

Modification of Cognitive Performance in Schizophrenia by Complexin 2 Gene Polymorphisms

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Context: Schizophrenia is the collective term for a heterogeneous group of mental disorders with a still obscure biological basis. In particular, the specific contribution of risk or candidate gene variants to the complex schizophrenic phenotype is largely unknown.

Objective: To prepare the ground for a novel “phenomics” approach, a unique schizophrenia patient database was established by GRAS (Göttingen Research Association for Schizophrenia), designed to allow association of genetic information with quantifiable phenotypes. Because synaptic dysfunction plays a key role in schizophrenia, the complexin 2 gene (*CPLX2*) was examined in the first phenotype-based genetic association study (PGAS) of GRAS.

Design: Subsequent to a classic case-control approach, we analyzed the contribution of *CPLX2* polymorphisms to discrete cognitive domains within the schizophrenic population. To gain mechanistic insight into how certain *CPLX2* variants influence gene expression and function, peripheral blood mononuclear cells of patients, *Cplx*-null mutant mice, and transfected cells were investigated.

Setting: Coordinating research center (Max Planck Institute of Experimental Medicine) and 23 collaborating psychiatric centers all over Germany.

Participants: One thousand seventy-one patients with schizophrenia (*DSM-IV*) examined by an invariant in-

vestigator team, resulting in the GRAS database with more than 3000 phenotypic data points per patient, and 1079 healthy control subjects of comparable ethnicity.

Main Outcome Measure: Cognitive performance including executive functioning, reasoning, and verbal learning/memory.

Results: Six single-nucleotide polymorphisms, distributed over the whole *CPLX2* gene, were found to be highly associated with current cognition of schizophrenic subjects but only marginally with premorbid intelligence. Correspondingly, in *Cplx2*-null mutant mice, prominent cognitive loss of function was obtained only in combination with a minor brain lesion applied during puberty, modeling a clinically relevant environmental risk (“second hit”) for schizophrenia. In the human *CPLX2* gene, 1 of the identified 6 cognition-relevant single-nucleotide polymorphisms, rs3822674 in the 3′ untranslated region, was detected to influence microRNA-498 binding and gene expression. The same marker was associated with differential expression of *CPLX2* in peripheral blood mononuclear cells.

Conclusions: The PGAS allows identification of marker-associated clinical/biological traits. Current cognitive performance in schizophrenic patients is modified by *CPLX2* variants modulating posttranscriptional gene expression.

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DETAILED KNOWLEDGE OF the complex gene-environment interactions underlying the etiology and pathogenesis of the schizophrenias¹ is required to understand the causes of the disease and to establish causal therapies. However, the search for genetic markers of vulnerability for schizophrenia has been hampered by extensive interfering effects of patient heterogeneity and environmental fac-

tors.² Indeed, many of the numerous genetic association studies have yielded poorly reproducible data, likely because corresponding patient cohorts were too small and/or too heterogeneous with regard to clinical characteristics. One way to circumvent the problems that are posed by the lack of exact phenotyping in the available schizophrenia samples, and thus confound genetic studies, is to analyze large patient cohorts. Three recent studies of this type³⁻⁵ showed that several com-

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mon genetic variations contribute to the risk of schizophrenia, but the functional consequences of these variations are still completely unknown.

In the present study, we opted for an alternative approach to study genetic causes of the schizophrenic phenotype: a phenotype-based genetic association study (PGAS). This approach is different from and complementary to the genome-wide association studies on schizophrenia as a disease. With the PGAS, we are not looking for major disease genes in schizophrenia because such genes may not exist. Rather than searching for schizophrenia genes, we seek to learn more about the contribution of genetic variants of certain candidate genes to the schizophrenic phenotype. Obviously, traits of interest in schizophrenia can never be explained only by a single modifier gene. However, a particular gene may co-determine (with other trait-relevant genes) the outcome of an individual with schizophrenia.

Based on the assumption that valuable information about relevant genetic disease mechanisms can be obtained by association studies on patient cohorts of at least 1000 patients, if performed on very detailed clinical data sets and quantifiable biological readouts of schizophrenia rather than the end point diagnosis in comparison with healthy controls, we generated a new schizophrenia patient database, the GRAS (Göttingen Research Association for Schizophrenia) data collection. For this purpose, 1071 patients with schizophrenia were recruited between July 21, 2005, and July 7, 2008, by one and the same team of traveling investigators in a cross-sectional field study, consisting of 23 German psychiatric hospitals (listed in the supplementary Appendix; <http://www.archgenpsychiatry.com>). The corresponding data set includes biographical and family information, disease history, environmental risk factors, comorbidities, treatments, and the results of cross-sectional psychopathological, neuropsychological, and neurological examinations. With more than 3000 data points per subject, this unique database of living patients, who are accessible for follow-up studies, provides a comprehensive and standardized phenotype characterization of as yet unprecedented detail.

Neurocognitive impairments, including deficits in executive functions, attention, and memory, are core symptoms of schizophrenia and the main cause of disease-related disability.⁶ With regard to these and other schizophrenia symptoms, synaptic dysfunctions and disruptions of functional synaptic connectivity have been proposed to play a key role, and some authors even call schizophrenia a disease of the synapse.⁷⁻⁹ Numerous reports have implicated genes encoding synaptic proteins in the etiology of schizophrenia.^{10,11} Postmortem studies on the brain tissue of schizophrenic patients have consistently observed alterations in the expression of several synaptic proteins. In this context, the complexin (CPLX) family of presynaptic regulatory proteins is particularly interesting.¹²⁻¹⁸ This family consists of 4 members (CPLX1-CPLX4), of which only *Cplx1* and *Cplx2* are strongly expressed in rodent forebrain.¹⁹ They play an essential role in the regulation of synaptic transmitter release by controlling assembly and stability of exocytotic soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes and thereby

influence synaptic signaling,^{20,21} synaptic plasticity,^{22,23} and neuronal network function.²⁴

Whereas previous genetic association studies exploring *CPLX1* (OMIM 605032) or *CPLX2* (OMIM 605033) as classic genetic risk factors for schizophrenia have yielded inconclusive results,^{25,26} interesting findings were described regarding *CPLX1* and *CPLX2* messenger RNA (mRNA) and protein analysis in postmortem brain tissue. Although no systematic overall analysis of *CPLX1* and *CPLX2* expression patterns in normal human brain has been published, a number of reports are available on *CPLX* mRNA and protein changes in discrete brain areas of schizophrenic patients and control subjects, providing evidence of a decreased *CPLX2* mRNA expression in regions of high relevance for schizophrenia (eg, dorsolateral prefrontal cortex, superior temporal cortex, and certain regions of the hippocampus). A correspondence to decreased *CPLX2* protein levels in these regions is difficult to establish because the literature is scarce.²⁷

In addition to schizophrenia, altered *CPLX/CPLX* levels were measured in different brain areas of patients with diverse other neuropsychiatric disorders and implicated in the disease process, perhaps as determinants of a final common pathological pathway. Among these neuropsychiatric disorders are Huntington disease,^{28,29} Alzheimer disease,³⁰ and bipolar disorder,^{14,17} all of them characterized, among others, by cognitive impairments. Interestingly, *CPLX1* and *CPLX2* expression changes in the hippocampus were found to be associated with cognitive deficits in schizophrenia,³¹ still leaving unanswered whether the altered tissue levels reflect the cause or the consequence of disease-related cognitive dysfunction.

In this regard, behavioral consequences observed in null mutant mice have to be considered. Although *Cplx1*-null mutant mice show severe ataxia, they have no clear cognitive phenotype and only subtle alterations in social behavior.³² The *Cplx2*-null mutant mice have an even milder phenotype with slight motor abnormalities and equivocal results regarding cognition under unchallenged conditions.³³⁻³⁵ However, when maternal deprivation stress presents a second hit, cognitive dysfunction and decreased induction of hippocampal long-term potentiation in *Cplx2*-null mutant mice become evident.³⁴ A similar second hit effect may be involved in the disease-related function of another presynaptic protein, 25-kDa synaptosome-associated protein (SNAP-25).³⁶

Taking all these observations together, we hypothesized that common genetic variants of *CPLX2* may play an important role as modifiers of cognition in situations of additional challenge. Such a second hit to the brain, which ultimately leads to the disease, may cause a schizophrenic phenotype of variable severity depending on the genetic variant present. Searching for mechanistic insight, we hypothesized that the respective variants, if located in the noncoding region of the gene, may influence quantitative gene expression, for example, by modulating binding of microRNAs (miRs), which are increasingly recognized as significant contributors to the adaptive fine-tuning of synaptic functions in the brain.^{37,38} To lend further support to our second hit concept, we investigated the effect of a mild peripubertal neuro-trauma on cognition in *Cplx2*-null mutant mice.

A more comprehensive description of the patient population and all methods and materials applied is provided in the supplementary Methods section (see also eTable 1).

SCHIZOPHRENIC SUBJECTS

The GRAS data collection was approved by the Ethics Committee of the Georg-August-University (master committee) and the local internal review boards of the collaborating centers. The project complied with the Helsinki declaration.³⁹ Patients fulfilling DSM-IV criteria for schizophrenia^{40,41} (all types, eg, paranoid, disorganized, catatonic, and undifferentiated,⁴² proven or suspected) or schizoaffective disorder were included (N = 1071), regardless of the stage of the disorder (acute, chronic, residual, or remitted). A total of 792 patients (73.9%) were diagnosed as having schizophrenia; 159 (14.8%), schizoaffective disorder; and 120 (11.2%), suspected schizophrenic psychosis (other psychotic disorder or yet to be confirmed). The mean (SD) age was 39.6 (12.8) years, with a range from 18 to 83 years. Seven hundred fourteen (66.7%) were men (age, 37.6 [12.0] years) and 357 (33.3%) were women (age, 43.7 [13.3] years). Subjects (all older than 18 years) and, if applicable, their legal representatives, gave written informed consent.

Patients were recruited in the 23 German psychiatric hospitals listed in the supplementary Appendix, and almost all of them were of European Caucasian descent (Caucasian, 95.3%; other, 1.6%; unknown, 3.1%). European Caucasians are a genetically homogeneous group with low average levels of genetic differentiation when compared with other human populations (no strong influence on association results to be expected).⁴³⁻⁴⁵ Specifically, the German population is very homogeneous, with a low genetic differentiation along a north-south gradient within Germany. In fact, population substructure within Germany is too low to be detectable without prior information on subpopulation membership.⁴⁶

CONTROL SUBJECTS

The control subjects were voluntary blood donors recruited by the Department of Transfusion Medicine at the Georg-August-University according to national guidelines for blood donation. As such, they widely fulfill health criteria, ensured by a broad pre-donation screening process containing standardized questionnaires, interviews, hemoglobin levels, and blood pressure, pulse, and body temperature determinations. Of the 1079 controls, 635 (58.9%) were men and 444 (41.1%) were women. The mean (SD) age was 34.7 (12.3) years, with a range from 18 to 69 years. Comparable to the patient population, almost all controls were of European Caucasian descent (Caucasian, 97.8%; other, 2.0%; unknown, 0.2%). All donors gave written informed consent.

PHENOTYPING

Comprehensive interviews and testing were performed by the same traveling team of trained examiners (psychiatrists and psychologists) using the GRAS Manual described in the supplementary Methods. Briefly, structured interviews were conducted to explore biographical and family information, level of education, quality-of-life indicators, disease history, and exposure to prenatal, perinatal, and/or postnatal environmental risk factors. Likewise, the psychopathological profile, psychiatric comorbidities, and current/former treatments were assessed. Psychometric rating, neuropsychological tests, and neurological examinations were also performed.

GENOTYPING

Standard methods were used for DNA extraction from peripheral blood cells (Genomed GmbH, Löhne, Germany). Sequencing was performed using the dideoxy chain termination method (BigDye Terminator version 3.1 cycle sequencing kit on a 3730XL DNA analyzer; Applied Biosystems, Foster City, California). Genotyping was performed with simple probes (TIB Molbiol, Berlin, Germany) on a real-time polymerase chain reaction instrument (LightCycler 480; Roche Diagnostics GmbH, Mannheim, Germany).

EXPRESSION ANALYSIS

Expression analysis was conducted in Neuro-2a (N2a) cells (LGC Standards GmbH, Wesel, Germany) with the dual-luciferase reporter system (Promega, Mannheim, Germany). Briefly, the first 274 base pairs (bp) of the 3' untranslated region (3'UTR) of *CPLX2*, containing single-nucleotide polymorphism (SNP) rs3822674 with a C or a T allele, were amplified from respective human samples and cloned into Renilla luciferase vector phRL-SV40 (Promega). The N2a cells were plated in 96-well plates, cultured for 16 to 18 hours, and transfected using a commercially available reagent (Lipofectamine 2000; Invitrogen, Karlsruhe, Germany). A total of 1 ng of phRL-SV40 (T or C construct or the vector without the insert) and 1 pg of pCMV-FFluc control vector (Promega) were cotransfected with/without 1 pmol of hsa-miR-498 (Ambion, Foster City, California). The dual-luciferase reporter assay was performed 24 hours after transfection according to the manufacturer's protocol (Promega). Measurements were conducted with a commercially available microplate reader (Mitras LB 940; Berthold Technologies GmbH, Regensburg, Switzerland). Renilla values were divided by the corresponding firefly readings producing values expressed as relative luciferase units.

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Blood was collected in citrate phosphate dextrose adenine tubes from schizophrenic patients with different genotypes (CC, CT, and TT) at SNP rs3822674 in the 3'UTR. Six patients had the CC genotype (mean [SD] age, 48.5 [5.3] years); of these, 3 were men (age, 52.3 [4.7] years) and 3 were women; (age, 44.6 [10.1] years). Four had the CT genotype (age, 41.8 [3.4] years), and all 4 were men. Six had the TT genotype (age, 47.5 [5.8] years); of these, 5 were men (age, 45.6 [6.7] years) and 1 was a woman (age, 57 years). Peripheral blood mononuclear cells (PBMCs) were isolated applying a standard isolation procedure (Ficoll-Paque Plus; GE Healthcare, München, Germany). The RNA was prepared using a commercially available kit (miRNeasy Mini Kit; Qiagen GmbH, Hilden, Germany). The RNA samples were used to synthesize complementary DNAs (SuperScriptIII; Invitrogen). The quantitative real-time polymerase chain reaction analysis was performed using the fluorescent dye SYBR Green (LightCycler 480; Roche Diagnostics GmbH). The cycle threshold values were standardized to the cycle threshold values of glyceraldehyde-3-phosphate dehydrogenase. Primers are listed in the supplementary "Methods" section.

ANIMAL BEHAVIOR

Male *Cplx2*-null mutant mice vs wild-type littermates with or without juvenile parietal cortical cryolesion⁴⁷ underwent behavioral testing, including the Morris water maze, at 10 months of age.

Table. Neurocognitive Performance Associated With *CPLX2* SNPs^a

Phenotype	SNP										Permutation Test P Value (95% CI) ^e	
	rs6868608	rs2443541 ^b	rs2243404	rs4242187	rs10072860 ^b	rs4868539	rs1366116 ^b	rs3892909 ^b	rs3822674 ^{b,c}	rs56934064		+ 251 Ex ⁶
Target (Modifier) Variables												
Combined ^f F(P) values	0.310 (.733)	2.827 (.059)	3.705 (.025)	0.679 (.507)	2.254 (.105)	0.868 (.420)	4.288 (.014)	5.073 (.006)	3.288 (.037)	0.423 (.655)	0.517 (.596)	4 .020 (.017-.023)
Individual ^g Exec F(P) values	1.554 (.212)	4.238 (.015)	3.621 (.027)	2.000 (.136)	3.690 (.025)	0.673 (.510)	1.753 (.174)	1.808 (.165)	1.043 (.353)	0.459 (.632)	0.279 (.759)	
Reasoning F(P) values	0.670 (.512)	1.562 (.210)	1.722 (.179)	1.361 (.257)	2.567 (.077)	1.357 (.258)	5.327 (.005)	5.434 (.004)	4.409 (.012)	0.374 (.688)	0.366 (.694)	9 .012 (.010-.014)
Verbal L/M F(P) values	1.186 (.306)	3.321 (.037)	4.437 (.012)	0.520 (.594)	0.202 (.817)	0.310 (.733)	2.218 (.109)	3.239 (.040)	1.294 (.275)	1.012 (.364)	0.603 (.548)	
Constitutive Variable												
Premorbid intelligence F(P) values	1.500 (.224)	1.159 (.314)	2.185 (.113)	0.494 (.610)	2.107 (.122)	0.076 (.927)	2.309 (.100)	4.511 (.011)	0.754 (.471)	0.867 (.420)	0.481 (.618)	1 .357 (.348-.366)
Control Variable												
GAF F(P) values	0.442 (.643)	1.875 (.154)	1.579 (.207)	0.404 (.668)	0.131 (.877)	1.059 (.347)	1.413 (.244)	0.143 (.867)	1.068 (.344)	0.633 (.531)	0.803 (.448)	0 1.000 (1.000-1.000)

Abbreviations: CI, confidence interval; Exec, executive functioning; GAF, Global Assessment of Functioning Scale; L/M, learning/memory; SNP, single-nucleotide polymorphism.

^aSingle locus association analyses of phenotypes with *CPLX2*, adjusted for sex and age and, in the case of premorbid intelligence, additionally for nonnative German speakers with language problems (8.6% of the total sample; 1.9% had to be taken out completely owing to severe language difficulties [described in the supplementary "Methods" section]). Significant test statistics (*F* values, 2 *df*) and *P* values are displayed in boldface type (*P* < .05).

^bMarkers that underwent haplotypic analysis.

^cThe SNP in the 3' untranslated region, affecting the miR-498 binding site.

^dThe observed number of *P* values less than .05 on all tests for the multivariate phenotype, for the 3 univariate phenotypes, for the premorbid intelligence, as well as for the control variable (GAF).

^eMultiple testing adjusted *P* values (with 95% confidence interval to characterize estimation quality) were obtained by permutation test (described in the supplementary Methods section).

^fMultivariate model.

^gUnivariate phenotypes.

STATISTICAL ANALYSES

Statistical analyses were performed with commercially available software (GraphPad Prism, version 5.01; GraphPad Software Inc, La Jolla, California) for experimental data (animal study and expression experiments). For transfection studies and for PBMC analysis, 2-tailed pairwise Mann-Whitney test was applied; for mouse behavior studies, 2-way analysis of variance for repeated measures was used. For human data, haplotype association analyses of binary categorical variables was performed with UNPHASED (version 3.0.13; Frank Dudbridge, <http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/>), performing likelihood ratio tests in a log-linear model through unconditional logistic regression adjusting for age and sex.⁴⁸ Correlations of the 3 quantitative modifier variables (executive functioning, reasoning, and verbal learning/memory) and the quantitative constitutive variable (premorbid intelligence) were assessed using Pearson product-moment correlation,⁴⁹ determining internal consistency of the 3 target variables by the Cronbach α coefficient.⁵⁰ All metric phenotypic variables were standardized to be normally distributed with zero mean and variance 1 (by Blom Transformation⁵¹ [described in the supplementary Methods section]). Subsequently, linear models with covariate adjustment were used for single-marker association analyses (R, version 2.8.1; <http://cran.r-project.org>) and for haplotype association analyses of the standardized quantitative traits (SAS, version 9.1⁵²), with PLINK (version 1.06)⁵³ for estimation and tabulation of all possible individuals' haplotype phases and their posterior probabilities (given the genotype data). Multivariate analysis modeled a target phenotype vector,

accounting for individual correlation between vector entries. For single-marker association analyses, a permutation test was used to estimate multiple testing adjusted *P* values to obtain by chance no less than the observed amount of significances for a given set of tests (**Table**). All other *P* values are nominal, with *P* less than .05 indicating significance. Details for quantitative trait analyses and for the permutation test are given in the supplementary Methods section.

DATABASES

Information on *CPLX2* sequences was obtained from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and the University of California–Santa Cruz (<http://genome.ucsc.edu/>) (GeneID, 10814). Accession numbers for the 2 *CPLX2* transcripts are NM_006650.3 (T1) and NM_001008220.1 (T2).

RESULTS

CPLX2 GENOTYPING AND ASSOCIATION STUDIES

Case-Control Study

We analyzed first the genetic variability of the *CPLX2* gene⁵⁴ in 1071 schizophrenic patients of the GRAS sample vs 1079 healthy control subjects with comparable ethnicity. The *CPLX2* coding region (exons 4-6), adjacent introns, and

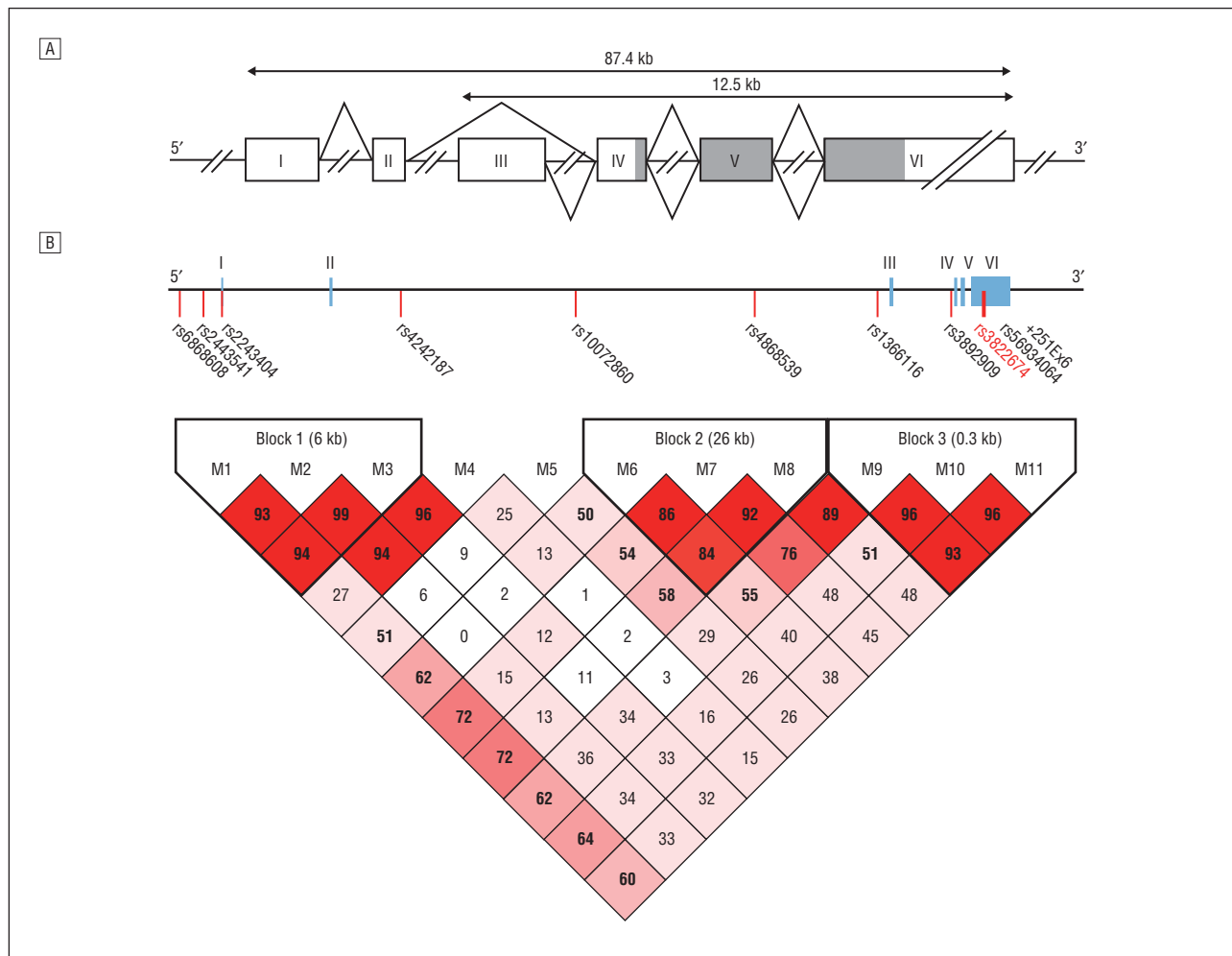


Figure 1. Genomic organization of the *CPLX2* gene. A, The *CPLX2* gene is located at 5q35.2 and spans 87.4 kilobases (kb). The 6 exons (boxes) encode 2 different splicing variants (indicated by triangles) leading to 1 protein (coding region is shaded). Introns are shown as interrupted solid lines; size of transcripts is indicated. B, Overview of the analyzed markers (Ms) in the *CPLX2* gene. The exons (blue boxes) are numbered and the single-nucleotide polymorphisms (SNPs) (red lines) are named (described in eTable 2). The SNP affecting the microRNA-498 binding site is labeled in red. Linkage disequilibrium (LD) analysis was performed in Haploview⁶⁵ (n=2027 cases and control subjects). The numbers in the squares are the measure of LD, D'. The darker the red is, the higher the linkage; all D values of 50 or higher are in boldface type. The gene shows 3 main blocks of high LD.

part of the 3'UTR were sequenced, revealing the presence of 4 SNPs in this region. The putative promoters, upstream of or within exons 1 and 3, and adjacent introns were analyzed by direct genotyping of 7 selected SNPs covering this region (**Figure 1** and eTable 2).

On sequencing of the coding region of *CPLX2*, no informative mutations were found in schizophrenic or healthy control subjects (eTable 3). A simple case-control association study, based on end point diagnosis and single markers, did not yield significant differences between cases and controls regarding genotypic or allelic frequencies (eTable 4). Three main haploblocks, closely resembling those described for this region by the HapMap Project (<http://www.hapmap.org>), were also identified in our population (Figure 1). Here, a haplotypic combination covering these 3 haploblocks and consisting of SNPs rs2443541/rs3892909/+251Ex6 appeared to be increased in cases (14.6%) vs controls (12.0%) (odds ratio [OR], 1.31; 95% confidence interval [CI], 0.98-1.76; $\chi^2_1=3.92$; $P=.048$). This result, however, is of borderline significance and will need further confirmation. In contrast, a low-frequency haplotype

(2.3% in cases vs 0.9% in controls) within haploblock 2, including SNPs rs1366116 and rs3892909, showed higher association with schizophrenia (OR, 2.47; 95% CI, 1.41-4.34; $\chi^2_1=11.15$; $P<.001$).

Phenotype-Based Genetic Association Study

In a first analysis of associations between genetic signatures and specific biological readouts within the GRAS group of schizophrenic patients, we focused on cognitive performance. Regarding the cognitive tests used, we followed our hypothesis of *CPLX2* influencing current higher brain functions but not premorbid intelligence. This hypothesis was mainly derived from our *Cplx2*-null mutant mouse study, exploring the effect of a second hit to the brain. Other neuropsychological tests performed in the frame of the GRAS project (which we looked at later for exploratory reasons) turned out to be less or not affected by *CPLX2* gene variants (data not shown).

To examine the role of *CPLX2* in cognition, we constructed a phenotypic intercorrelation network consist-

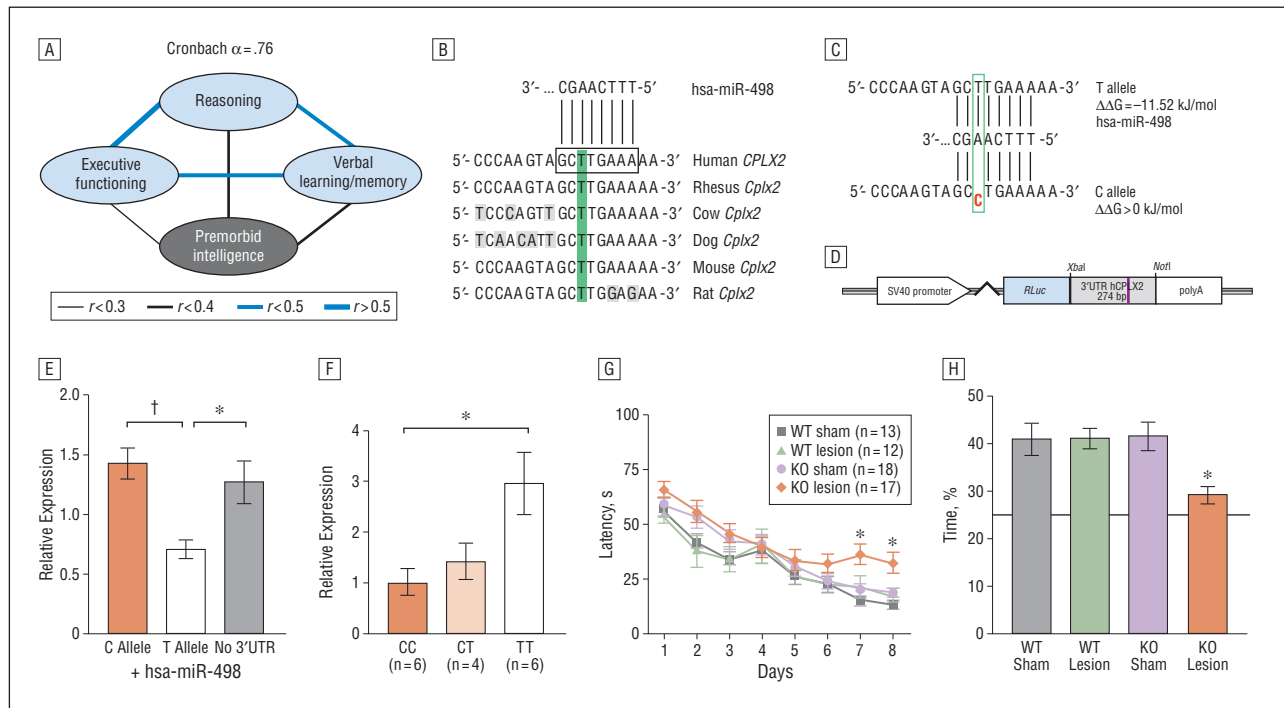


Figure 2. *CPLX2* in schizophrenia: genotype-phenotype interactions. A, Intercorrelation network of target (light blue) and constitutive (gray) variables to demonstrate phenotype coherence regarding higher cognitive functions. The Cronbach α of .76 shows a high internal consistency of the 3 target variables. Lines of different thickness and color indicate strengths of correlation between different tests. B, The single-nucleotide polymorphism (SNP) rs3822674 (green shading) lies in the seed region (black box) of hsa-microRNA [miR]-498. Mismatches are indicated with gray shading. C, $\Delta\Delta G$ values for the different alleles (green box) indicate the binding probabilities for hsa-miR-498 to the *CPLX2* 3' untranslated region (3' UTR). D, pHRL-*CPLX2*-3' UTR contains an SV40 promoter, the Renilla luciferase (*RLuc*) gene, the first 274 base pairs (bp) of the 3' UTR, and a polyA signal. The restriction sites are indicated and the pink line shows the predicted binding site for hsa-miR-498. E, pHRL-*CPLX2*-3' UTR (containing C or T allele) or pHRL-SV40 (without insert), internal control pCMV-FFLuc, and hsa-miR-498 were cotransfected into Neuro-2a (N2a) cells. Relative luciferase expression (normalized to the data without miR) is displayed for the different conditions. For 6 independent experiments, means were obtained of 6 replicates each. With pairwise Mann-Whitney 2-tailed test, $^*P = .015$ ($U_{n1=n2=6}=3$); $^\dagger P = .002$ ($U_{n1=n2=6}=0$). F, Spontaneous *CPLX2* messenger RNA expression in peripheral blood mononuclear cells is modulated by the genotype. With pairwise Mann-Whitney 2-tailed test, $^*P = .026$ ($U_{n1=n2=6}=4$). G and H, A "second hit" (cryolesion) is required to provoke a cognitive phenotype in *Cplx2*-null mutant mice in the Morris water maze test. G, Only *Cplx2*-null mutant mice with lesions show elevated escape latency in the hidden platform task (2-way analysis of variance [ANOVA] for repeated measures applied for the last 2 test days; significant effect of factor lesion in *Cplx2*-null mutant mice: $^*F_{1,33}=10.83$; $P = .002$). KO indicates knockout; WT, wild-type. H, Again, only *Cplx2*-null mutant mice with lesions show absence of preference for the target quadrant in the probe trial (2-way ANOVA; significant interaction effect between factors genotype and lesion: $^*F_{1,56}=4.82$; $P = .032$). In parts E through H, limit lines represent SEM.

ing of test results for executive functioning, reasoning, and verbal learning/memory as target (modifier) variables that are subject to potential influence of disease-modifying factors. Premorbid intelligence, representing the development dependent intellectual state at disease onset, was selected as the constitutive variable, expected to influence the cognitive phenotype and to correlate with the target variables but to be essentially independently regulated (**Figure 2A**). Determination of premorbid intelligence assists in estimation of the cognitive decline in brain diseases such as schizophrenia. The most frequently used tests (including the one applied herein, the Mehrfachwahl-Wortschatz Test B⁵⁶) measure vocabulary skills, which essentially depend on the level of education reached at the time point of disease onset.⁵⁷ This vocabulary knowledge, once acquired, tends to stay on (frozen) even if other cognitive skills decline owing to the disease process. As expected, there is a highly significant correlation between the number of years in school according to the final degree as a measure of level of education and the test results of the Mehrfachwahl-Wortschatz Test B (in the GRAS sample at present, $r=0.45$; $P < .001$).

The cognitive target variables together yielded a phenotypic intercorrelation network of high quality (Cron-

bach $\alpha = .76$), providing a solid basis for multivariate analyses. After correction for age and sex, 4 SNPs in the *CPLX2* gene (among them 2 markers belonging to the haplotypes of risk described in the case-control approach) correlated with overall neurocognitive function as evaluated by our multivariate model. Tests of univariate phenotypes—executive functioning, reasoning, and verbal learning/memory—showed this association even for 6 SNPs (Table). As expected, the constitutive variable premorbid intelligence was not significantly associated with single SNPs. The Table provides a synopsis of all tested SNPs of *CPLX2* for the multivariate model and the univariate phenotypes, as well as for the constitutive variable. Global functioning is presented as a disease-relevant control variable. Raw data and the influence of specific genotypes on cognition are shown in eFigure 1 and eTable 5.

Multilocus analysis, including all SNPs with any effects on cognition in individual neuropsychological tests (Table), showed that the combination of markers has a statistically significant effect on cognitive performance in the multivariate model and the univariate phenotypes (eTable 6). The haplotypic combination CTC at markers rs1366116/rs3892909/rs3822674 predicts the

worst cognitive outcome for all target phenotypes and even for the constitutive variable. In fact, the CTC haplotype shows significantly decreased cognitive performance compared with all other haplotypic combinations (eTable 7).

SEARCH FOR MECHANISMS OF SNP FUNCTION

Allele-Dependent Reporter Gene Expression

To gain a first insight into the mechanisms by which the identified SNPs might modulate certain domains of cognition, we performed an extensive analysis of the relevant genomic sequences (see the supplementary Methods section). One of the cognition-relevant SNPs that we identified (rs3822674) is located within a predicted binding site of the hsa-miR-498 in the 3'UTR of the *CPLX2* mRNA. This binding site is highly conserved among species (Figure 2B and C as well as eFigure 2 and eTable 7). Allele-dependent structural predictions and $\Delta\Delta G$ values indicated that the C-to-T exchange affects miR binding and might therefore modulate *CPLX2* expression. To test this, we cloned the first 274 bp of the 3'UTR of *CPLX2* downstream of a luciferase reporter gene (phRL-SV40; Figure 2D) and transfected this construct into N2a cells. On addition of hsa-miR-498, luciferase expression was significantly (>50%) reduced in the presence of the T allele ($U=0.00$; $P=.002$), whereas the presence of the C allele yielded an expression comparable to that of the control (vector lacking 3'UTR insert) (Figure 2E). These results suggest a role of SNP rs3822674 in posttranscriptional regulation of *CPLX2* expression that may become relevant on miR profile changes in specific neuronal subsets in response to, for example, brain injury.³⁸

Spontaneous Genotype-Dependent *CPLX2* mRNA Expression in PBMCs

To test the influence of genotype on baseline *CPLX2* mRNA levels, PBMCs from patients with the CC, CT, or TT genotype at SNP rs3822674 were analyzed. There was a significant genotype effect, with the TT genotype having the highest levels of the transcript (Figure 2F).

MODELING COGNITIVE EFFECTS OF ALTERED *Cplx2* EXPRESSION IN MICE

Based on the hypothesis that altered *CPLX2* expression influences cognitive performance, we investigated mice with a *Cplx2*-null mutation.⁵⁸ These mice develop essentially normally and lack major behavioral abnormalities. To test for a potential influence of a second hit to their brains, a right parietal cortical cryolesion was applied stereotactically at a vulnerable time (ie, puberty [day 28 of life]). This lesion paradigm had originally been developed to model the neurodegenerative processes of schizophrenia⁴⁷ spreading from the initiation site, the parietal lobe, to other cortical areas.⁵⁹ Specific deficits in spatial memory (Morris water maze, escape latency) became evident only in *Cplx2*-null mutant mice that had

received a peripubertal lesion ($F_{1,33}=10.83$; $P=.002$), but not in identically treated wild-type littermates ($F_{1,23}=2.27$; $P=.146$) (Figure 2G). Also, only lesioned *Cplx2*-null mutant mice showed absence of any preference for the target quadrant (Morris water maze, probe trial, $F_{1,56}=4.82$; $P=.032$) (Figure 2H). Hence, a cognition modifier role of *Cplx2* was revealed only upon combination of a *Cplx2*-null mutation with an environmental cofactor (ie, parietal cryolesion as a second hit), leading to a remarkable deterioration of cognitive performance.

COMMENT

Collectively, these data support a modifier role of *CPLX2* variants on cognitive performance in schizophrenia. Whereas our conventional case-control study revealed schizophrenia-at-risk haplotypes of the *CPLX2* gene, constituting several of the investigated markers, only the PGAS allowed the specific identification of 6 cognition-related SNPs. These SNPs in turn may become relevant mainly on additional (environmental) cofactors, that is, second hits. The effects of *CPLX2* genotypes on current cognition of schizophrenic patients observed herein do not exclude a comparable role in other neuropsychiatric disorders or even in healthy individuals in which, for instance, aging would be an inevitable second hit. In this regard, further extensive studies on different populations are required. Among the mechanisms mediating genotype-dependent *CPLX2* expression upon second hit may be the binding of hsa-miR-498 to SNP rs3822674 in the 3'UTR of the *CPLX2* mRNA that leads to its subsequent downregulation.

Measuring spontaneous *CPLX2* mRNA expression in PBMCs of schizophrenic patients reveals different levels dependent on the genotype, with the best cognitive performers (TT at SNP rs3822674) showing the highest expression. In contrast, the CC carriers have lower mRNA expression and reduced cognitive capabilities. Most important, however, CC and TT carriers are not different when compared with respect to their premorbid intelligence, reflecting cognitive abilities before they were "hit by the disease." Correspondingly, *Cplx2*-null mutant mice are severely cognitively impaired only after a second hit, delivered in the present study by mild neurotrauma (juvenile parietal cortical cryolesion) and in a prior report by maternal deprivation stress.³⁴ Intriguingly, only the T allele at SNP rs3822674 (resulting in higher baseline levels of *CPLX2* mRNA and better cognition) allows binding of hsa-miR-498 and thus regulability, that is, subsequent downregulation of *CPLX2* mRNA.

Current models of a dichotomous *Cplx* function assume that *Cplx*s have a facilitatory role in transmitter release, by stabilizing SNARE complexes in a highly fusogenic state, as well as an inhibitory role, by clamping SNARE complexes and thus preventing them from executing synaptic vesicle fusion until triggered by an action potential and the concomitant increase in the intrasynaptic calcium ion concentration. Indeed, calcium ion-regulated exocytosis in many different preparations is inhibited to similar degrees by increased or decreased *Cplx* activity.^{21,27}

Based on these facts and the cognitive data from our patients, we conclude that the regulability necessary for maintaining or adjusting the homeostasis of *CPLX2* expression may constitute a key factor in the fine tuning of synaptic function. This concept has similarly been suggested earlier on the basis of in vitro studies⁶⁰ and is in agreement with the notions that (1) loss as well as overexpression of *CPLXs/Cplx*s can perturb presynaptic secretory function, and that (2) *CPLXs* combine facilitatory and inhibitory functions with respect to synaptic secretion. This functional combination has been delineated in a recent study also demonstrating that in different *CPLXs* the balance between facilitatory and inhibitory activities may be different.⁶¹

Considering the large number of human postmortem studies describing abnormalities in the absolute amount or the ratios of the different *CPLX* proteins and *CPLX* mRNAs,^{13-18,31} these dichotomous *CPLX/Cplx* functions and their tight regulation under physiological conditions may be of major relevance for pathological cognition seen in several neuropsychiatric diseases. The mechanistic basis for the observed alterations in tissue concentration, however, is presently far from clear.

Analyses of mouse models failed to provide evidence of simple compensatory changes in *Cplx* expression in the corresponding *Cplx1*-, *Cplx2*-, *Cplx3*-, or *Cplx4*-single-null mutant mice.^{58,62,63} Expression levels of all other presynaptic proteins tested so far were also found to be unaltered in *Cplx1*-null mutant brains (α -SNAP, Munc13-1, Munc13-2, Munc13-3, Munc18-1, N-ethylmaleimide-sensitive factor, SNAP-25, synapsin I/IIa, synapsin IIb, synaptobrevin 2, synaptophysin, synaptotagmin 1, syntaxin 1, and vesicular γ -aminobutyric acid transporter), in *Cplx2*-null mutant brains (α -SNAP, Munc13-1, Munc13-2, Munc13-3, Munc18-1, SNAP-25, synapsin I/IIa, synapsin IIb, synaptobrevin 2, synaptophysin, synaptotagmin 1, and syntaxin 1), and in *Cplx1/2*-double-null mutant brains (SNAP-25, synaptobrevin 2, synaptotagmin 1, syntaxin 1, and vesicular γ -aminobutyric acid transporter).⁵⁸ In *Cplx3/4*-double-null mutant retina, only Ribeye expression was reduced among 17 presynaptic proteins tested.⁶² Based on these findings, we would argue that even loss of *CPLX2/Cplx2* is unlikely to be compensated for by robust changes in the expression levels of other related presynaptic proteins. Functionally relevant alterations, however, may be subtle and escape detection by the available methods. Also, given that pathological states were not explored and that not all of the many dozens of proteins were tested that might act in a compensatory manner on loss of *Cplx2*, the possibility remains that such compensatory changes occur. However, it is almost impossible to investigate this systematically with the currently available technology and tools/reagents.

In addition to SNP rs3822674, 5 other SNPs were also associated with cognitive performance of schizophrenic patients in the present study. The mechanisms underlying the impact of these other intronic genetic variants on cognition are still unclear, but several possibilities may be considered. (1) Because of the linkage disequilibrium between these genetic markers, the

influence of the 3'UTR regulatory mechanism mediated by SNP rs3822674 may be detected by several other markers along the gene. (2) Effects of the intronic variants, largely independent of the rs3822674 3'UTR mechanism, cannot be excluded at this point, for example, on transcription factor binding sites or other regulatory elements in the gene affecting expression level or splicing of transcripts.

To summarize, we propose that neurons in brain regions that control cognitive abilities require—depending on the situation—an exact control of *CPLX2* expression. Subtle disturbances in the optimal amount of *CPLX2/Cplx2* levels would therefore influence cognitive functions. Our data indicate (1) that this control is, at least in part, mediated by the binding of hsa-miR-498 to the 3'UTR of the *CPLX2* mRNA, and (2) that a polymorphism in the binding region of hsa-miR-498 (SNP rs3822674) influences *CPLX2* gene expression and thus modulates cognitive performance. Together with other genetic and environmental mechanisms that affect cognition, this genetic variant may be an important code-determinant of cognitive outcome in schizophrenia.

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Online-Only Material: The supplementary Appendix, Methods section, eTables, and eFigures are available at <http://www.archgenpsychiatry.com>.

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Correction

Errors in Text. In the Original Article titled “Modification of Cognitive Performance in Schizophrenia by Complexin 2 Gene Polymorphisms” by Begemann et al, published in the September issue of the *Archives* (2010; 67[9]:879-888), 3 errors occurred in the text. On page 879, in the “Objective” section of the “Abstract,” the last sentence should have read, “Because synaptic dysfunction plays a key role in schizophrenia, the complexin 2 gene (*CPLX2*) was examined in the first phenotype-based genetic association study (PGAS) of GRAS.” On page 880, the first sentence of the first full paragraph should have read, “In the present study, we opted for an alternative approach to study genetic causes of the schizophrenic phenotype: a phenotype-based genetic association study (PGAS).” On page 883, the subheading in the right column should have read, “Phenotype-Based Genetic Association Study.”