REPORT

Schizophrenia and Oxidative Stress: Glutamate Cysteine Ligase Modifier as a Susceptibility Gene

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Oxidative stress could be involved in the pathophysiology of schizophrenia, a major psychiatric disorder. Glutathione (GSH), a redox regulator, is decreased in patients' cerebrospinal fluid and prefrontal cortex. The gene of the key GSH-synthesizing enzyme, glutamate cysteine ligase modifier (*GCLM*) subunit, is strongly associated with schizophrenia in two case-control studies and in one family study. *GCLM* gene expression is decreased in patients' fibroblasts. Thus, GSH metabolism dysfunction is proposed as one of the vulnerability factors for schizophrenia.

Schizophrenia (MIM 181500) is a major and frequent chronic psychiatric disorder with a strong genetic component.[1,2] Converging evidence points to the involvement of oxidative stress[3–7] and N-methyl D-aspartate (NMDA)–receptor hypofunction[8,9] in the pathophysiology of the disease. As a main cellular nonprotein antioxidant and redox regulator,[10] glutathione (GSH) plays a major role in protecting nervous tissue against reactive oxygen species[11] and in modulating redox-sensitive sites, including NMDA receptors (NMDA-R).[12,13] It was shown elsewhere that the GSH levels were decreased in patients' cerebrospinal fluid (−27%), in medial prefrontal cortex in vivo (−52%),[14,15] and in striatum postmortem tissue.[16] GSH-deficient models reveal morphological, electrophysiological, and behavioral anomalies similar to those observed in patients.[17–21] Here, we present strong evidence for an association between schizophrenia and the gene of the key GSH-synthesizing enzyme, glutamate cysteine ligase modifier (*GCLM*) subunit. The functional role of the *GCLM* gene variance in schizophrenia is supported by its low expression in patients' fibroblasts and by the decreased stimulation of the enzyme activity when challenged by an oxidative stress.[22] These findings are consistent with the concept that an abnormal GSH metabolism is a risk factor for schizophrenia.

To identify candidate gene(s) responsible for the low level of GSH observed in patients with schizophrenia, we studied steady-state levels of mRNA for 14 genes (data not shown) involved in GSH metabolism (fig. 1). Since GSH is ubiquitously present in cells, gene expression was studied in cultured skin fibroblasts. Two enzymes are responsible for GSH synthesis: glutamate cysteine ligase (GCL), also known as γ-glutamyl cysteine synthetase (Enzyme Commission number 6.3.2.2), and glutathione synthetase (GSS [Enzyme Commission number 6.3.2.3]).[10] GCL, the first and rate-limiting enzyme,[10] is composed of two subunits—GCL modifier (GCLM [light: 27.7 kDa])[23] and GCL catalytic subunit (GCLC [heavy: 73 kDa])[24]—each encoded by separate genes.[25] The specific mRNA steady-state levels were measured in fibroblasts obtained from 32 patients and 53 controls from a Swiss population (table 1). The subjects were recruited with fully informed written consent and guidelines for ethical treatment given by the University of Lausanne. All subjects were assessed using the Diagnostic Interview for Genetic Studies (DIGS) developed by the National Institute of Mental Health (NIMH).[27] Additional measures of psychopathology of patients included the Positive and Negative Syndrome Scale (PANSS), which assessed the presence of symptoms within the same week as the blood collection and skin biopsy. Specific mRNA steady-state levels were measured in cultured skin fibroblasts grown for three passages, with the use of TaqMan chemistry and ABI Prism 7000 sequence detection system. cDNA corresponding to 10 ng of reverse-transcribed total RNA was amplified using TaqMan gene expression assays (Hs00155249 m1, Hs00157694 m1, and Hs00609286 m1) at the following amplification condition: 1 cycle for 2 min at 50°C, 1 cycle for 10 min at 95°C, and 50 cycles for 15 s at 95°C, followed by 1 min at 60°C. Human glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystem 4333764F) was used as endogenous control.

Case-control comparisons showed significant differences in GCLM ($t = 1.989$; $P = .037$) and GSS ($t = 1.997$; $P = .030$) mRNA levels between patients and control subjects, without effect from sex or age. For the GCLC mRNA,

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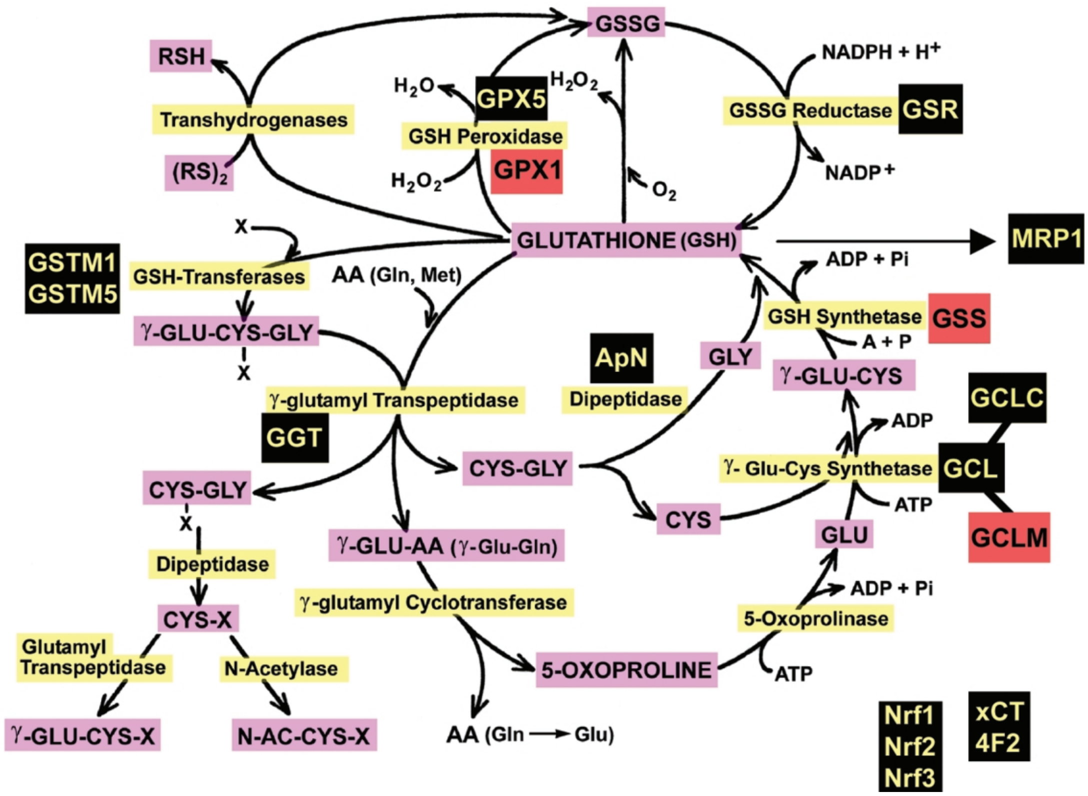


Figure 1. GSH metabolic pathway. The genes for which expression was studied in fibroblast cultures from skin biopsies are highlighted. Black indicates no difference between controls and patients, and red indicates lower expression in patients compared with controls. These genes code for the enzymes highlighted in yellow. Substrates and products are highlighted in purple. Genes not directly involved in GSH metabolism but included in the expression study: *Nrf1, Nrf2,* and *Nrf3* = NF-E2–related transcription factors 1, 2, and 3, respectively; *xCT* and *4F2* = genes encoding proteins involved in cystine/glutamate exchange; and *MRP1* = multidrug resistance protein 1. ADP = adenosine diphospate; GSSG = glutathione disulfide; RSH = reduced thiols; RS = disulfide; N-AC-CYS-X = N-acetyl-cysteine conjugate; Pi = inorganic phosphate.

a trend toward a decrease could be observed ($t = 2.000$; $P = .064$) in patients.

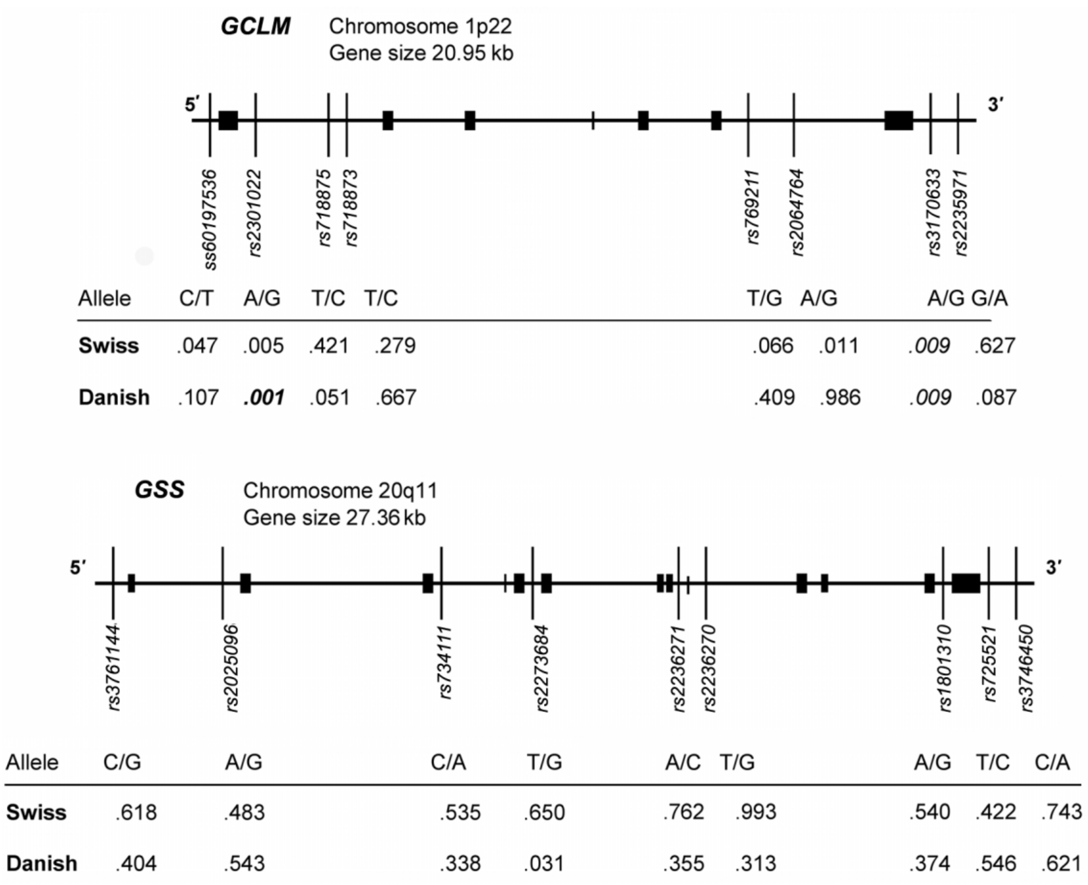
We tested, therefore, whether the reduced GCLM and GSS mRNA levels observed in patients with schizophrenia could be due to a primary defect. We studied eight SNPs in the *GCLM* gene and nine SNPs in the *GSS* gene for possible association with schizophrenia (fig. 2). Sixteen SNPs were chosen from a group of 60 SNPs selected from publicly available databases (SNP Consortium and dbSNP) on the basis of the estimated level of polymorphism in our population. One SNP (*ss60197536*) at the *GCLM* 5′ end, described by Nakamura et al.,[28] is being submitted to dbSNP. The dbSNP-annotated SNP numbers and their positions in each gene are shown in figure 2. Genotyping was performed with DNA extracted from peripheral blood by the use of either Sequenom technology[29] or sequencing. The list of specific primers is shown in table 2.

A pilot association study was performed with a relatively small sample (40 patients and 31 controls from a Swiss population) (fig. 2). Most, although not all, of the subjects from the Swiss population were used in both gene expression and association studies. The details about subject groups are given in the tables: demographic data in table 1, genotype and allele frequencies for each SNP in table 3, and the allelic frequencies compared with those known for other populations in table 4. All SNPs were in Hardy-

Table 1. Demographic Characteristics of the Samples Used in Gene Expression and Association Studies

Study and Population	N	Age[a] (years)	Sex[b]	Diagnostic Tool
Expression study:				
Switzerland:				
Patient	32	35.5 ± 10.5	3.0	DSM-IV[26]/DIGS[27]
Control	53	37.2 ± 13.4	.9	DIGS
Association study:				
Switzerland:				
Patient	40	35.9 ± 11.5	3.5	DSM-IV/DIGS
Control	31	37.7 ± 13.31	1.6	DIGS
Denmark:				
Patient	349	38.8 ± 12.1	1.5	ICD-10[c]
Control	348	40.2 ± 10.5	1.5	...

[a] Expressed as the mean and range.
[b] Ratio of males to females.
[c] International Classification of Diseases, 10th Revision.



	ss60197536	rs2301022	rs718875	rs718873	rs769211	rs2064764	rs3170633	rs2235971
Allele	C/T	A/G	T/C	T/C	T/G	A/G	A/G	G/A
Swiss	.047	.005	.421	.279	.066	.011	*.009*	.627
Danish	.107	***.001***	.051	.667	.409	.986	*.009*	.087

	rs3761144	rs2025096	rs734111	rs2273684	rs2236271	rs2236270	rs1801310	rs725521	rs3746450
Allele	C/G	A/G	C/A	T/G	A/C	T/G	A/G	T/C	C/A
Swiss	.618	.483	.535	.650	.762	.993	.540	.422	.743
Danish	.404	.543	.338	.031	.355	.313	.374	.546	.621

Figure 2. Map of *GCLM* and *GSS* genomic structures, with the positions of SNP markers and results of two case-control studies. Tables show *P* values for each SNP in case-control studies of subjects from two independent populations, from Switzerland and Denmark. Values in italics indicate a significant difference, and the value in bold italics indicates a significant difference after correction for multiple testing.

Weinberg equilibrium (HWE) in both subject groups (data not shown). No association was observed with the *GSS* gene. Two *GCLM* markers, *rs2301022* in intron 1 and *rs3170633* at the 3′ end, had *P* values that were low, but not significant after correction for multiple testing. However, these data suggested that a functional region in *GCLM* may be associated with schizophrenia. Consequently, we proceeded with a second case-control study of a larger sample from an independent population.

The second study included 349 patients with schizophrenia from the Danish Psychiatric Biobank and 348 unrelated anonymous blood donors serving as unaffected control subjects (table 1). Both control and affected groups were tested for HWE. Whereas the control group was in equilibrium for all markers, the SNP *rs2301022* showed a deviation from HWE ($P = .026$) in patients. This SNP also showed an association in our Swiss population. Genotype frequency analysis confirmed a strong association of SNP *rs2301022* ($\chi^2 = 13.2$, 2 df; $P = .023$) after correction for multiple testing (fig. 2). Thus, the deviation from HWE in patients but not in controls must be viewed as additional evidence of association. Here again, no association with *GSS* SNPs was found; thus, we excluded this gene from further studies.

Our results showed that *rs2301022* is strongly associated with schizophrenia. This marker is localized between markers *rs718875* and *ss60197536,* which showed weaker association with this disease in one of two populations that we studied (fig. 2). This fact suggests that there is a functional variant associated with the disease in the proximity of these three markers. SNP *ss60197536* is 343 bp upstream of the transcription initiation site, and *rs718875*

Table 2. Primers Used in Genotyping Studies

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 3. Genotype and Allele Frequencies for Each SNP Studied in Two Populations

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 4. Frequencies of the Rare Allele in Swiss and Danish Populations Compared with Reported Frequencies in Other White Populations

The table is available in its entirety in the online edition of *The American Journal of Human Genetics.*

is localized, like *rs2301022,* in intron 1. We analyzed different genotype pattern classes for these three markers (*ss60197536, rs2301022,* and *rs718875*) in a region of ~3,000 bp that includes *GCLM* exon 1. We identified 14 different genotype patterns (table 5), among which 2 were specifically present in affected individuals. Nine patterns of these three SNPs showed different frequencies between patients and unaffected controls, with $\chi^2 = 30.39$ and $P = .004$. Two particular combinations, TT/GG/TC and CC/GG/TT, had odds ratios (ORs) of 4.89 and 4.17, respectively.

At present, we cannot completely exclude the possibility of a second functionally associated region in the vicinity of SNP *rs3170633*. The association test for this SNP showed low *P* values in both populations ($P = .009$) (fig. 2). However, these values are not significant after correction for multiple testing.

Genotype frequency distribution in the Danish population suggests a dominant mode of transmission for the two SNPs of interest (*rs2301022* and *rs3170633*), with the G allele dominant in each of them (fig. 3). For *rs2301022,* genotypes AA, AG, and GG show respective ORs of 0.37, 1.22, and 1.12. Thus, we pooled AG and GG genotypes, since both are associated with disease, to approximately the same degree. The corresponding OR for disease then was 2.72 ($P = .0005$). For *rs3170633,* which showed weaker association, OR = 1.77 ($P = .002$).

Thus, case-control studies of two independent populations provided strong evidence of an association of the *GCLM* gene and schizophrenia. These data were supported with an additional linkage study of the families from the NIMH cohort, shown in table 6. Genotyping of 275 individuals from 72 families for seven SNPs in the *GCLM* gene showed supportive (although by no means significant) evidence of linkage between schizophrenia and two *GCLM* markers. The highest LOD score, 1.382 (corresponding to $P = .012$), was obtained under assumed dominant inheritance for *rs2064764,* which is located within 2 kb (~0.002 cM) of *rs3170633.*

Table 5. Diplotype Analysis of the SNP Patterns Associated with Schizophrenia

Genotype Pattern			Frequency		
rs718875	*rs2301022*	*ss60197536*	Patients	Controls	OR
CC	GG	TT	.035	.009	**4.17**
TC	GG	TT	.006	.014	.4
TT	GG	CC	.263	.282	.91
TT	GG	TC	.041	.009	**4.89**
TT	AG	TC	.05	.035	1.46
TT	AG	CC	.339	.3	1.2
TC	GG	TC	.137	.138	.99
TT	AA	CC	.044	.121	.33
TC	AG	TC	.067	.078	.85
Five rare patterns			.018	.014	1.22

NOTE.—$\chi^2 = 30.40$, 9 df; $P = .000375$. Cells highlighted in bold show significantly different values.

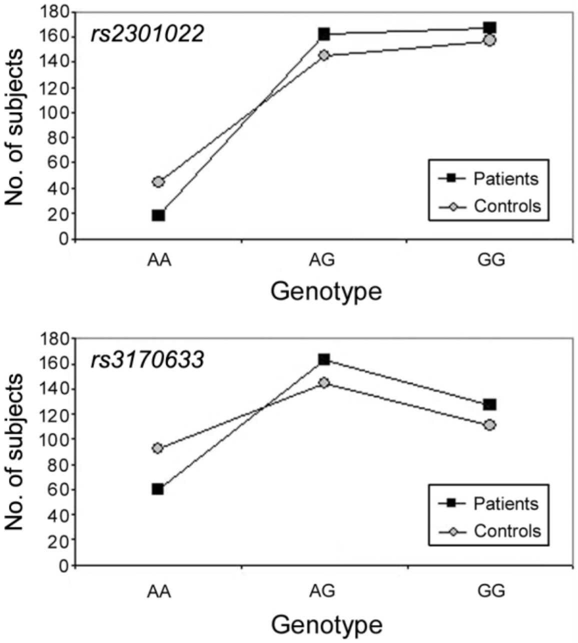


Figure 3. Genotype distribution of the two most common SNPs at the *GCLM* gene in the controls and patients sampled from a Danish population. The graph shows a dominance of the G allele for both markers.

To test whether there is association in the presence of linkage, we ran a family-based association statistic test (FBAT v. 1.7.2) in NIMH families (table 7). The results showed that there is significant evidence ($Z = 3.247$; $P = .0012$) to accept the alternative hypothesis of association in the presence of linkage between SNP marker *rs2301022* and the schizophrenia phenotype: allele G at the marker appeared as significantly overtransmitted to the affected offspring. Moreover, the *P* value remains significant, experiment-wise, after correction for multiple testing (table 7).

To define possible functional variants associated with the disease, we estimated pairwise linkage disequilibrium (LD) for all 17 SNPs of *GCLM* and *GSS* genes in 348 unaffected subjects from Denmark. The resulting LD map showed very strong association among all markers within each of the two genes (fig. 4). Thus, any association with this region might suggest the presence of a functional variant associated with schizophrenia.

On the basis of the strong association of two SNPs (*rs2301022* and *rs718875*) in the 5′ region of the *GCLM* gene and schizophrenia, we examined the relationship of these variants and the *GCLM* expression level in the Swiss

Table 6. Family Linkage Analysis of the *GCLM* Gene

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

population. The mRNA steady-state level in cultured fibroblasts showed a significant correlation with these two SNPs ($P = .040$). This result confirms the functional effect of the *GCLM* variants on the schizophrenia phenotype. However, it is still unknown in which way these variants affect the *GCLM* gene expression, since their localization is not directly related to any of the currently known regulatory sequences. It is worth noting that the *GCLM* gene is localized on chromosome 1p21, the region shown by previous linkage studies to be one of the several regions critical for schizophrenia.[30,31]

Several genes involved in GSH metabolism have already been considered as potential candidates for schizophrenia. Association of the glutathione-S-transferase M 1 gene was shown in a subgroup of patients with schizophrenia in Japanese[32] and Korean[33] populations. Case-control studies of glutathione-S-transferase P1 and glutathione peroxidase 1 showed no association.[34,35]

Although GCLM is not essential for survival, its interaction with the GCLC subunit increases, by four- to fivefold, the catalytic efficiency of the holoenzyme.[36] The GCLM knockout (KO) mice exhibit an increased sensitivity to oxidative stress[37]; mouse fetal fibroblasts are 10-fold more sensitive to an oxidative stress than are the wild type. Similarly, we observed lower GCL activity in patients' fibroblasts exposed to an oxidative stress, compared with that in control fibroblasts.[22] Thus, a GCLM defect could lead to GCL-activity dysregulation, consistent with the involvement of oxidative stress–induced impairment of neuronal processes and mitochondrial function[3–7] reported for schizophrenia.

GCLM KO mice showed an increased feedback inhibition of GCL activity, apparently resulting in brain GSH levels ~40% of normal.[36,37] This is strikingly similar to the levels 52% below normal reported for patients with schizophrenia.[15] Thus, GCLM KO mice can be used as a model for further studies of GSH deficit in schizophrenia.

A GCL dysregulation could lead to cellular alterations in the surroundings of dopaminergic terminals, affecting the synaptic contacts on dendritic spines of prefrontal cortical neurons that are particularly rich in dopamine innervations. Indeed, the metabolism of dopamine generates reactive oxygen species (e.g., hydrogen peroxide and quinones), which, in GSH-deficit conditions, are not adequately neutralized and, thus, induce cellular damage.[11] Interestingly, in rat models, GSH deficit and excess dopamine during development mimic structural and functional anomalies observed in patients: they exhibited a decrease in spine density of pyramidal neurons (F. Gheorghita, unpublished data) and, selectively, in GABA-parvalbumin immunoreactivity of prefrontal cortex,[21] similar to patients.[38,39] The same rat model presented an impairment in object recognition[18,19] and in integration of olfactory information,[40] reproducing some cognitive deficits of schizophrenia.

Table 7. Family-Based Association Test for SNP Markers Linked to *GCLM*

Marker	Allele	Allele Frequency	Z	P
rs2301022	**G**	.567	**3.247**	**.0012**
rs718875	T	.892	.202	.8399
rs718873	T	.862	.258	.7963
rs769211	T	.209	.784	.4332
rs2064764	A	.428	.935	.3496
rs3170633	A	.441	.577	.5637
rs2235971	A	.266	.832	.4054

NOTE.—Cells highlighted in bold show significantly different values.

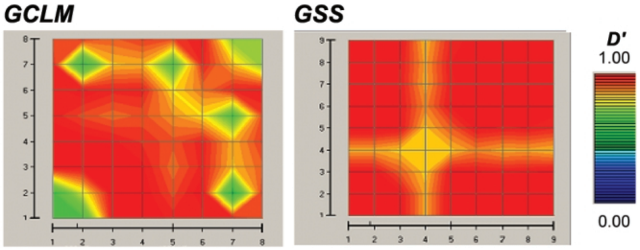


Figure 4. GOLD plot of pairwise LD for 17 SNPs that belong to genes *GCLM* and *GSS*. The 17 SNPs used in association and linkage studies and graphically presented in figure 2 are labeled on both axes. The color differences represent regions of the local map that are in disequilibrium and the magnitude of that disequilibrium. Regions of high and low LD are presented in red and blue, respectively. GOLD plot shows very strong LD within each gene.

Furthermore, NMDA-R hypofunction is implicated in schizophrenia, since the NMDA-R antagonist phencyclidine induces a psychotic syndrome.[41] In the case of GSH deficit, NMDA-R activity could be depressed through interaction at their redox sites.[12,13] Similarly, in rat hippocampal slices, GSH depletion impaired NMDA-dependent synaptic plasticity.[20]

In conclusion, these studies provide converging evidence of a link between schizophrenia and *GCLM* genetic variations, which affect the function of the encoded protein in its ability to promote GSH synthesis when challenged by an oxidative stress. They support the new concept that a dysregulation of GSH metabolism is one of the vulnerability factors contributing to the development of the disease.

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Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
International Classification of Diseases (ICD-10), http://www3.who.int/icd/currentversion/fr-icd.htm
Online Mendelian Inheritance in Man (OMIM) http://www.ncbi.nlm.nih.gov/Omim/ (for schizophrenia)
SNP Consortium, http://snp.cshl.org/

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