 3 (PK3). Hexokinase, phosphofructokinase, and pyruvate kinase all catalyze essentially irreversible reactions in the glycolytic pathway. Hexokinase catalyzes the first step in glucose metabolism, the utilization of adenosine triphosphate (ATP) for the phosphorylation of glucose to glucose-6-phosphate. Phosphofructokinase, which is the rate-limiting enzyme in the pathway, utilizes ATP to phosphorylate glucose-6-phosphate to fructose-6-phosphate. Pyruvate kinase utilizes adenosine diphosphate (ADP) to catalyze the transfer of a phosphoryl group from phosphoenolpyruvate to yield pyruvate and ATP. PFKFB2 is the heart isozyme of a family of enzymes that catalyzes the synthesis and degradation of fructose-2,6-bisphosphate, which is a regulatory molecule that controls opposing glycolytic and gluconeogenic pathways [Okar et al., 2001]. The genetic locations for the genes that regulate these enzymes were identified through the Online Mendelian Inheritance in Man (OMIM) website (www3.ncbi.nlm.nih.gov/OMIM), and are shown in Table II.

Linkage Analyses

The multipoint linkage analysis program, Genehunter 2.0, was run using schizophrenia affection status as the qualitative trait. A positive affection status was defined as any individual with a DSM-III-R diagnosis of schizophrenia or schizoaffective disorder, depressed. All affected sibling pairs were used. Maximum likelihood estimates of the marker allele frequencies, which are required by the Genehunter program, were calculated using SOLAR. These estimates were calculated separately for each ethnic group to account for the locus heterogeneity found previously in this sample. The non-parametric LOD (NPL) scores resulting from the Genehunter analysis were then used to calculate empirical $P$-values using permutation testing.

Permutation testing is used to determine accurate $P$-values for our hypothesis: is there significant linkage to the five glucose regulating genes in schizophrenic families? To answer this question, we constructed five empirical distributions using permutation testing. $P$-values for each candidate gene were calculated from these distributions, allowing us to assess the significance of our finding at the five locations accurately [Faraone et al., submitted].

To assess the individual significance of the NPL scores for the five glucose-related candidate genes corrected for multiple comparisons, empirical distributions were created using the following procedure. Five NPL scores were selected randomly from a 1 cM scan, sampling NPL scores at 1 cM intervals. These five NPL scores were then sorted from highest to lowest. This procedure was repeated 50,000 times, resulting in five empirical distributions: one for each of the five NPL scores. As this procedure accounts for multiple testing within each ethnic sample, it is appropriate to evaluate the genome-wide level of statistical significance at the 0.05 level. The $P$-value for each candidate gene was calculated as the percentile of its NPL score in the appropriate empirical distribution (i.e., our highest NPL score was referenced to the empirical distribution of highest NPL scores, our second highest NPL score was referenced to the empirical distribution of second highest NPL scores, and so forth). This analysis was performed separately on each ethnic group.

Although the procedure described above accounts for multiple testing within ethnic samples, it does not adjust for multiple testing of each gene in the two ethnic groups together. To deal with this issue, we used the following procedure to assess the overall significance of each of the five glucose-related candidate genes corrected for multiple comparisons. Five positions along the genome were randomly selected from a 10 cM scan, sampling NPL scores at 1 cM intervals. The five NPL scores that were chosen via this procedure were then sorted from highest to lowest. As with the previous sets of analyses, this technique was repeated 50,000 times, resulting in five empirical distributions: one for each of the five NPL scores. In this approach, however, only the most significant NPL score for each of the five candidate genes across the two samples (African-American and European-American) was matched to the empirical distribution that corresponded to its rank. The resultant $P$-value for each candidate gene was calculated as the percentile of its NPL score in the appropriate empirical distribution. As above, in each

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**TABLE II. Genes Relevant to Glucose Metabolism**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location</th>
<th>Previous schizophrenia findings</th>
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<tbody>
<tr>
<td>PFKFB2</td>
<td>1q32.2</td>
<td>1q21-p22; Brzustowicz et al. [2000]</td>
</tr>
<tr>
<td>HK3</td>
<td>5q35.3</td>
<td>5q11.2-q13.3; McGuffin et al. [1990]</td>
</tr>
<tr>
<td>PFKP</td>
<td>10p15.1</td>
<td>10p12.31; Faraone et al. [1998]</td>
</tr>
<tr>
<td>HK1</td>
<td>10q22.1</td>
<td>10q12.31; Faraone et al. [1998]</td>
</tr>
<tr>
<td>PK3</td>
<td>15q23</td>
<td>15q15; Freedman et al. [2001]</td>
</tr>
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</table>
distribution, the appropriate level for significant evidence of linkage is $P \leq 0.05$.

**RESULTS**

The empirical $P$-values for the five candidate genes are presented in Table III. The analyses conducted on each ethnic sub-group showed the following results. For the European-American sample, three of the glucose-related genes were significant at the 0.05 level: HK3, PK3, PFKFB2. None of the genes showed significance in the African-American sample. In the combined sample, presented in the last column of Table III, PFKFB2 was significant at the 0.05 level ($P = 0.044$) and HK3 was nearly significant ($P = 0.097$).

**DISCUSSION**

These findings demonstrate evidence for linkage between enzymes that control glycolysis, and schizophrenia. In the overall sample, PFKFB2 showed linkage with schizophrenia in a multiplex sample of subjects who participated in the NIMH Genetics Initiative for Schizophrenia. In the European-American sample alone, PFKFB2, HK1, and HK3 all demonstrated linkage, but none of the five enzymes assessed in this study showed linkage in the African-American sample alone. These findings, which are not in previously identified schizophrenia susceptibility regions, are novel and raise the possibility of evaluating PFKFB2 in particular, but also HK1 and HK3, as candidate genes for schizophrenia. Particularly because PFKFB2 is a rate-limiting enzyme in glycolysis, and hexokinase is another regulatory enzyme in the metabolic pathway, the results demonstrate a possible relationship between glucose regulation and schizophrenia. In light of the biological ubiquity of these enzymes, and if these findings are correct, they highlight the view that multiple, common genes are involved in producing a vulnerability to schizophrenia [Gottesman, 2001].

At the same time, it is necessary to consider the limitations of the present results. First, these results need to be replicated with other samples, as this is the first finding implicating these genes. It will also be important to determine whether forms of these enzymes are associated with other psychiatric disorders, and in diabetes with subjects without mental disorders. Second, the small sample size limited the power of this study. If PFKFB2, HK1, and/or HK3 do contribute to the liability for schizophrenia, their influence is likely to be modest, as most cases of schizophrenia are likely to result from multiple factors including combinations of genes and adverse biological consequences of environmental exposures (e.g., head trauma, in-utero complications, and illicit substance abuse). Third, a qualitative trait is an imprecise way to measure a complex phenotype such as schizophrenia. It is unlikely that even the narrow definition of affection status will clearly delineate the multiple etiologies of schizophrenia. Therefore, a more efficient approach would use a highly refined quantitative trait that is related to glucose functioning. Our intention in using a qualitative trait was to compare the initial findings with this sample. Fourth, these findings used only European- and African-American samples, so the results should not be generalized to other ethnic groups. Fifth, the current findings do not identify which specific genes actually produced the positive evidence of linkage. To do so, it will be important to genotype the genes that actually contributed to these results, and then perform association studies on those genes to determine which alleles might be involved. Thus, the present findings support the view that glucose regulation and schizophrenia are related, but do not provide direct evidence for it. Sixth, only enzymes that are involved in glucose regulation and are near identified schizophrenia susceptibility regions were assessed here. Thus, other enzymes involved in glucose regulation that might be involved in schizophrenia, were not explored in this study. Despite these limitations, the current results are encouraging, and are significant for several reasons. First, they provide genetic support for the view that abnormalities in glucose regulation and/or availability in some patients are not only epiphenomena related to medication effects, but might reflect inherent dysfunction associated with the disorder. The results are consistent with those from older studies showing poor glucose regulation and often, insulin resistance, in un-medicated patients [Braceland et al., 1945; Schimmelbusch et al., 1971]. The present findings add to the previous ones, however, both because a very different methodology was used here, and also because methodological shortcomings (e.g., not accounting for relationships between body weight and glucose or insulin regulation; less reliable psychiatric diagnosis) cloud the interpretation of many earlier findings. Results from more recent studies that also show a vulnerability to poor glucose regulation in patients with schizophrenia,

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>cM</th>
<th>African-American</th>
<th>European-American</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK3</td>
<td>5q35</td>
<td>203</td>
<td>0.139 (4)</td>
<td>0.0057 (5)</td>
<td>0.097 (5)</td>
</tr>
<tr>
<td>PK3</td>
<td>15q23</td>
<td>76</td>
<td>0.497 (1)</td>
<td>0.0152 (4)</td>
<td>0.284 (2)</td>
</tr>
<tr>
<td>PFKFB2</td>
<td>1q32.2</td>
<td>233</td>
<td>0.212 (3)</td>
<td>0.0328 (3)</td>
<td>0.044 (4)</td>
</tr>
<tr>
<td>HK1</td>
<td>10q22.1</td>
<td>90</td>
<td>0.371 (5)</td>
<td>0.169 (2)</td>
<td>0.189 (3)</td>
</tr>
<tr>
<td>PFKP</td>
<td>10p15.1</td>
<td>3</td>
<td>0.354 (2)</td>
<td>0.187 (1)</td>
<td>0.480 (1)</td>
</tr>
</tbody>
</table>

All $P$-values are empirically derived as described in the “Materials and Methods.” Thus, they are corrected for multiple comparisons.
however, have not yet addressed the issue of whether the vulnerability is produced by the drugs, the disorder, or by a combination of both factors [Newcomer et al., 2002].

The present findings are also of interest because of the difference in outcome in the European-American and the African-American samples. In light of higher rates of diabetes and related health problems in African-Americans [Davis et al., 2003] and the significant findings for three enzymes in the European-American sample, one or more of the enzymes assessed here might have been expected to show evidence of linkage in the African-American sample. The group differences likely reflect multiple etiological factors, and emphasize the need for multiple analytical strategies (e.g., the assessment of population differences in admixture [Pfaff et al., 2001; Smith et al., 2001]). While the underlying reasons for the differences still require further investigation, it should be noted that the present results replicate, in principal, previous analyses showing evidence for linkage on chromosome 10p in the European-American, but not the African-American, sample [Cloninger et al., 1998; Faraone et al., 1998].

The mechanisms underlying impaired glucose regulation, non-insulin dependent diabetes mellitus (NIDDM) and/or ketoacidosis in schizophrenia are mainly unknown, but are likely to be multifactorial. For example, antipsychotic medications such as clozapine and olanzapine often produce weight gain and increased adiposity [Haupt and Newcomer, 2001], which may result in higher levels of insulin resistance [Haupt and Newcomer, 2001; Steppan et al., 2001; Taniguchi et al., 2002]. Possible mechanisms by which atypical antipsychotic medications increase insulin resistance include direct actions at receptors, such as interference with glucose transporters [Henderson, 2001]. Hormones such as resistin may mediate these effects partially [Steppan et al., 2001]. However, antipsychotic medication is also associated with hyperglycemia and other gluco-regulatory abnormalities independent of increased adiposity [Baptista et al., 2001a; Newcomer et al., 2002], which indicates the likelihood that multiple mechanisms contribute to poor regulation. Abnormal insulin secretion and/or abnormal gluconeogensis, in addition to insulin resistance, are also factors that may contribute to impaired glucose regulation and the development of NIDDM.

While speculative, it is possible in light of the present findings that antipsychotic medications add to one or more pre-existing vulnerabilities, at least in some patients, to impair glucose regulation in schizophrenic individuals. Such a view is consistent with a recent model proposing that effects of antipsychotic medications on carbohydrate metabolism (including, among other effects, insulin resistance, increased appetite, and related physiological changes that leads to body weight gain in some but not all individuals) and possibly on dyslipidemia, are additive with the effects of genetic predisposition and environmental factors [Baptista et al., 2001a]. While glucose dysregulation is not specific to psychiatric disorders in general, nor is schizophrenia the only psychiatric disorder associated with impaired regulation (e.g., it occurs in affective disorders [Cassidy et al., 1999; Eaton, 2002; Regenold et al., 2002]), the present findings suggest, nevertheless, that impaired glucose regulation contributes to the genetic liability for schizophrenia.

A second, related, reason the current findings are significant, is that they raise the question of whether glucose regulation in non-psychotic (and un-medicated), first-degree biological relatives of patients might be impaired. Consistent with this possibility, Mukherjee et al. [1989] found that 32% of a sample of relatives (7 out of 22) was diagnosed with non-insulin dependent diabetes mellitus. In light of the enzymes implicated in schizophrenia in the current study, it will be important to determine whether other gluco-regulatory enzymes (e.g., hepatic PFKFB2) also show linkage to schizophrenia. The activity of gluco-regulatory enzymes provides a potential way to relate the results of this study to hyperglycemia and poor regulatory control. If, for example, an allele of (hepatic) PFKFB2 showed lowered activity in some patients with schizophrenia, then lower levels of fructose 2,6-bisphosphate would be produced. This would simulate a fasting state in which glycolysis would be inhibited and hepatic gluconeogensis facilitated, which would raise blood glucose levels. In susceptible individuals (e.g., in European-Americans), lowered activity in hexokinase and pyruvate kinase might have similar effects. Interactions with various hormones might also exacerbate the effect, including stress-related hormones such as glucocorticoids, which themselves facilitate gluconeogensis and are associated with hyperglycemia and increased insulin resistance [e.g., Nyirenda et al., 1998]. Thus, glycemic status, considered alone or in combination with drugs or hormones that modulate its expression, may become a potentially useful indicator of the liability to develop schizophrenia. Eventually, it may also provide a significant target for prevention or early intervention strategies.

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