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Journal

Schizophrenia Research, 170(1)

ISSN

0920-9964

Authors

Greenwood, Tiffany A
Lazzeroni, Laura C
Calkins, Monica E
et al.

Publication Date

2016

DOI

10.1016/j.schres.2015.11.008

Peer reviewed

HHS Public Access

Author manuscript

Schizophr Res. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Schizophr Res. 2016 January ; 170(1): 30–40. doi:10.1016/j.schres.2015.11.008.

Genetic Assessment of Additional Endophenotypes from the Consortium on the Genetics of Schizophrenia Family Study

Tiffany A. Greenwood, Ph.D.[1,*], **Laura C. Lazzeroni, Ph.D.**[2], **Monica E. Calkins, Ph.D.**[3], **Robert Freedman, M.D.**[4], **Michael F. Green, Ph.D.**[5,6], **Raquel E. Gur, M.D., Ph.D.**[3], **Ruben C. Gur, Ph.D.**[3], **Gregory A. Light, Ph.D.**[1,7], **Keith H. Nuechterlein, Ph.D.**[5], **Ann Olincy, M.D.**[4], **Allen D. Radant, M.D.**[8,9], **Larry J. Seidman, Ph.D.**[10,11], **Larry J. Siever, M.D.**[12,13], **Jeremy M. Silverman, Ph.D.**[12,13], **William S. Stone, Ph.D.**[10,11], **Catherine A. Sugar, Ph.D.**[14], **Neal R. Swerdlow, M.D., Ph.D.**[1], **Debby W. Tsuang, M.D.**[8,9], **Ming T. Tsuang, M.D., Ph.D., D.Sc.**[1,15,16], **Bruce I. Turetsky, M.D.**[3], and **David L. Braff, M.D.**[1,7]

[1]Department of Psychiatry, University of California San Diego, La Jolla, CA

[2]Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA

[3]Department of Psychiatry, University of Pennsylvania, Philadelphia, PA

[4]Department of Psychiatry, University of Colorado Health Sciences Center, Denver, CO

[5]Department of Psychiatry and Biobehavioral Sciences, University of California Los Angeles, Los Angeles, CA

[6]VA Greater Los Angeles Healthcare System, Los Angeles, CA

[7]VISN-22 Mental Illness, Research, Education and Clinical Center (MIRECC), VA San Diego Healthcare System

[8]Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA

[9]VA Puget Sound Health Care System, Seattle, WA

Address correspondence to: Tiffany A. Greenwood, Ph.D.; University of California San Diego, Department of Psychiatry; 9500 Gilman Drive, MC 0689; La Jolla, CA 92093; tgreenwood@ucsd.edu.

Conflict of Interest

Drs. Braff, Calkins, Greenwood, RE Gur, RC Gur, Olincy, Radant, Seidman, Siever, Silverman, Stone, Sugar, DW Tsuang, MT Tsuang, and Turetsky report no financial relationships with commercial interests. Dr. Freedman has a patent through the Department of Veterans Affairs on DNA sequences in CHRNA7. Dr. Green has been a consultant to AbbVie, Biogen, DSP, EnVivo/Forum, and Roche; he is on the scientific advisory board of Mnemosyne; and he has received research funds from Amgen. Dr. Lazzeroni is an inventor on a patent application filed by Stanford University on genetic polymorphisms associated with depression. Dr. Light has served as a consultant for Astellas, Forum, and Neuroverse. Dr. Nuechterlein has received unrelated research support from Janssen Scientific Affairs, Genentech, and Brain Plasticity, Inc., and has consulted to Genentech, Otsuka, Janssen, and Brain Plasticity, Inc. Dr. Swerdlow has been a consultant for Genco Sciences, Ltd.

Contributors

Dr. Greenwood performed the discriminability, heritability, association, and linkage analyses and drafted and critically revised the manuscript. Dr. Lazzeroni developed the bootstrap Total Significance Test and performed the analyses using this test. All authors participated in aspects of study design, data validation, and interpretation. All authors provided valuable edits to the text and approved the final manuscript.

[10]Department of Psychiatry, Harvard Medical School, Boston, MA

[11]Massachusetts Mental Health Center Public Psychiatry Division of the Beth Israel Deaconess Medical Center, Boston, MA

[12]Department of Psychiatry, The Mount Sinai School of Medicine, New York, NY

[13]James J. Peters VA Medical Center, New York, NY

[14]Department of Biostatistics, University of California Los Angeles School of Public Health, Los Angeles, CA

[15]Center for Behavioral Genomics, and Institute for Genomic Medicine, University of California San Diego, La Jolla, CA

[16]Harvard Institute of Psychiatric Epidemiology and Genetics, Boston, MA

Abstract

The Consortium on the Genetics of Schizophrenia Family Study (COGS-1) has previously reported our efforts to characterize the genetic architecture of 12 primary endophenotypes for schizophrenia. We now report the characterization of 13 additional measures derived from the same endophenotype test paradigms in the COGS-1 families. Nine of the measures were found to discriminate between schizophrenia patients and controls, were significantly heritable (31 to 62%), and were sufficiently independent of previously assessed endophenotypes, demonstrating utility as additional endophenotypes. Genotyping via a custom array of 1536 SNPs from 94 candidate genes identified associations for *CTNNA2, ERBB4, GRID1, GRID2, GRIK3, GRIK4, GRIN2B, NOS1AP, NRG1*, and *RELN* across multiple endophenotypes. An experiment-wide p value of 0.003 suggested that the associations across all SNPs and endophenotypes collectively exceeded chance. Linkage analyses performed using a genome-wide SNP array further identified significant or suggestive linkage for six of the candidate endophenotypes, with several genes of interest located beneath the linkage peaks (e.g., *CSMD1, DISC1, DLGAP2, GRIK2, GRIN3A*, and *SLC6A3*). While the partial convergence of the association and linkage likely reflects differences in density of gene coverage provided by the distinct genotyping platforms, it is also likely an indication of the differential contribution of rare and common variants for some genes and methodological differences in detection ability. Still, many of the genes implicated by COGS through endophenotypes have been identified by independent studies of common, rare, and *de novo* variation in schizophrenia, all converging on a functional genetic network related to glutamatergic neurotransmission that warrants further investigation.

Keywords

endophenotype; genetics; schizophrenia; association; linkage; heritability

1. Introduction

Schizophrenia is a severe psychotic disorder with a lifetime prevalence of approximately 1% and an estimated heritability of 60–80% (Karayiorgou and Gogos, 1997; Sullivan, 2005; Wray and Gottesman, 2012). The genetic heterogeneity and polygenicity associated with

schizophrenia are substantial and have hindered many attempts to confirm initial candidate gene associations and to replicate linkage regions across studies (Baron, 2001; Gogos and Gerber, 2006; Harrison and Weinberger, 2005; Lewis et al., 2003; Owen et al., 2004). Increasingly large genome-wide association studies (GWAS) have begun to provide insight into common genetic variants associated with schizophrenia risk, yet the neurobiological significance of these variants remains largely unexplored (O'Donovan et al., 2008; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Shi et al., 2009). While most common and rare variants confer small increases in risk for schizophrenia, it is likely that risk variants will cluster within a limited number of pathways (Purcell et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Schizophrenia is a profoundly clinically heterogeneous disorder with patients exhibiting a broad range of neurobiological deficits and symptom severity, which has further complicated efforts to identify genetic risk variants. Recent studies have demonstrated that employing more specific phenotype definitions in genetic studies of complex diseases, including schizophrenia, is even more important than large sample sizes for detecting true genetic associations (Liang and Greenwood, 2015; Manchia et al., 2013). The use of endophenotypes as objective measurements related to specific neurobiological functions may be particularly useful in reducing the heterogeneity associated with the considerably more subjective diagnosis, facilitating the detection of risk variants and aberrant molecular pathways (Braff et al., 2007; Gottesman and Gould, 2003; Insel and Cuthbert, 2009). Many endophenotypes are also amenable to human neuroimaging and translational animal model studies, allowing for direct evaluations of neural circuit dysfunctions and neurobiological substrates (Swerdlow et al., 2008; Young et al., 2013).

The Consortium on the Genetics of Schizophrenia Family Study (COGS-1) previously reported significant heritability for 12 endophenotypes for schizophrenia, with candidate gene association and genome-wide linkage analyses that demonstrate their utility for resolving the genetic architecture of schizophrenia (Greenwood et al., 2007; Greenwood et al., 2011; Greenwood et al., 2013c). Other analyses of the COGS-1 sample suggested additional measures for several endophenotype domains that may provide complementary information (Horan et al., 2008; Olincy et al., 2010; Stone et al., 2011; Swerdlow et al., 2007; Turetsky et al., 2008), yet these measures have remained uncharacterized for their genetic contributions in this sample. We now report the significant heritability of nine new candidate endophenotypes derived from the same original endophenotype test paradigms that provide complementary information. These measures include pulse-alone startle magnitude, P50 conditioning amplitude, N100 conditioning amplitude, Degraded-Stimulus Continuous Performance Test (DS-CPT) hit rate, CPT Identical Pairs (CPT-IP) 3-digit d', Letter-Number Span (LNS) forward, California Verbal Learning Test, Second Edition, (CVLT-II) list B and delayed recall, and Logical Memory Stories total recall. For these measures, we also evaluated association using the COGS SNP Chip, a custom array that incorporates common variants in genes involved in pathways hypothesized to underlie schizophrenia risk, and linkage using a genome-wide SNP linkage panel to assess the joint impact of rare and common variation on the candidate endophenotypes.

2. Methods

Ascertainment, genotyping, and analysis methods are provided in brief below with full methods available in the Supplement and elsewhere (Calkins et al., 2007; Greenwood et al., 2011; Greenwood et al., 2013c).

2.1 Subjects

Families were ascertained at seven sites through probands who met DSM-IV-TR criteria for schizophrenia (American Psychiatric Association, 2000). Each family minimally consisted of a proband with schizophrenia, an unaffected sibling, and both parents. Unrelated community comparison subjects without personal or family history of psychosis were also recruited. Only those without history of any Axis I or Cluster A personality disorder were considered as controls here. All subjects underwent a standardized clinical assessment using the Diagnostic Interview for Genetic Studies (DIGS) (Nurnberger et al., 1994). Details of the ascertainment, diagnostic, and screening procedures are provided elsewhere (Calkins et al., 2007). Written informed consent was obtained for each subject per local IRB protocols. The final COGS-1 dataset of 296 families consisted of 1,364 subjects, 1,004 of whom were characterized for the endophenotype paradigms. While most families (62%) consisted of the minimum discordant sibling pair and both parents, the remaining 38% represented larger families. The majority of subjects (89%) were confirmed to be of European ancestry.

2.2 Neurophysiological and Neurocognitive Measures

Detailed descriptions of the rationale and assessment procedures for all COGS-1 test paradigms and the heritability assessments of the 12 primary endophenotypes have been published (Greenwood et al., 2007; Gur et al., 2007; Turetsky et al., 2007). The two neurophysiological and three neurocognitive test paradigms administered yielded various quantitative measures in addition to the primary endophenotypes, from which 13 measures were selected for further validation as candidate endophenotypes as described below. These measures had previously shown promise as endophenotypes in COGS-1, and most have also demonstrated good test-retest reliability in an independent sample (Light et al., 2012).

While prepulse inhibition of startle at 60 msec was our primary endophenotype, we assessed pulse-alone startle magnitude on non-prepulse trials and both the difference and percent startle habituation from the first to final block of testing as additional measures (Swerdlow et al., 2007). The primary endophenotype of P50 suppression was the difference in amplitudes of the event-related potentials generated in response to the conditioning (S1) and test stimuli, and the S1 amplitude was considered an additional measure (Olincy et al., 2010). N100 amplitude was also derived from the P50 paradigm and measured as the minimum trough occurring 75–125 ms post-stimulus. Only the N100 conditioning (C1) amplitude was considered based on initial investigations in a subset of this sample (Turetsky et al., 2008).

We used two forms of the CPT to measure sustained, focused attention, one with a high perceptual load (DS-CPT) (Nuechterlein et al., 1983) and one with a working-memory load (CPT-IP) (Cornblatt et al., 1988). For the DS-CPT, the primary endophenotype was a signal/

noise discrimination index (d') derived from correct target detections (hit rate) and incorrect responses to nontargets, and hit rate was considered an additional measure. For the CPT-IP, 3-digit d' was considered an additional measure. The LNS was used to assess working memory with the primary endophenotype considered as the correct reordering of intermixed numbers and letters and a simple repetition in the order dictated (forward) considered as an additional measure (Horan et al., 2008). We used the CVLT-II to assess verbal learning and memory (Stone et al., 2011), and considered the immediate recall of items from list A summed over 5 trials (list A total score) as the primary endophenotype. Additional measures included list B immediate recall, the free recall of list A after a 20-minute delay, and recall of list A items via sematic and serial clustering. The Logical Memory Test from the Wechsler Memory Scale was added midway through the COGS-1 study as an verbal learning and memory task, and total story recall was considered an additional measure (Wechsler, 1997).

2.3 Genotyping

A subset of 534 subjects from 130 families was previously genotyped for the COGS SNP Chip, which contains 1,536 SNPs within 94 candidate genes for schizophrenia and is described in detail elsewhere (Greenwood et al., 2011). The final set of 1,380 SNPs had an average gene-centric physical spacing of 10kb with variance due to linkage disequilibrium. The complete sample of 296 COGS-1 families were genotyped in two phases for the Illumina Infinium HumanLinkage-12 and −24 panels and underwent an extensive quality control process. The final 6,023 SNPs had an average physical spacing of 512 kb and an average genetic spacing of 0.65 cM.

2.4 Statistical Analyses

Assessments of mean differences between schizophrenia probands and controls used covariate adjusted residuals for age at interview, sex, and site of ascertainment as required based on endophenotype correlations and verified by the heritability analyses. Effect sizes were calculated using Cohen's *d* (i.e., standard deviation units).

Association analyses were conducted using the variance components method in MERLIN v. 1.1.2 with adjustment for age, sex, and ancestry, consistent with previous methods (Abecasis et al., 2002; Greenwood et al., 2011). Data were available on average for 395±53 subjects across the measures. Stories recall could not be evaluated for association because it was added midway through the study, and data was only available for 98 genotyped subjects. The effective number of independent SNPs tested was determined to be 977, with a corresponding Bonferroni correction for multiple comparisons of $p=5\times10^{-5}$ for a given endophenotype (Nyholt, 2004). A similar Bonferroni correction for multiple phenotypes would be overly conservative, given the observed between-endophenotype correlations. We therefore implemented the bootstrap Total Significance Test to evaluate whether the observed associations for all SNPs and endophenotypes combined significantly exceeded what would be expected by chance, given the 11,040 total tests (1,380 SNPs and 8 candidate endophenotypes). The resultant p-value was designed to collectively evaluate the strongest results in the data and provide an *a posteriori* predictive value for each genotype-endophenotype association. The Total Significance Test conditions simultaneously on all

observed correlations among endophenotypes; among SNPs; and among related individuals within each family to correct for multiple testing using a null-resampling form of the bootstrap, (Greenwood et al., 2011). This method has been demonstrated to appropriately control type I error, while reducing type II error (Hall and Wilson, 1991; Martin, 2007).

The heritability and linkage analyses were conducted according to previously established methods (Greenwood et al., 2013c). Briefly, heritability estimates, genetic correlations, and two-point log of the odds ratio (LOD) scores were calculated for each candidate endophenotype using the variance components method in SOLAR v.4.3.1 (Almasy and Blangero, 1998; Almasy et al., 1997). Multipoint LOD scores were computed using both variance components and pedigree-wide regression methods in SOLAR and MERLIN, as each has favorable properties (Almasy and Blangero, 1998; Schork and Greenwood, 2004; Sham et al., 2002). Empirical p values were estimated from 10,000 replicates (Blangero et al., 2000). All analyses used normalized trait values, an ascertainment correction (see Supplement), and covariate adjustment for age, sex, and/or site as appropriate. Only regions of convergent linkage between the two methods were considered, where at least one met standard criteria for significant or suggestive linkage (LOD >3.6 or 2.2, respectively) (Lander and Kruglyak, 1995) and the other either produced a LOD ≥1.0 within 5cM or a significantly overlapping 1-LOD interval.

3. Results

3.1 Discriminability, Heritability, and Genetic Relationships of the Additional Measures

Table 1 displays the means and standard deviations for each additional measure in the schizophrenia probands and control subjects. Large effect sizes (>0.8) were observed for CPT-IP-3d and all verbal learning measures, with LNS-fwd displaying a medium effect size. Significant heritability estimates were observed for all 13 additional measures, with most in the moderate to substantial range (25–62%). Since many measures are derived from the same test paradigm, we used a combination of strength of the heritability estimate and effect size to reduce the number of candidate endophenotypes for further study. Four measures were thus eliminated for poor discriminability (Hab-diff and CVLT-serial) and/or low heritability (Hab-pct and CVLT-semantic). Startle displayed a minimal effect size (0.16) but had highest heritability (62%) of all additional measures and was thus retained for further evaluation.

Table 2 shows the observed genetic correlations between the selected nine candidate endophenotypes and their primary counterparts. Startle was not significantly correlated with PPI, nor was N100-C1 correlated with the P50 difference score, so these candidate endophenotypes represent independent measures. P50-S1 and DS-CPT-hr were highly correlated with P50 difference and DS-CPT d', respectively, which is expected as these candidate endophenotypes are used to calculate their primary endophenotype counterparts and therefore not independent measures, although they may capture novel information. However, CPT-IP-3d was not significantly correlated with either measure from the DS-CPT, validating that the CPT-IP is an independent measure of attention. The two LNS endophenotypes were highly correlated, and the CVLT total score was correlated with all three candidate verbal learning endophenotypes, which were also significantly correlated.

3.2 Candidate Gene Association Analyses

The COGS SNP Chip provides excellent coverage of most pre-GWAS schizophrenia candidate genes and many genes from putatively important pathways (Greenwood et al., 2011). Analysis of the candidate endophenotypes collectively revealed associations to 40 of the 94 genes with a cluster in the glutamate pathway, one of seven biological pathways specifically targeted by the custom array (see Figure S1). Figure 1 provides a gene-wise association summary and highlights associations across multiple domains (see Table S1 for individual SNP p values). Ten genes displayed extensive evidence for pleiotropy with associations to three or more candidate endophenotypes, including *ERBB4*, *NRG1*, *RELN*, and several genes related to glutamate signaling.

The most significant finding was for rs4646316 in *COMT* with CPT-IP-3d, which gave a p value of 4.6×10^{-5} and explained 4.7% of the variation. An additional 20 SNPs had p values <0.001, and 124 SNPs had p values <0.01. Association was observed to three nonsynonymous SNPs: *GRM1* Gly884Glu with CVLT-delay (p=0.003, 2.6% of the variation), *NRG1* Arg38Gln with CVLT-delay and CVLT-B ($p=9.1\times10^{-4}$ and 0.004, respectively; 3.2% and 2.4% of the variation, respectively), and *TAAR6* Val265Ile with DS-CPT-hr ($p=3.6\times10^{-4}$, 3.7% of the variation). Given the prior associations of the *GRM1* and *NRG1* variants with CVLT total score and the *TAAR6* variant with DS-CPT d', these associations likely reflect a portion of the shared genetic component between the primary and candidate endophenotypes (Greenwood et al., 2011). Of the 40 genes on the COGS SNP Chip with prior evidence of association with schizophrenia, 17 were associated with at least one of the candidate endophenotypes: *COMT*, *DAOA*, *DGCR2*, *DISC1*, *DRD3*, *DTNBP1*, *ERBB4*, *GRID1*, *GRIK3*, *GRIK4*, *GRIN2B*, *GRM4*, *NRG1*, *PRODH*, *SLC1A2*, *SP4*, *TAAR6*, and *ZDHHC8*, including five SNPs with prior association to schizophrenia (Fallin et al., 2005; Funke et al., 2004; Liu et al., 2006; Mukai et al., 2004; Shifman et al., 2006; Stefanis et al., 2007).

The collective results across all SNPs and candidate endophenotypes were highly significant according to the bootstrap Total Significance Test analysis. After controlling for linkage disequilibrium patterns, phenotypic correlations, family structure, gene size, and multiple testing of both SNPs and endophenotypes, an experiment-wide omnibus p value of 0.003 was obtained. Furthermore, 247 SNP-endophenotype associations involving 59 genes and eight candidate endophenotypes were strong enough to satisfy the omnibus 0.05 significance level (see Table S2). These results demonstrate that the findings in Figure 1 exceed what would be expected by chance alone.

3.3 Genome-wide SNP Linkage Analyses

As shown in Figure 2 and summarized in Table 3, the linkage analyses collectively identified 12 regions of convergent linkage between the two methods (see complete results in Table S3). Note that all linkage peaks identified for the candidate endophenotypes represent novel findings within COGS-1 that were not identified by the primary endophenotypes, with the exception of 5p15 observed for PPI and Stories-recall (see Table S4).

Significant evidence for linkage was observed for CVLT-B on 9q34, with several neuronally expressed genes located in this gene-dense region. *DBH* and *GRIN1* are excellent functional candidates that have shown association with neurophysiological or neurocognitive endophenotypes in our prior studies of two independent samples (Greenwood et al., 2011; Greenwood et al., 2012). Although *DBH* was only nominally associated ($p<0.05$) with CVLT-B in this study, this association did involve four SNPs (see Table S2). *NTNG2* promotes neurite outgrowth and provides an interesting alternative, as does *CACNA1B*, given the implication of calcium channels in psychosis (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Highly suggestive linkage to 9q22–31 was observed for CPT-IP-3d. Several interesting genes are located beneath this peak, including *NTRK2* and *CTNNAL1*, a paralog of *CTNNA2*, which revealed associations with CPT-IP-3d and several endophenotypes in our prior studies of two independent samples. Finally, *GRIN3A* is located beneath this peak and provides an excellent candidate gene, given the associations with several endophenotypes for schizophrenia across studies (Greenwood et al., 2011; Ohi et al., 2015), although it was only nominally associated with CPT-IP-3d in this study.

Suggestive evidence for linkage under both models was observed for four regions. *CNIH3* and *DISC1* are located beneath the peak on 1q41-42 for N100-C1. *DISC1* is of obvious relevance, given its history as a candidate gene for schizophrenia, and SNPs in this gene were associated with several endophenotypes in our prior studies of two independent samples. While *DISC1* was only nominally associated with N100-C1 in this study, the association signal derived from five SNPs. *CNIH3* is an auxiliary receptor subunit that regulates the trafficking and gating of AMPA-selective glutamate receptors, with upregulated expression in schizophrenia patients (Drummond et al., 2012). Interestingly, *CNIH3* is found in a complex with *CACNG2*, which is associated with several neurocognitive endophenotypes (Greenwood et al., 2012). Although the region on 5p15 with linkage to Stories-recall is very gene-dense, the dopamine transporter (*SLC6A3*) lies closest to the peak and has shown evidence of association and linkage with PPI and startle habituation (Greenwood et al., 2012; Greenwood et al., 2013c), schizophrenia (Stober et al., 2006), bipolar disorder (Greenwood et al., 2001; Greenwood et al., 2006), and several neurocognitive endophenotypes (Greenwood et al., 2011). Unfortunately, we were unable to evaluate Stories-recall for association with the custom array. *ADORA2A* and *ADRBK2* lie closest to the peak on 22q11-12, but neither was associated with any endophenotype in this study, nor in our previous assessments, suggesting that either rare variants in these genes or other genes in the region are contributing to the linkage signal. *PREP* and *GRIK2* are located beneath the peak on 6q21-22 observed for both CVLT-B and DS-CPT-hr. *PREP* encodes prolyl endopeptidase, a serine proteinase with lower activity in patients with major depression and increased activity in patients with mania and schizophrenia (Maes et al., 1995). Interestingly, CPT-IP-3d produced suggestive linkage under the regression model to 6q16-21 with *GRIK2* as the nearest gene of interest. While *GRIK2* was not evaluated for association, functionally related genes *GRIK3* and *GRIK4* were associated with DS-CPT-hr, CPT-IP-3d, and CVLT-B. Furthermore, *GRIK2* interacts with both *DLG4* (Garcia et al., 1998; Mehta et al., 2001), which was associated with DS-CPT-hr, and *GRID2* (Kohda et al., 2003), which was associated with CVLT-B and CPT-IP-3d.

Four other regions yielded suggestive linkage under one model with modest support from the other. *DLGAP2* and *CSMD1* are located beneath the 8p23 peak for LNS-fwd. *DLGAP2* may play a role in synapse organization and signaling in neuronal cells and interacts with *DLG4*, which was associated with LNS-fwd. *DGKH* and *HTR2A* are located beneath the 13q13 peak for DS-CPT-hr. *HTR2A* was associated with several neurocognitive endophenotypes (Greenwood et al., 2011).

4. Discussion

Investigations of endophenotypes that quantitatively measure crucial neurobiological processes that are deficient in schizophrenia may facilitate the identification of genes contributing to risk for the disorder. We have further validated nine of the 13 additional measures that were assessed, demonstrating behavioral deficits in schizophrenia patients versus controls and significant heritability. The heritability estimates for these additional schizophrenia endophenotypes range from moderate to substantial (25–62%), consistent with our previous reports of heritability for the primary endophenotypes for COGS-1 and with the heritability of schizophrenia itself in this cohort (Light et al., 2014). The additional endophenotypes also produced independent genetic signals in both the association and linkage analyses (see Figure S2 and Table S4), confirming their utility to further explore the genomic influences on the aberrant neurobiology of schizophrenia by providing complementary information.

We expected that some genes would contribute to the variance in multiple endophenotypes, particularly those that are genetically correlated. Additionally, some genes, like *NRG1*, are involved in neurodevelopment and may impact more than one domain. Eight genes displayed pleiotropic associations in both the primary and additional endophenotype analyses and were also pleiotropic in our independent case-control study of many of the same endophenotypes: *CTNNA2*, *ERBB4*, *GRID2*, *GRIK3*, *GRIK4*, *NOS1AP*, *NRG1*, *and RELN* (Greenwood et al., 2011; Greenwood et al., 2012). The consistent observation of pleiotropic associations across multiple endophenotypes in two independent samples suggests a role for these genes in schizophrenia risk.

The linkage analyses identified 12 regions of genome-wide significant or suggestive linkage, with candidate genes *DBH*, *DISC1*, *GRIN1*, *GRIN3A*, *HTR2A*, and *SLC6A3* located beneath the linkage peaks, all of which displayed also pleiotropic associations across the COGS-1 primary endophenotypes (Greenwood et al., 2011). Other genes beneath the peaks, including *CNIH3*, *CSMD1*, *DGKH*, *DLGAP2*, *GRIK2*, *NTNG2*, and *NTRK2*, have been implicated in schizophrenia or bipolar disorder (Aoki-Suzuki et al., 2005; Baum et al., 2008; Drummond et al., 2012; Greenwood et al., 2013a; Greenwood et al., 2013b; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Smith et al., 2009). Several of these linkage regions have repeatedly been implicated in schizophrenia, including 1q42, 6q21-22, and 22q11-12 (Blackwood et al., 2001; Cao et al., 1997; Coon et al., 1994; DeLisi et al., 2002; Ekelund et al., 2000; Gill et al., 1996; Hamshere et al., 2005; Levinson et al., 2000; Lewis et al., 2003; Martinez et al., 1999; Millar et al., 2000).

Most of the genes displaying pleiotropic associations across endophenotype domains are involved either directly or indirectly in glutamate signaling, and several of the genes identified through linkage also relate to glutamate signaling. Figure 3 details the molecular interactions of a subset of the genes present on the custom array, as well as those implicated by linkage, revealing a functional network of genes related to glutamate and neuregulin signaling. The association results from the primary COGS-1 endophenotypes (Greenwood et al., 2011) and those of our independent case-control sample (Greenwood et al., 2012) provide additional support for this gene network. Recent studies of both common and rare variants in schizophrenia have also implicated genes involved in glutamatergic neurotransmission and synaptic plasticity (Kirov et al., 2012; Ohi et al., 2015; Purcell et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Walsh et al., 2008), most of which converge on the functional gene network in Figure 3. For example, common variants in genes involved in glutamatergic signaling were implicated both by a recent large GWAS of schizophrenia conducted by the Psychiatric Genomics Consortium and a GWAS of cognitive endophenotypes for schizophrenia, with specific associations to *ATXN7*, *CSMD1*, *CHRNA4*, *CHRNA3*, *GRIN2A*, *GRIN3A*, and *GRM3* (Ohi et al., 2015; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Other studies have shown a significant burden of rare variants in *DGKH*, *GRIN1*, *GRIN3A*, and *SLC1A2* in schizophrenia patients (Fiorentino et al., 2014; Purcell et al., 2014), a disproportionate disruption of genes in the neuregulin and glutamate pathways in schizophrenia (Walsh et al., 2008), and *de novo* variants in *CSMD1*, *CTNNA2*, *DBH*, *DISC1*, *DLGAP2*, *GRID2*, *GRIN2B*, *HTR2A*, *RELN*, and *SLC6A3* in schizophrenia and related disorders (de Ligt et al., 2012; Fromer et al., 2014; Guilmatre et al., 2009; Iossifov et al., 2012; Li et al., 2014; Neale et al., 2012; O'Roak et al., 2012; Rauch et al., 2012; Sanders et al., 2012). These studies of rare and *de novo* variation thus provide independent evidence in support of many of the same genes identified by COGS-1 using common variants and endophenotypes. Collectively, these results support a strong role for genes involved in glutamate signaling in mediating schizophrenia susceptibility, consistent with the glutamate hypothesis (Coyle, 2006; Sodhi et al., 2008). Combined with a growing body of literature, the repeated associations of *NRG1* and *ERBB4* with multiple endophenotypes suggest the importance of neuregulin-mediated ErbB4 signaling in the pathophysiology of schizophrenia (Corvin et al., 2004; Hall et al., 2006; Silberberg et al., 2006; Stefansson et al., 2002; Williams et al., 2003).

The partial convergence between genes implicated by association and linkage likely reflects a number of factors. First, the gene coverage provided by the two platforms differed notably, with an average gene-centric density of 10kb for the custom array versus an average of 500kb for the linkage array. Thus, the linkage array generally did not provide adequate coverage of the candidate genes. Additionally, the regions implicated by linkage are very large, and the true signal may derive from another gene in the region, despite our efforts to prioritize genes of interest based on two-point linkage results and prior evidence for involvement in schizophrenia. This is a common problem in the interpretation of linkage data and can be resolved through the use of a higher density genome-wide array. Alternatively, the divergence for some genes may be an indication of the differential contributions of rare and common variants in different families or differences in the ability

of association and linkage methodologies to detect such variants. One would expect to find both rare and common variants in genes and pathways impacting SZ risk, and we indeed find evidence for this here with linkage and association results converging on the same functional network, a finding that is supported by independent studies of common, rare, and de novo variation in schizophrenia.

There are a number of applicable caveats. First, the two COGS-1 ascertainment requirements of siblings discordant for schizophrenia, which was intended to increase variation in the endophenotypes, and intact families of willing participants may have produced a sample with less genetic loading for pathological endophenotype values, resulting in an underestimation of heritability. Second, while we used the Total Significance Test to provide a robust correction for multiple comparisons in the association analyses, similar corrections for linkage are less straightforward and are complicated by the phenotypic correlations. Third, these studies suggest additional endophenotypes for schizophrenia that will require validation in other samples. Finally, our sample of nuclear families lacks sufficient power to reliably detect loci with smaller effects, independent of heritability. Still, we identified several genes and genomic regions related to these new endophenotypes, many of which have been previously implicated in studies of common and rare variation in schizophrenia and thus warrant further investigation.

Our data thus provide significant evidence of discriminability and heritability for nine novel neurophysiological and neurocognitive endophenotypes for schizophrenia. Using these additional endophenotypes, we demonstrated association and linkage with many functionally relevant genes. The degree of genetic heterogeneity associated with schizophrenia is substantial, with contributions of common, rare, and *de novo* variants, as well as epigenetic and environmental factors. However, results across many studies are beginning to converge on genetic pathways and associated neural circuits leading to the dysfunction associated with illness. This endophenotype strategy can thus lead to a better understanding of the underlying causes of schizophrenia and ultimately to optimal treatment strategies by placing genomic variation in a neurobiologically relevant context (Braff, 2015; Glahn et al., 2014; Insel and Cuthbert, 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors wish to thank all of the participants and support staff that made this study possible. This study was supported by grants R01-MH065571, R01-MH065588, R01-MH065562, R01-MH065707, R01-MH065554, R01-MH065578, R01-MH065558, R01 MH86135, and K01-MH087889 (TAG) from the National Institute of Mental Health. Genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the National Institutes of Health to The Johns Hopkins University, Contract Number HHSN268200782096C. COGS website: http://www.npistat.com/cogs/.

Role of Funding Source: Other than proving support, the NIH had no further role in this manuscript.

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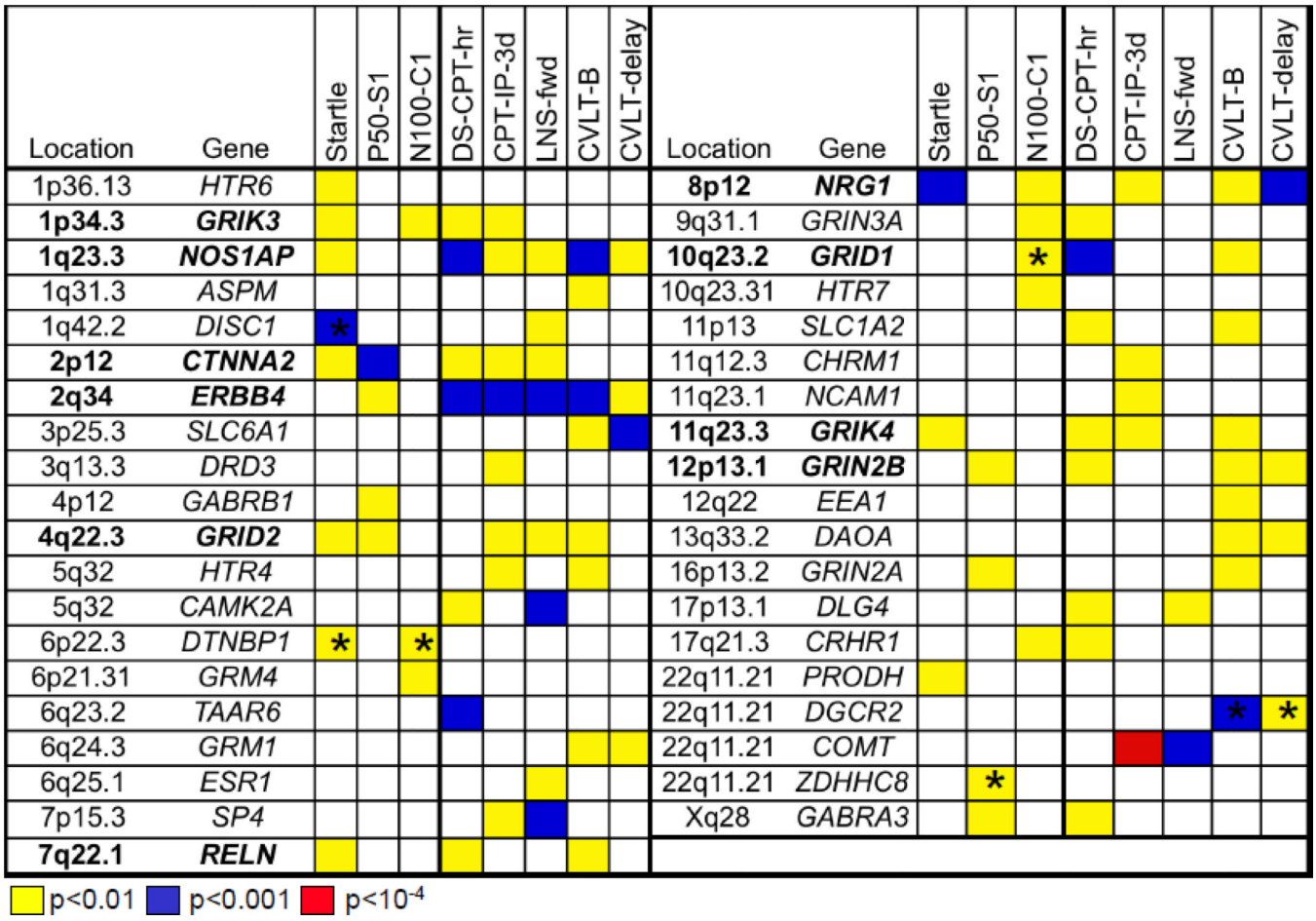

Figure 1.

Summary of the candidate gene association results in the 130 families. The most significant p-value observed for each of the 39 genes with each of the eight candidate endophenotypes is shown using a minimum p-value of <0.01 as a threshold. Note that not all associations to the same gene across endophenotypes reflect associations to the same SNP, although many do. Genes associated with three or more endophenotypes are indicated in bold. An asterisk (*) indicates that at least one SNP in the gene associated with the specified phenotype has been previously associated with schizophrenia as follows: rs807759 in *DGCR2* (Shifman et al., 2006), rs2793092 in *DISC1* (Liu et al., 2006), rs1018381 in *DTNBP1* (Funke et al., 2004; Stefanis et al., 2007), rs2814351 in *GRID1* (Fallin et al., 2005), and rs175174 in *ZDHHC8* (Mukai et al., 2004). The two SNPs in *DTNBP1* are >100kb apart and represent two independent associations with startle but not N100-C1 where only rs1040410 is associated. Note that Stories-recall could not be evaluated for association with the custom array because data for this endophenotype was only present in 98 subjects from 29 of the 130 genotyped families.

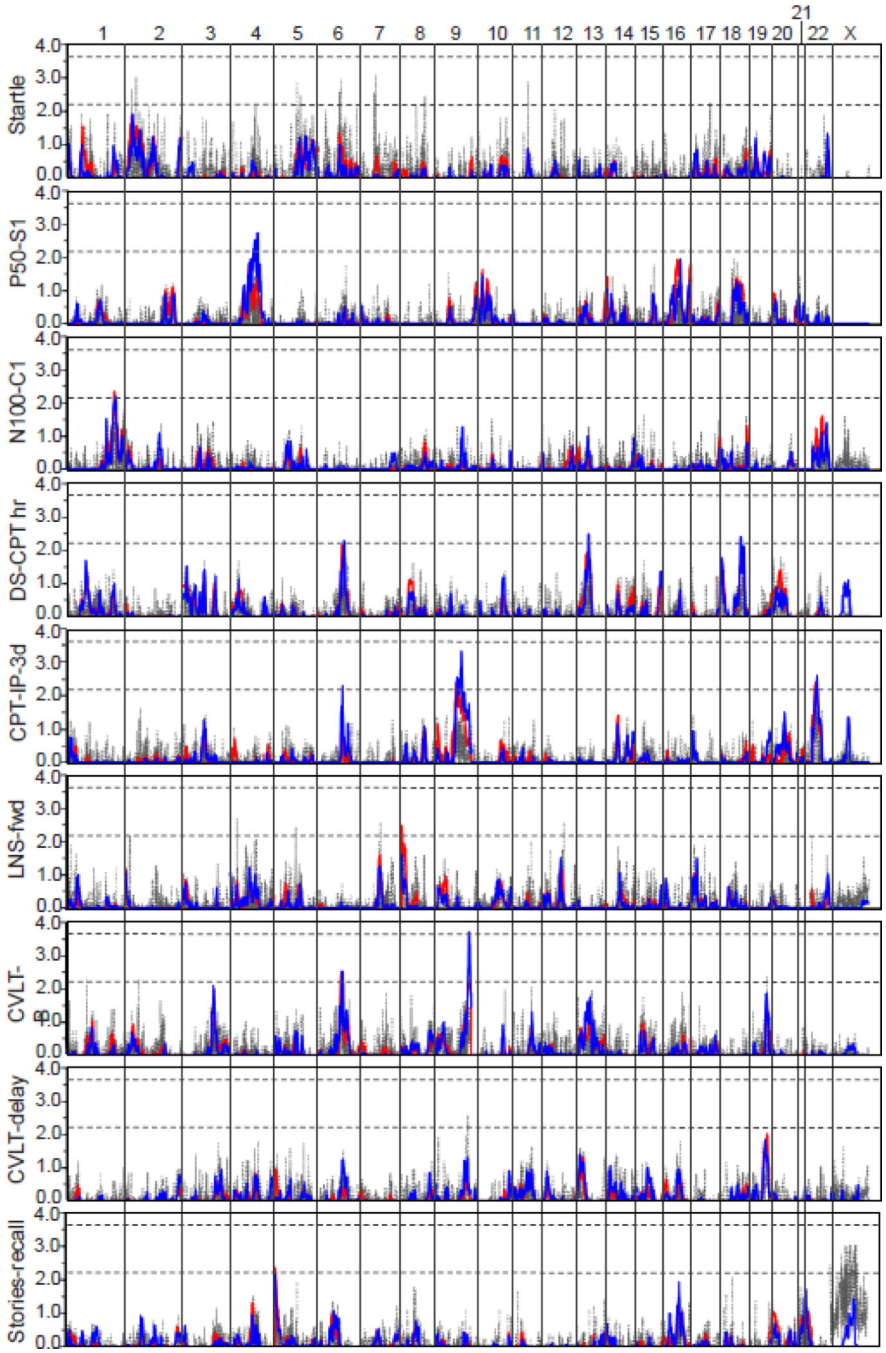

Figure 2.

Results of the genome-wide SNP linkage scan in the 296 families for each of the 9 candidate endophenotypes. The variance components multipoint results are shown in red, the pedigree-wide regression multipoint results are shown in blue, and the variance components two-point results are shown in grey. LOD scores are indicated on the *y*-axis, along with the name of the corresponding endophenotype. Chromosomes are aligned along the *x*-axis end to end with the *p*-terminus on the left and locations indicated at the top of the figure. Dashed

horizontal lines indicate genome-wide significant and suggestive LOD scores of 3.6 and 2.2, respectively.

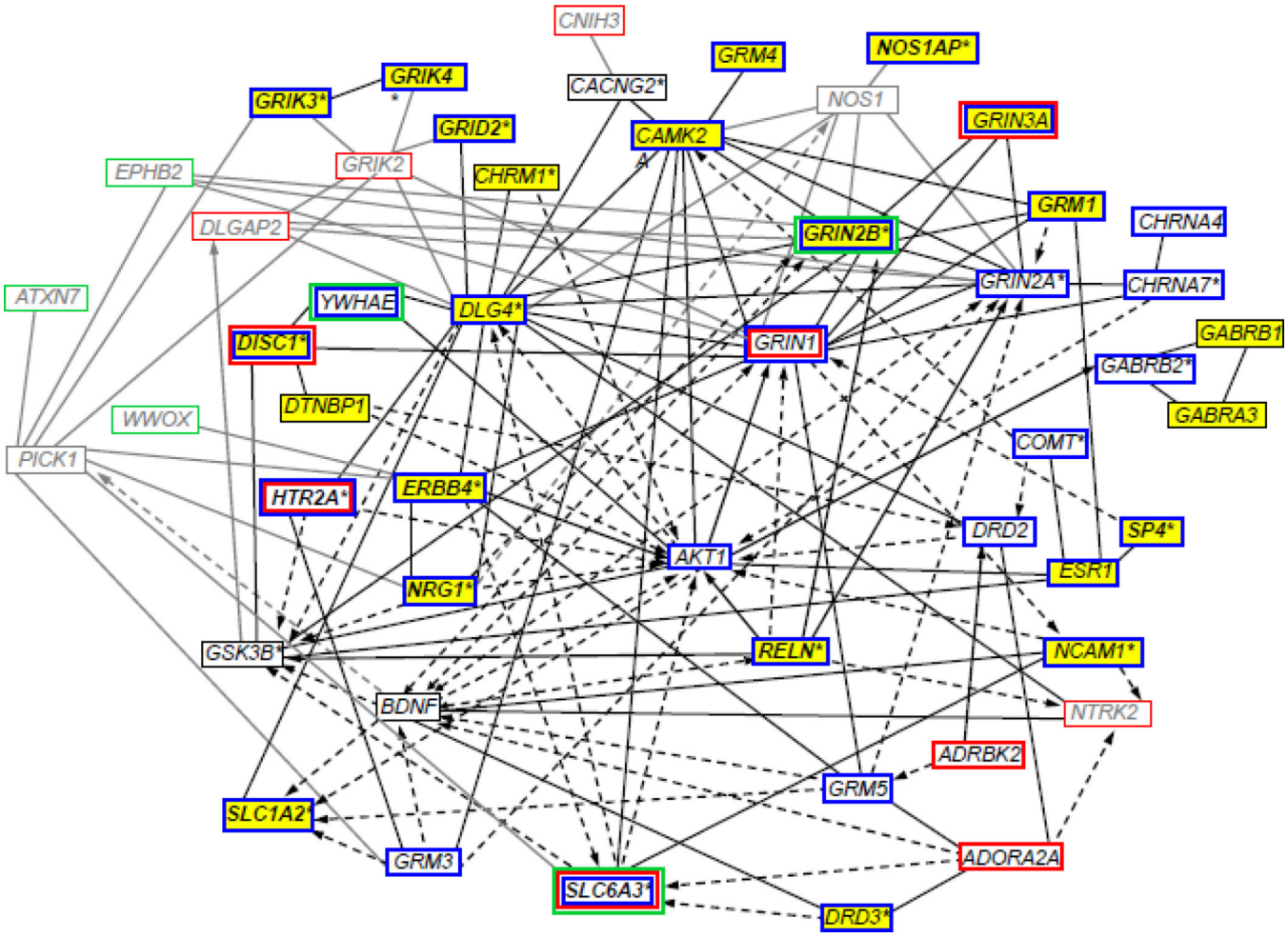

Figure 3.

Pathway analysis of the genes identified through association and linkage. Genes are represented as nodes, and the molecular interactions between nodes are represented by lines and arrows, with solid lines representing direct protein-protein or protein-DNA interactions, solid arrows representing phosphorylation, and dashed arrows representing indirect effects on expression, activation, or inhibition. Gene functions and relationships were determined by Ingenuity Pathway Analysis. Genes and interactions shown in black represent those included on the custom array and directly evaluated for association, while those in gray represent those derived from the linkage studies or other interacting genes. Candidate genes from the custom array associated ($p<0.01$) with at least one additional endophenotype are highlighted in yellow, and genes identified through linkage analysis are indicated with a red box. Genes from the custom array associated ($p<0.01$) in with a primary endophenotype in our previous study are indicated with a blue box (Greenwood et al., 2011), and genes identified through linkage analysis of a primary endophenotype are indicated in a green box (Greenwood et al., 2013c). Genes from the custom array associated with ≥3 additional endophenotypes or ≥3 primary endophenotypes are shown in bold, and those further

associated ($p<0.01$) in our independent case-control sample are identified with an asterisk (*) (Greenwood et al., 2012).

Table 1

Discriminability of the additional measures in the schizophrenia probands and controls and heritability in the 296 families.

	Discriminability					Heritability		
	Probands		Controls					
	N	Mean ± SD	N	Mean ± SD	*d*	N	h^2_r ± SE	P value
Baseline Startle Magnitude (Startle)[a]	**241**	**105.0 ± 70.4**	**355**	**95.8 ± 66.6**	**0.16**	**821**	**0.62 ± 0.07**	**<0.0001**
Startle Habituation Difference (Hab-diff)	241	55.9 ± 42.3	354	58.6 ± 45.5	0.03	814	0.37 ± 0.07	<0.0001
Startle Habituation Percent Change (Hab-pct)[a]	236	0.6 ± 0.2	352	0.6 ± 0.3	0.21	806	0.16 ± 0.08	0.016
P50 Conditioning Amplitude (P50-S1)[a]	**168**	**2.7 ± 1.5**	**252**	**3.2 ± 1.8**	**0.21**	**564**	**0.39 ± 0.10**	**<0.0001**
N100 Conditioning Amplitude (N100-C1)[a]	**187**	**−8.0 ± 3.8**	**156**	**−8.1 ± 4.0**	**0.33**	**702**	**0.31 ± 0.08**	**<0.0001**
CPT, Degraded Stimulus hit rate (DS-CPT-hr)[a]	**259**	**0.7 ± 0.2**	**376**	**0.7 ± 0.2**	**0.22**	**881**	**0.25 ± 0.06**	**<0.0001**
CPT, Identical Pairs 3-digit d' (CPT-IP-3d)[b]	**245**	**2.1 ± 0.8**	**369**	**3.0 ± 0.8**	**1.00**	**866**	**0.25 ± 0.06**	**<0.0001**
LNS Immediate Recall (LNS-fwd)[b]	**292**	**13.0 ± 2.8**	**385**	**14.3 ± 2.9**	**0.51**	**955**	**0.50 ± 0.07**	**<0.0001**
CVLT-2 List B Recall (CVLT-B)[b]	**288**	**4.6 ± 2.0**	**385**	**6.5 ± 2.2**	**0.81**	**949**	**0.29 ± 0.07**	**<0.0001**
CVLT-2 Long Delay Free Recall (CVLT-delay)[b]	**288**	**8.8 ± 3.8**	**385**	**12.6 ± 3.0**	**0.97**	**949**	**0.32 ± 0.06**	**<0.0001**
CVLT-2 Semantic Clustering (CVLT-semantic)[b]	288	0.4 ± 1.5	384	2.0 ± 2.5	0.68	947	0.17 ± 0.08	0.005
CVLT-2 Serial Clustering (CVLT-serial)	285	0.7 ± 0.8	383	0.5 ± 1.0	0.09	939	0.18 ± 0.07	0.005
Logical Memory Stories Recall (Stories-recall)[b]	**157**	**31.4 ± 13.0**	**240**	**46.6 ± 11.6**	**1.06**	**432**	**0.51 ± 0.09**	**<0.0001**
Age (in years)	296	34.3 ± 10.9	393	35.3 ± 12.5				
Education (in years)[b]	295	13.6 ± 2.1	391	15.4 ± 2.3				
Wide Range Achievement Test (WRAT)-3 Reading Standard Score[b]	289	102.4 ± 11.3	383	107.5 ± 10.7				

Note that the sample size of each endophenotype varied due to differences in endophenotype-specific exclusion criteria, interpretable data, and availability of data for each measure, and the smaller N for Stories-recall is due to its inclusion midway through the study. While raw data values are presented for the discriminability analyses, all comparisons between schizophrenia probands and controls are based on covariate-adjusted residuals for age, sex, and site as appropriate, except for the comparisons of age, education, and WRAT.

Significant group differences between probands and controls of $p<0.05$ ([a]) and $p<0.001$ ([b]) are indicated. The nine endophenotypes selected for further analysis are indicated in bold. Key: N = number; SD = standard deviation; *d* = effect size as Cohen's *d*; h^2_r = residual heritability after variance due to covariates is removed; SE = standard error.

Table 2

Genetic correlations observed between the primary and additional endophenotypes derived from five test paradigms.

	PPI[a]	**Startle**	**P50-diff**[a]	**P50-S1**	**N100-C1**	**DS-CPT-d**[a]	**DS-CPT-hr**	**CPT-IP-3d**	**LNS-reord**[a]	**LNS-fwd**	**CVLT-total**[a]	**CVLT-B**	**CVLT-delay**
Startle	0.11 (0.17)												
P50-diff[a]	−0.03 (0.30)	−0.12 (0.19)											
P50-S1	−0.05 (0.24)	−0.03 (0.15)	**0.82**[b] **(0.11)**										
N100-C1	**−0.60**[b] **(0.19)**	−0.12 (0.14)	−0.22 (0.28)	−0.28 (0.22)									
DS-CPT-d[a]	0.03 (0.18)	0.18 (0.13)	0.30 (0.22)	**−0.40 (0.17)**	−0.14 (0.17)								
DS-CPT-hr	0.02 (0.21	0.10 (0.14)	−0.17 (0.27)	−0.33 (0.20)	−0.11 (0.20)	**0.96**[b] **(0.03)**							
CPT-IP-3d	−0.18 (0.23)	**0.35 (0.13)**	−0.18 (0.25)	0.04 (0.20)	**−0.39 (0.20)**	0.22 (0.16)	0.10 (0.18)						
LNS-reord[a]	−0.10 (0.19)	0.11 (0.12)	**−0.46 (0.26)**	−0.28 (0.17)	0.01 (0.16)	0.22 (0.14)	0.18 (0.16)	**0.32 (0.14)**					
LNS-fwd	0.00 (0.17)	0.03 (0.11)	−0.36 (0.20)	−0.15 (0.15)	0.20 (0.15)	**0.32 (0.12)**	0.28 (0.14)	0.25 (0.14)	**0.90**[b] **(0.06)**				
CVLT-total[a]	−0.10 (0.21)	**0.33 (0.13)**	0.18 (0.25)	−0.05 (0.19)	0.03 (0.18)	0.30 (0.15)	0.10 (0.18)	0.24 (0.17)	**0.31 (0.06)**	0.06 (0.14)			
CVLT-B	−0.30 (0.20)	0.13 (0.13)	−0.25 (0.26)	−0.22 (0.19)	0.28 (0.18)	**0.38 (0.15)**	0.24 (0.18)	0.09 (0.18)	0.29 (0.16)	0.15 (0.14)	**0.84**[b] **(0.11)**		
CVLT-delay	−0.12 (0.20)	0.17 (0.13)	0.29 (0.24)	0.01 (0.18)	0.11 (0.18)	0.29 (0.15)	0.26 (0.17)	0.22 (0.17)	0.18 (0.16)	0.01 (0.4)	**0.87**[b] **(0.07)**	**0.65**[b] **(0.14)**	
Stories-recall	−0.05 (0.21)	**0.38**[b] **(0.12)**	−0.11 (0.14)	0.15 (0.8)	0.06 (0.19)	0.30 (0.16)	0.26 (0.18)	0.36 (0.18)	**0.48**[b] **(0.16)**	0.12 (0.15)	**0.75**[b] **(0.14)**	**0.79**[b] **(0.16)**	**0.55**[b] **(0.14)**

Key: PPI = prepulse inhibition at 60 ms; P50-diff = P50 difference score; DS-CPT-d = DS-CPT d' measure; LNS-reord = LNS reordered condition; CVLT-total = CVLT list A total score. All genetic correlations (standard error) values significant at $p<0.05$ are listed in bold text.

[a] Indicates the primary endophenotypes.

[b] Indicates correlations meeting a Bonferroni correction of $p=0.004$.

Table 3

Summary of all chromosomal regions with multipoint LOD scores reaching at least suggestive evidence for linkage.

		SOLAR				MERLIN			
Chr	**Phenotype**	**Location (cM)**	**Peak LOD**	**P_{emp}**	**1-LOD Interval (cM / Mb)**	**Location (cM)**	**Peak LOD**	**1-LOD Interval (cM / Mb)**	**Genes of Interest**
1q41-42	N100-C1	226	2.3	0.0003	216–233 / 211.0–227.8	229	2.2	214–238 / 208.7–231.1	*CNIH3, DISC1*
4q26	P50-S1	117	1.4	0.0062		120	2.7	118–120 / 114.4–120.1	
5p15	Stories-recall	0.6	2.4	0.0001	0–12 / 0.6–4.9	0.6	2.2	0–9 / 0.6–3.3	*SLC6A3/DAT*
6q16-21	CPT-IP-3d	102	1.0	0.016		108	2.3	102–111 / 97.2–107.6	*GRIK2*
6q21-22	CVLT-B	111	2.5	0.0004	106–118 / 102.8–115.9	113	2.5	109–119 / 106.2–117.2	*GRIK2, PREP*
6q21-22	DS-CPT-hr	108	2.1	0.0007		114	2.3	107–119 / 104.5–117.2	*GRIK2, PREP*
8p23	LNS-fwd	0	2.5	0.0007	0–18 / 0.4–7.4	2	1.6		*DLGAP2, CSMD1*
9q22-31	CPT-IP-3d	89	2.4	0.0003	83–119 / 86.3–115.7	111	3.3	105–115 / 104.2–112.8	*NTRK2, NXNL2, GRIN3A GABBR2, CTNNAL1*
9q34	CVLT-B	155	2.2	0.0007	139–160 / 131.4–140.1	150	3.7	148–155 / 135.7–137.2	*NTNG2, DBH, GRIN1 CACNA1B*
13q13	DS-CPT-hr	37	1.9	0.0014		43	2.5	35–52 / 37.1–48.3	*DGKH, HTR2A*
18q21-22	DS-CPT-hr	90	2.0	0.0010		89	2.4	85–94 / 56.8–63.6	
22q11-12	CPT-IP-3d	19	2.4	0.0003	9–29 / 17.8–25.5	24	2.6	13–28 / 20.2–25.5	*ADORA2A, ADRBK2*

Key: 1-LOD interval = genetic and physical boundaries of suggestive LOD scores within one unit of the maximum; P_{emp} = empirical p value based on 10,000 simulations; Genes of Interest = genes within the 1-LOD interval prioritized by proximity to SNPs with two-point LOD scores >1.5 and evidence of functional significance, expression in brain, and/or implication in previous studies of psychiatric

illness; *CNIH3* = cornichon homolog 3; *DISC1* = disrupted in schizophrenia 1; *SLC6A3/DAT* = dopamine transporter; *GRIK2* = glutamate receptor ionotropic, kainate 2; *PREP* = prolyl endopeptidase; *DLGAP2* = discs large (Drosophila) homolog-associated protein 2; *CSMD1* = CUB and Sushi multiple domains 1; *NTRK2* = neurotrophic tyrosine receptor kinase 2; *NXNL2* = nucleoredoxin-like 2; *GRIN3A* = glutamate receptor ionotropic, NMDA 3A; *GABBR2* = GABA receptor B2; *CTNNAL1* = catenin, alpha-like 1; *NTNG2* = netrin G2; *DBH* = dopamine beta-hydroxylase; *GRIN1* = glutamate receptor ionotropic, NMDA 1; *CACNA1B* = calcium channel, voltage-dependent, N type, alpha 1B subunit; *DGKH* = diacylglycerol kinase eta; *HTR2A* = serotonin receptor 2A; *ADORA2A* = adenosine receptor A2a; *ADRBK2* = beta-adrenergic receptor kinase 2. Note that the region on 4q26 included *CAMK2D* (calcium/calmodulin-dependent protein kinase II) and 18q21-22 included many genes of the serine proteinase inhibitor (SERPIN) and cadherin (CDH) families that are not of obvious functional significance.