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RELATIONSHIP BETWEEN GENETIC VARIATION IN THE GLUTAMINASE GENE *GLS1* AND BRAIN GLUTAMINE/ GLUTAMATE RATIO MEASURED *IN VIVO*

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Abstract

Background—Abnormalities in glutamatergic neurotransmission are implicated in several psychiatric disorders, but *in vivo* neurochemical studies of the glutamate system have been hampered by a lack of adequate probes. On the other hand, glutamine and glutamate can be quantified separately in proton magnetic resonance spectroscopy studies *in vivo*. Accumulating evidence suggests that the glutamine/glutamate (Gln/Glu) ratio is a putative index of glutamatergic neurotransmission but interpretation of changes in the Gln/Glu ratio depends on the conditions of the system, including ammonia levels.

Methods—Here, we explored whether variation in *GLS1* (the gene encoding the brain isoform of glutaminase which catalyzes Gln-to-Glu conversion) is associated with Gln/Glu measured *in vivo* in two brain regions (anterior cingulate cortex (ACC), parieto-occipital cortex (POC)).

Results—A specific haplotype of 4 single nucleotide polymorphisms within *GLS1* was significantly associated with Gln/Glu in the POC in an MRS-genetics dataset optimized for Gln/Glu detection (N=42). This finding was replicated in a second MRS dataset that was optimized for GABA detection where Gln and Glu measurements could still be extracted (N=40).

Conclusions—These findings suggest that genetic variation in a key component of glutamatergic machinery is associated with a putative *in vivo* index of glutamatergic neurotransmission. Thus, *GLS1* genotype may provide insight into normal brain function and into the pathophysiology of many psychiatric conditions where glutamatergic neurotransmission has been implicated. It might also serve as a biomarker for predicting response to existing and novel therapeutic interventions in psychiatry that target glutamatergic neurotransmission.

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Keywords

MRS; J-resolved; MEGAPRESS; neurotransmission

INTRODUCTION

Glutamate (Glu) plays several crucial roles in brain function, including as the main excitatory neurotransmitter. After release into the synaptic cleft, Glu is taken up by adjacent astrocytes, converted to glutamine (Gln), shuttled to neurons, and converted back to Glu (1,2). Activity-dependent changes in neurotransmission may affect Gln-Glu cycling rate and concentrations of Gln and Glu. Both of these metabolites can be measured using proton magnetic resonance spectroscopy (^1H MRS) with the major caveat that ^1H MRS measures not only synaptic but all Gln and Glu. Many previous studies have quantified Gln and Glu together in the composite measure Glx, but this carries limited utility as compared with separate Gln and Glu quantification (3–5). Given the enrichment of Gln in glia and Glu in neurons, the Gln/Glu ratio is one potentially useful index of neurotransmission. In this context, Gln/Glu elevation and reduction are thought, in part, to correlate with increased and decreased neurotransmission, respectively. Gln/Glu elevations are reported during somatosensory stimulation (6) and experimentally induced ischemia in rodents (7,8), and with infusion of NMDA-receptor blockers phencyclidine in rodents (9) and ketamine in humans (10). Finally, blockade of Glu-to-Gln conversion in glial cells via glutamine synthetase inhibition in an animal model of amyotrophic lateral sclerosis leads to 41–48% reductions in Gln/Glu in two brain regions (11). Thus, the experimental evidence supports a role for the Gln/Glu ratio as a putative index of glutamatergic activity, although the details of its physiological determinants are not completely understood. Interpretation of changes in Gln/Glu should be approached with some caution since metabolic factors such as hyperammonemia can also modify the index. Thus, Gln/Glu alterations will be pathology- and/or activity-sensitive and changes will likely not apply in all cases or may have opposite interpretations in different pathologies.

Abnormalities in glutamatergic neurotransmission have been implicated in several psychiatric conditions including schizophrenia and mood disorders (12–16). There has been great interest in developing novel therapeutic agents that modulate the glutamate system (17,18), or in testing existing agents with glutamatergic properties (19). In parallel with therapeutic developments, there are an increasing number of clinically-oriented *in vivo* neuroimaging studies focusing on glutamatergic neurotransmission. Using ^1H MRS, we reported elevated Gln/Glu in anterior cingulate (ACC) and posterior cingulate (POC) cortices in bipolar mania (20), and with antidepressant treatment in bipolar depression (21). Others have reported reduced Gln/Glu in major depression (22) and elevated Gln/Glu in first-break schizophrenia in ACC (23,24).

Disruption in glutamatergic neurotransmission is also suggested by *postmortem* studies of the neuronal enzyme glutaminase (which catalyzes Gln-to-Glu conversion) and the glial enzyme glutamine synthetase (which catalyzes the reverse reaction) in psychiatric conditions. Elevation in mRNA/protein levels and enzymatic activity have been reported for both glutaminase and glutamine synthetase in schizophrenia and mood disorders *postmortem* (25–29) and with lithium treatment (30). By contrast, glutaminase knockdown mice have 50% reduced Gln-to-Glu conversion and show 30% elevation of Gln/Glu ratios in MRS studies in frontal cortex, and 20% elevation in the hippocampus and thalamus. These mice also display a series of schizophrenia-resilience phenotypes in behavioral, pharmacologic, and neuroimaging studies (31). These findings demonstrate that interference with a key part of the synaptic glutamatergic machinery leads to concurrent changes in Gln/Glu and in

CNS-related phenotypes. There are no published genetic association studies of the glutaminase gene *GLS1* in bipolar disorder or major depressive disorder. One study in a Chinese population was negative for *GLS1* association with schizophrenia, although this study had negative findings across the board (32). Another study found no association between *GLS1* genotype and serum glutamate levels in schizophrenia patients (33).

Of the two main isoforms, the kidney-type glutaminase accounts for most brain glutaminase activity although other isoforms may also be expressed in the brain. This predominant isoform is encoded by the *GLS1* gene on human chromosome 2. Glutaminase is localized on the outer face of the inner mitochondrial membrane (i.e. in the inter-membraneous space) (34) and is expressed almost exclusively in glutamatergic neurons *in vivo* (35). The catalytic rate of glutaminase is modulated by neuronal activity, mediated by phosphate (inhibition) and calcium (stimulation) ions (36), and is strongly inhibited by its reaction product Glu. Cytosolic variations in Glu can modulate glutaminase activity by 10-fold (35).

In this study, we sought to determine whether Gln/Glu measured using MRS is related to genetic variation at the *GLS1* locus. We first used an ¹H MRS dataset optimized for Gln-Glu detection obtained at our center on a 4 Tesla MR scanner (3) and largely overlapping with a previously published MRS study (20). In this primary analysis, we examined the relationship between Gln/Glu and *GLS1*. In a secondary dataset also overlapping with a previously published MRS study (37) which is optimized for GABA detection but nonetheless provides Gln and Glu data, we tested whether the association between Gln/Glu and *GLS1* genotype observed in the primary study would replicate.

METHODS

The MRS data used in this study came from 2 separate datasets which have already been presented elsewhere (20,37) but the use of the data is different from what was already published. Specifically, data on Gln and Glu quantification was presented in what we call Study 1 here (20) but not in what we call Study 2 here (37). Therefore, we present additional details concerning Gln and Glu quantification relevant to the current report in the Supplement. We also present example spectra used for Gln and Glu quantification in this study in Figures S1 and S2 in the Supplement. The subject genotypes and results of current MRS-genetics analyses have not been previously reported. We will describe below the details of participant composition and MRS data acquisition and analysis separately for these datasets. Study 1 used an MRS sequence optimized for detection of Gln and Glu. Therefore, we consider it the primary analysis in this work. Study 2 used an MRS sequence optimized for GABA detection. It provides Gln and Glu data which were of acceptable quality based on Cramer-Rao lower bounds and coefficients of variation but these data are likely to be of lower quality than those in Study 1. Therefore, we consider Study 2 a secondary analysis that is nonetheless of interest.

In both cases, following approval by the McLean Hospital IRB, all participants underwent two magnetic resonance imaging (MRI) scans on the same or consecutive days (depending on scanner availability): first, a structural MRI in a Siemens 3 Tesla Trio scanner (Erlangen, Germany). These scans were read by a radiologist and participants with structural abnormalities were excluded. Second, participants underwent an MRS scan on a 4 Tesla full body MR scanner (Varian/UnityInova, Varian Inc., Palo Alto, California). Blood samples were obtained from all participants as part of another IRB-approved genetics study, and further permission was obtained from the IRB to combine the MRS and genetics data in a single analysis. Because race can be an important variable in genetic analyses, we collected self-report of race from all participants and included this information as a covariate in the analyses (see Table 1).

Study 1

The details of participant recruitment/characterization, and MRI/MRS studies (including data acquisition and analysis) for Study 1 have been described in detail previously (20). After DNA extraction and quality control, 42 had usable genetic samples; these participants were a mixture of healthy controls (N=9 out of 21 total), and participants with schizophrenia (N=18 out of 18 total) or bipolar disorder (N=15 out of 15 total). Some subjects from the prior report were excluded because we added the blood sample collection for genetic studies after they had completed the MRI/MRS study. Demographic information is presented in Table 1.

As described previously (20), data were acquired from two $2 \times 2 \times 2$ cm midline voxels in the ACC and POC in this study. A point-resolved spectroscopy sequence (PRESS) modified for *J*-resolved proton MRS was used to collect 48 TE-stepped spectra with the echo time ranging from 30 msec to 500 msec in 10-msec increments; this sequence provides high quality data for resolving and quantifying Gln and Glu on our system (3). Total participant time in the scanner was 75 to 90 minutes.

All MRS data processing was blinded to diagnosis and genotype. Post-processing included zero-filling, Gaussian filtering, and Fourier transformation. The commercial spectral fitting package LCModel (version 6.0–1) was used for spectral fitting with gamma-simulated basis sets. We also carried out segmentation of T1-weighted images into grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF). The Gln/Glu ratio does not require an internal reference as it is the ratio of two metabolites and not affected by between-subject variation in overall signal detection. See Supplement for additional details.

Study 2

The details of participant recruitment/characterization, and MRI/MRS studies (including data acquisition and analysis) for Study 2 have been described in detail previously (37). After DNA extraction and quality control, this study included 40 participants consisting of healthy controls (N=11 out of 19 total), and participants with schizophrenia (N=15 out of 21 total), or bipolar disorder (N=14 out of 14 total). This sample was overlapping but not identical to that in the original publication (37); some subjects from the prior report were excluded because we added the blood sample collection for genetic studies after they had completed the MRI/MRS study. Demographic information on these participants is presented in Table 1.

Data were acquired from two $23 \times 22 \times 33$ mm midline voxels in the ACC and POC in this study. A MEGAPRESS (38) sequence was implemented for optimized GABA detection. The MEGAPRESS sequence collected two subsets of spectra: one PRESS acquisition at TE=68msec with the selective Gaussian refocusing RF pulses centered at the 1.9 ppm GABA resonance ('on' scan) and one with the editing RF pulses centered 20000 Hz off-resonance ('off' scan). The subtraction of off from on spectra reveals resolvable GABA peaks. Although this sequence is optimized for GABA detection, Gln and Glu levels can be quantified from the 68msec subspectrum within the MEGAPRESS sequence (see Supplement). Total time in the scanner was 75 to 90 minutes.

All MRS data processing was blinded to diagnosis and genotype. Post-processing included zero-filling, Gaussian filter, and Fourier transformation. The commercial spectral fitting package LCModel (version 6.0–1) was used for spectral fitting with phantom-acquired basis sets. We also carried out segmentation of T1-weighted images into grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF). See Supplement for additional details.

Genetic Analyses

Genotyping was performed at the MGH Center for Human Genetic Research Psychiatric and Neurodevelopmental Genetics Unit, following approval by the McLean Hospital and Massachusetts General Hospital IRBs and transfer of frozen whole blood samples. Mass spectroscopy genotyping was performed using the Sequenom MassArray system and iPLEX assay design. The major steps included the following: DNA amplification through PCR, removal of unincorporated dNTPs using shrimp alkaline phosphatase, single base extension for allele differentiation, and molecular determination through mass spectroscopy using SpectroCHIP analysis. SNPs were analyzed only if they meet the following quality control criteria: 1) >90% of attempted genotypes for any SNP successful, 2) Hardy-Weinberg equilibrium $p > 0.001$, and 3) >1% minor allele frequency.

To select the *GLSI* SNPs, we used the UCSC Genome Browser to identify the base pair region for our genes of interest (plus 10 kb on either side) and downloaded the CEPH SNP genotype data for this region from the International HapMap Project database (<http://hapmap.org>). To identify SNPs tagging the gene region, we uploaded the CEPH genotype data into Haploview and used the Tagger module within Haploview to run a pairwise tagging approach using an r^2 threshold of 0.80 and a minor allele frequency threshold of 5%. (39). This process identified 16 tagging SNPs across the *GLSI* locus, but one did not survive the quality control criteria and one had a minor allele frequency <5%. Association analyses for the 16 SNPs (rs16832963, rs7578618, rs4853750, rs3771311, rs6743496, rs13000464, rs17748089, rs13414554, rs12185688, rs6434430, rs6712504, rs10437, rs17748980, rs16824035, rs13395505, rs880253) and Gln/Glu ratios were conducted using PLINK (40) in a linear regression model with self-reported race and diagnosis as covariates. We also ran haplotype linear regression analyses testing a 4-SNP sliding window across the *GLSI* gene vs. the Gln:Glu ratio for both the ACC and PCC. In the haplotype analyses race was included as covariate but diagnosis was not (because all interaction terms were negative in all of the single marker analyses). See Supplement for additional details.

RESULTS

Study 1

We describe assessments of single SNPs first followed by a sliding window haplotype analysis across *GLSI*. We first examined association between the 16 *GLSI* SNPs and Gln/Glu for both the ACC and PCC brain regions. Using Bonferroni correction for multiple testing controlling for all of our single marker (16 SNPs) and haplotype tests (11 haplotype blocks) for two brain regions, the study-wide significant p-value was $0.05 / [(16+11) \times 2] = 9 \times 10^{-4}$. Four of the 16 SNPs showed nominally significant association with Gln/Glu in the ACC and five in the POC, but none of these survived correction for multiple comparisons (not shown). All of the nominally significant SNPs are intronic. In addition to our base models controlling for race, we also ran models that added diagnosis as a covariate to check for a possible interaction between diagnosis and SNP and found no significant interaction.

We next ran haplotype analyses using 4-marker sliding windows across the 16 SNPs (yielding 11 haplotype blocks). Three haplotypes comprising overlapping SNPs demonstrated association with Gln/Glu in the POC after Bonferroni correction (Table 2). The most significant haplotype (shaded in yellow) from the analysis is shown in Figure 1. For this haplotype, $\text{Beta} = 0.117$ (i.e. a rise in Gln/Glu of 0.117 for each copy of the associated haplotype) and $p = 5 \times 10^{-4}$. The 4-marker windows associated with Gln/Glu in the POC comprised several SNPs spanning exons 10–14 that were nominally significant in single marker analyses. Thus, it appeared that when the several nominally significant SNPs

in the center of the gene are tested together as haplotypes, three displayed study-wide statistical significance in their relationship with Gln/Glu.

Study 2

We next turned to a second dataset in Study 2. In order to preserve statistical power, we did not carry out a SNP-based analysis in this study. Instead, this secondary analysis focused only on the three 4-SNP haplotypes with study-wide significant findings in Study 1. All SNPs in these three haplotypes passed data quality thresholds in this sample. In a linear regression model using self-reported race as a covariate, we confirmed association of one haplotype with POC Gln/Glu (Table 2). As shown in Figure 2, Gln/Glu ratio was linearly related to haplotype dose for this specific haplotype. Of note, the other two specific haplotype windows showed trends in the same direction as in Study 1 in the POC. Table 2 also shows the allele frequencies of the relevant haplotypes in both studies. The Gln/Glu ratio varied as expected in both the original and replication samples depending on whether individuals carried 0, 1, or 2 copies of the target haplotype (Figure 2). That is, individuals carrying 2 copies of the haplotype had higher Gln/Glu ratios than those carrying 1 copy, while those carrying 0 copies had the lowest Gln/Glu ratios.

DISCUSSION

Here we report the first evidence of association between genetic variants in the glutaminase gene *GLS1* and brain Gln/Glu ratios measured *in vivo*. In a primary analysis, we observed association between three four-marker haplotypes completely spanning exons 10–14 in *GLS1* and Gln/Glu ratios in the POC. In a secondary dataset, we replicated this significant association for one of the three haplotypes of interest. Our findings are particularly compelling because they relate genetic variation in a key enzyme to its substrate/product ratio. It should be noted that *in vivo* measures of Gln/Glu do not necessarily reflect synaptic levels of the metabolites and that non-synaptic factors (such as ammonia levels) can modify the Gln/Glu ratio.

The major limitations of this study are sample size and composition. First, both samples in the analyses included a mix of healthy participants and psychiatrically ill participants receiving pharmacotherapy; we cannot rule out medication effects or an interaction between psychiatric condition and *GLS1* genotype which affected our findings. However, such heterogeneity would be expected to bias a genotype/phenotype association toward the null. When we included diagnosis as a covariate we did not detect an interaction, but this analysis was limited in power. Second, both samples were relatively small, limiting our statistical power and increasing the likelihood of Type II error. Larger studies of healthy populations and genotype x diagnosis interactions are needed to address both limitations. Third, the MRS sequence used for data collection differed between Study 1 and Study 2. Study 1 used a *J*-resolved MRS sequence optimized for Gln and Glu detection. This sequence attempts to address some major challenges including resolving Gln and Glu signal from underlying macromolecule resonances as well as those from glutamate-conjugate compounds such as glutathione (see Supplement). Study 2 used MEGAPRESS, a *J*-editing approach which is optimized for GABA detection although it does collect Gln and Glu data. The reliability of Gln and Glu detection using this approach has not been previously reported (41, 42) but we provide those data in the Supplement. Differences in quantification and analysis (e.g. percent grey matter content, signal-to-noise ratios etc.) led to differences in mean Gln/Glu ratios between the two studies (Figure 2). Although it would be desirable to collect comparable data across the two studies so they could be pooled, the fact that we detected the same genotype effect by two methods in two distinct studies is reassuring. In fact, since sources of measurement noise differ between *J*-resolved MRS and MEGAPRESS, we are

more confident that systematic problems in MRS data acquisition and analysis across the two studies did not confound our Gln/Glu analyses.

It is notable that our findings were confined to the POC in the first study, although we studied both the POC and the ACC. In the second study we only examined the POC. A region-specific genotype effect on Gln/Glu is possible but seems unlikely. This pattern could be related to lower ACC data quality due to susceptibility artifacts. In fact, we acquired twice the number of repetitions in ACC as compared with POC in both of our studies to try to compensate for this phenomenon (20,42). Poorer ACC data quality may lead to greater noise in the Gln/Glu measures and obscure the genotype effect. However, we did not observe higher standard deviation/mean measures in ACC vs. POC in our analysis (37% vs. 42% in Study 1 and 37% vs. 28% in Study 2 in the ACC and POC, respectively). An alternative explanation is the low power of the current study and larger studies may indeed find an ACC effect.

The relevant *GLS1* variation in our study was restricted to intronic polymorphisms with no known functional significance. We did not observe significant association with the individual tagging SNPs we tested but did with haplotypes spanning exons 10–14. Thus, our results leave the identity of the causal variant(s) unclear but raise the possibility that unmeasured variants in or around exons 10–15 may underlie the associations we observed. Intronic polymorphisms have been reported to alter mRNA splicing and gene expression, potentially linking our data with the *postmortem* literature on abnormal glutaminase activity in psychiatric illness (25,28). However, we do not have any direct data on glutaminase expression or activity levels and further studies will be needed to evaluate the functional relevance, if any, of the associated haplotype. In studies of the glutaminase knockdown mice a 50% reduction in protein levels led to 20–30% elevation in Gln/Glu. Therefore, we would estimate that the 50% elevation in Gln/Glu we observe using MRS corresponds to greater than a 50% reduction in protein levels due to allelic variation in *GLS1*. Although two previous *GLS1* schizophrenia genetic association studies were negative (32,33), only one SNP in these two studies overlaps with those tested in our dataset (rs3771311), and this SNP was not in the associated haplotype in our study.

Our findings suggest that genetic variation in a key component of the glutamatergic machinery impacts a putative *in vivo* index of neurotransmission. To the extent that Gln/Glu is related to the pathophysiology of mood and psychotic disorders, our results support the power of intermediate phenotypes in genetic research. Despite our limited sample size, the proximal relationship between *GLS1* and Gln/Glu may have revealed an association that would have required much larger samples in studies of the relevant disorders themselves. A large literature suggests that hypofunction of the glutamatergic NMDA receptor is involved in the pathophysiology of schizophrenia. NMDA receptors are enriched on GABAergic interneurons in the cerebral cortex and NMDA hypofunction is thought to lead to pyramidal neuron disinhibition and ultimately the dysregulated information processing which characterizes schizophrenia (43,44). These abnormalities may also lead to a neurodegenerative process in schizophrenia (45). In bipolar disorder, the literature has focused on abnormalities in glial cells which play a critical role in Glu reuptake at the synapse (46–48). Glial cell function has also been implicated in other psychiatric conditions using MRS approaches (49). In animal models, glia-specific lesions are sufficient to reproduce a depressive-like phenotype (50,51), and this phenomenon is mediated at least in part by changes in glial Glu handling at the synapse (52). In short, systematic study of glutamatergic abnormalities may enable substantial progress in understanding the pathophysiology of both schizophrenia and bipolar disorder.

Although studying the Gln/Glu ratio has been fruitful, no experimental evidence proves that Gln/Glu directly reflects synaptic neurotransmission. Still, several lines of evidence reviewed above indicate that changes in Gln/Glu correlate with, and thus are a measure of, changes in glutamatergic activity. Our work on the effects of *GLS1* variation on Gln/Glu provides additional insight into sources of Gln/Glu variation. The functional information provided by Gln/Glu may be highly relevant to the pathophysiology of mental illness. We suggest that studies using Gln/Glu may improve our understanding of glutamatergic abnormalities and treatment interventions in psychiatric conditions (21).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Outline of *GLS1* on human chromosome 2. The very top panel shows the relevant stretch of DNA with the location of *GLS1* depicted. The locations of SNPs included in the analysis are marked with “rs” identifiers. The blue bars beneath SNPs represent the percent of sample genotyped at that SNP in Study 1. The next row lists the minor allele frequency of each SNP. The haplotype block structure is shown next, with the most significant haplotype from Study 1 shaded in yellow. The bottom panel shows the linkage disequilibrium (LD) structure of the SNPs within *GLS1* with red squares representing the highest degrees of LD between SNPs.

Figure 2.

Gln/Glu ratios measured in the POC for individuals carrying 0, 1, or 2 copies of the target haplotype starting with rs13000464 and ending with rs12185688 that was found to be significant in both Study 1 and Study 2. Values are from Study 1 (left) and Study 2 (right) presented as mean $\pm$ SE. Note that mean Gln/Glu values are different across the two studies because of differences in data acquisition and analysis but the same relative pattern is observed.

Table 1

Demographic information on study participants

	Group	Sex	Age	Race
Study 1	NC	7M/2F	40.1±10.3	6C; 2AfA; 1AsA
	BD	7M/7F	35.6±14.5	11C; 3AfA; 1AsA
	SZ	13M/5F	39.8±11.1	14C; 3AfA; 1AsA
Study 2	NC	7M/4F	34.7±9.3	9C; 1AsA; 1AfA
	BD	5M/9F	34.2±12.4	13C; 1AfA
	SZ	7M/8F	44.2±8.6	14C; 1AsA

C=Caucasian; AfA=African American; AsA= Asian American

Table 2

Description of haplotypes showing significant association with Gln/Glu in the POC (the full list and position of SNPs are shown in Fig 1)

Sliding Window		Haplotype	Study 1		Study 2	
First SNP	Last SNP		Frequency	p	Frequency	p
rs3771311	rs17748089	T-G-C-G	0.40	5×10^{-4}	0.45	0.13
rs6743496	rs13414554	G-C-G-G	0.40	5×10^{-4}	0.44	0.097
rs13000464	rs12185688	C-G-G-T	0.33	7×10^{-4}	0.35	0.035