Common genetic variation in Neuregulin 3 (NRG3) influences risk for schizophrenia and impacts NRG3 expression in human brain

Wee-Tin Kao, Yanhong Wang, Joel E. Kleinman, Barbara K. Lipska, Thomas M. Hyde, Daniel R. Weinberger, and Amanda J. Law

NRG3 is a neural-enriched member of the EGF family, a paralogue of NRG1, and a specific ligand for the ErbB4 receptor tyrosine kinase that plays pleotropic roles in neurodevelopment. Several genes in the NRG-ErbB signaling pathway including NRG1 and ErbB4 have been implicated in genetic predisposition to schizophrenia. Previous fine mapping of the 10q22-23 locus in schizophrenia identified genome-wide significant association between delusion severity and polymorphisms in intron 1 of NRG3 (rs10883866, rs10748842, and rs6584400). The biological mechanisms remain unknown. We identified significant association of these SNPs with increased risk for schizophrenia in 350 families with an affected offspring and confirmed association to patient delusion and positive symptom severity. Molecular cloning and cDNA sequencing in human brain revealed that NRG3 undergoes complex splicing, giving rise to multiple structurally distinct isoforms. RNA expression profiling of these isoforms in the prefrontal cortex of 400 individuals revealed that NRG3 expression is developmentally regulated and pathologically increased in schizophrenia. Moreover, we show that rs10748842 lies within a DNA ultraconserved element and homedomain and strongly predicts brain expression of NRG3 isoforms that contain a unique developmentally regulated 5' exon \((P = 1.097 \times 10^{-12} \text{ to } 1.445 \times 10^{-15})\). Our observations strengthen the evidence that NRG3 is a schizophrenia susceptibility gene, provide quantitative insight into NRG3 transcription traits in the human brain, and reveal a probable mechanistic basis for disease association.


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These results provide further evidence that NRG3 is a schizophrenia susceptibility gene and identify a molecular etiology underpinning the genetic association of NRG3 with neuropsychiatric disease.

Results

Clinical Genetic Association of NRG3 with Schizophrenia. Single-marker analysis in the family-based sample revealed evidence for association to 12 SNPs located in intron 1 of NRG3 (Fig. 1 and Table 1). This region includes the 13-kb interval (including rs10883866, rs10748842, and rs6584400) previously reported to show genome-wide significant and follow-up association with delusional behaviors in schizophrenia (4, 5). rs10883866 and rs10748842 are in allelic direction (\(r^2 = 1\)); therefore, we report \(P\) values for rs10748842 as representative of the association. We observed evidence of association to these SNPs with schizophrenia (Table 1)—rs10748842 (\(P = 0.02\)), rs6584400 (\(P = 0.01\)), and rs10399981 (\(P = 0.02\)), an additional tag SNP that resides in the 13-kb interval. A separate association analysis with independent probands (\(n = 445\)) and an independent control group (\(n = 488\)) replicated the signal at these three SNPs in the same allelic directionality (Fisher test: rs10748842, \(P = 0.01\); rs6584400, \(P = 0.03\); and rs10399981, \(P = 0.05\)). Interestingly, the opposite allelic association was observed to schizophrenia versus that in the dependent probands (\(n = 488\)).

Our most significant association in this region spanned an 80-kb interval of intron 1 (83942559–84023271), with the most significant SNPs being rs1336286 (\(P < 0.001\)) and rs1649960 (\(P < 0.001\)). This region is proximal to rs1080293, an SNP previously reported to be associated with schizophrenia (4). Family-based analysis also revealed nominal evidence of association in the 3′ region of the gene including rs671631 (\(P = 0.04\)), supporting previous observations in a UK case control sample (8), and rs7919853 (\(P = 0.035\)), rs17746658 (\(P = 0.032\)), and rs342386 (\(P = 0.04\)), a region where structural microdeletions have been reported (4). Finally, we failed to observe association to two SNPs (rs1937970 and rs677221; \(P = 0.85\) and \(P = 0.63\), respectively) previously associated with susceptibility to schizophrenia in a Chinese population (6). None of the significant SNPs violated Hardy-Weinberg equilibrium.

Association of NRG3 Risk Polymorphisms with Delusional Symptoms in Schizophrenia. Genome-wide significant association between rs10748842 and rs6584400 and delusion factor as a quantitative trait in patients with schizophrenia has been previously reported (4). We confirm association between rs10748842 (\(P = 0.01\)) and rs6584400 (\(P = 0.01\)) and the subscale for delusion severity derived from the Positive and Negative Syndrome Scale (PANSS), with higher symptom scores associated with the common alleles (T and G, respectively). The allelic directionality is consistent with our schizophrenia association findings, but opposite those reported by Chen et al. (4) and Morar et al. (5). We also observed association between these two SNPs and severity of positive symptom total scores, rs10748842 (\(P = 0.04\)) and rs6584400 (\(P = 0.02\)). Interestingly, rs10748842 was also associated with negative symptom load (\(P = 0.04\)).

Cloning and Characterization of NRG3 Transcripts in the Human Brain. We have isolated and comprehensively characterized full-length cDNA clones of the NRG3 gene in the adult human hippocampus and in whole brain and demonstrate that the gene generates numerous alternatively spliced transcripts, several of which have not previously been described in any human tissue as far as we are aware. We have classified families of transcripts based on exon homology and similarity to previously described human NRG3 sequences (Fig. 2).

Nucleotide sequencing of the clones and BLAST searching in the human genome using the National Center for Biotechnology Information (NCBI) University of California, Santa Cruz, Assembly (March 2006, NCBI36/hg18) and UniProt databases identifies the presence of a previously reported fetal brain–derived full-length NRG3 transcript in the adult human brain (9) (Fig. 2, transcript 1). This transcript contains a complete ORF of 720 aa, with a predicted Mr of 77.91 kDa. We also confirm the presence of NM_001010848 (NP_001010848; Fig. 2, transcript 2) in the adult human brain. This transcript contains a complete ORF of 696 aa, with a predicted Mr of 75 kDa. Consistently, we identified with Western blot a prominent approximately 75 kDa NRG3 protein band (and a weaker approximately 77-kDa band) in the adult and fetal brain (Fig. S1 A and B). Based on exon homology to NM_001010848, we have termed these variants class I NRG3 (Fig. 2).

To determine if the fetal brain–derived clone DQ857894; hFBNRG3 (15) is expressed in the adult human brain, we used primers specific to the 5′ N-terminal exon of DQ857894 (E2; Table S1) and the 5′ upstream region combined with reverse primers in the termination exon of NRG3. We document the presence of a transcript with high homology to DQ857894 (Fig. 2, transcript 4). Our transcript differs from that of Carteron et al. (15) because of splicing of exon 11 (amino acids IEVRK-TISHLPIQLWCVERPLDLK; Fig. 2). This observation demonstrates that variants homologous to DQ857894 (Fig. 2). Based on exon homology to NM_001010848, we have termed these variants class I NRG3 (Fig. 2).

We further report the identification of a family of NRG3 transcripts (termed class II or III) that are similar to class II NRG3, but with the presence of a 75 bp, GT, AG flanked extension to exon 2 (Fig. 2). Introduction of this exon is predicted to produce a greatly truncated protein as a result of the introduction of a premature TGA in the EGF-like domain. This TGA may render these transcripts for nonsense-mediated decay. Alternatively, there is an in-frame AUG in exon 6 (variant 10) that is predicted to produce a truncated N-terminal polypeptide. A homologous truncated protein is also predicted from a previously reported clone identified in human testis, AK098823. Interestingly, we confirm identification of this variant in human brain using primers to the 5′ UTR upstream of exon 6 (Fig. S2) and furthermore identify with Western blot an approximately 46 kDa NRG3 protein (Fig. S1B), consistent with the predicted Mr of AK098823. The biological relevance of these transcripts remains to be determined, given that their protein products would lack the bioactive EGF domain of NRG3.
We next assessed quantitative expression traits of NRG3 Splice Variant Expression: Development and Aging in the Human Brain.

Table 1. NRG3 SNP association in 356 families with an affected offspring

<table>
<thead>
<tr>
<th>SNP rs</th>
<th>Location (build NCBI 37.1)</th>
<th>Alleles</th>
<th>MAF</th>
<th>Empirical P value</th>
<th>Association</th>
<th>Risk</th>
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NRG3 Splice Variant Expression

NRG3 Splice Variant Expression Is Increased in the Dorsolateral Prefrontal Cortex in Schizophrenia and Associated with Clinical Risk Genetic Variation. We next assessed quantitative expression traits for NRG3 alternative isoforms (class I–IV; Table S2) in dorsolateral prefrontal cortex (DLPFC) tissue samples collected from a large cohort of individuals with schizophrenia, nonsympathetic controls, and fetal specimens during the second trimester of development. Expression of NRG3 variants containing the classic 5′ exon 1 (class I), including that of NM_001010848, were significantly elevated in individuals with schizophrenia compared with control individuals [Fig. 3; F(1,358) = 14.40; P = 0.0002; mean 40% increase]. NRG3 isoforms containing exon 3 contiguous with exon 4 (DO857894 and class IV) were also significantly increased in schizophrenia [Fig. 3; F(1,358) = 17.98; P = 0.0001; mean 50% increase]. No significant differences were observed between diagnostically groups for NRG3 transcripts of class II or III. Covariation for RNA integrity number (RIN), postmortem interval (PMI), and age were included in these analyses. No effects of race or race-diagnosis interactions were observed.

An inherited variant in the NRG3 gene, rs10748842, is significantly associated with schizophrenia as a discrete phenotype and delusion symptom severity in our family sample. This variant lies in an intronic risk region, approximately 12 kb proximal to an alternative promoter and transcription start site for a transcript cloned from human fetal brain (4, 15). Based on this evidence, we investigated in silico whether rs10748842 might alter protein binding properties and have potential functional consequences. Computational biology (MatInspector software; Genomatix), reveals that rs10748842 is centrally located within a classic homeodomain binding module (TAATTA), a core binding sequence for a family of transcription factors (TFs) including Hox, LIM, POU, and PAX domains. Consistently, there is a high degree of species conservation at this SNP locus, coherent with the observation that TAATTA binding modules are recognized ultraconserved genomic elements (UCEs) (19). The alleles of rs10748842 are predicted to differentially bind TFs; specifically, the common allele (T) is predicted to be associated with positive binding potential for at least 10 homeodomain TFs, including Pax6, compared with the nonrisk minor allele, which has no predicted TF binding potential.

Based on rs10748842’s physical location, computational predictions of functionality, and strength of association (4), we predicted that this polymorphism would be associated with NRG3 gene expression in human brain as a potential underlying mechanism for its disease association. We tested this hypothesis by examining association of allelic variation at rs10748842 and expression of NRG3 transcripts in the human DLPFC. A highly significant main effect of genotype was observed for rs10748842 in the DLPFC with NRG3 class II [F(2,315) = 38.16, P = 1.44E−13] and class III [F(2,315) = 27.37; P = 1.097E−12] expression, whereby individuals homozygous for the T allele had approximately 60% higher levels of expression compared with C-carriers (Fig. 4A and B). Because of the small number of homoyzogous CC individuals (n = 5), we grouped individuals into C-carriers. However, examination of the data for all three genotype groups revealed a significant allele dose effect for NRG3 class II and III expression [F(2,315) = 14.56; P = 8.95572E−5], with CC individuals having the lowest expression compared with C/T and T/T (Fig. S3). Remarkably, the impact of genetic variation on NRG3 class II and III expression was validated in the human fetal DLPFC in the same directionality as that in the adult brain [(F = 1.34) = 4.99; P < 0.05] and [F(1,34) = 4.0; P < 0.05, respectively; Fig. 4 C and D]. No genotype–diagnosis interactions were observed. Similar findings were observed for rs6584400, a risk SNP in strong LD with rs10748842.

NRG3 Splice Variant Expression: Development and Aging in the Human Brain. No correlations with age were observed for any NRG3 isoform class (during fetal or postnatal life). Class II and IV NRG3 variants were significantly more abundant in the fetal brain compared with postnatal life [0–85 y; F(1,221) = 41.39; P = 9.62E−11; and F(1,221) = 23.94; P = 5.40E−06, respectively; Fig. S4]. Fig. S5 shows relative expression levels of class I–IV NRG3 isoforms in normal control individuals.
different populations has been observed for a number of other diseases, such as autism (HTTLPR locus) and Alzheimer disease (apolipoprotein E e4-related polymorphisms) and has been suggested to be a valid biological phenomenon as a result of heterogeneous effects of the same variant related to multifocus interactions (i.e., effects of epistasis, environment, or LD, with causal variants that emerged on different genetic backgrounds (20). At present, it is unclear which of these factors account for the reversal of risk alleles in our study, but it is noteworthy that recent findings suggest association of the minor alleles (protective alleles in our study) at rs10883866 and rs6584400 with better cognitive performance in patients with schizophrenia (5), suggesting complex genetic association with the schizophrenia phenotype. The absence of association with schizophrenia in the Ashkenazi sample (4) also suggests that, in this population, the variants may be more closely related to certain phenotypic symptom traits than the disease itself. It is unclear why this might be, but modification of NRG3 allele effects by protective factors, other genes, or the environment is plausible. Nevertheless, in our family-based sample, we report consistent association of the common alleles with diagnosis and symptom severity.

We also identify association with schizophrenia and SNPs spanning an 80-kb interval of intron 1 (83942539–84023271). These SNPs reside in a block of LD separate from that of rs1074442, supporting two independent association signals within intron 1. This signal is located in the 3′ region of intron 1 and provides support for prior nominal association of this region with schizophrenia and disorganization severity (4). Intron 1 of NRG3 spans approximately 500 kb and represents one of the nine largest mammalian intronic elements in the genome (21). The enormity of such introns creates several molecular disadvantages, including transcriptional energy wastage, increased susceptibility to mutable events, and potential for errors in splicing (21). Such factors may contribute to the excess of disease association in this region. Nominal association was also observed with schizophrenia to SNPs in the 3′ region of NRG3, including variants previously reported in a UK case-control sample (8). Overall, these observations support the proposition that allelic heterogeneity is a characteristic of NRG3 association with schizophrenia. Interestingly, the physical distribution of risk associated variants in NRG3 is strikingly similar to that of NRG1, whereby genetic variation in the large intron 1, the 3′ exon–rich region, and interactions between the two have been associated with schizophrenia (8, 14, 22, 23)

Alternative splicing enables one gene to encode numerous proteins and is often regulated in a tissue-specific and developmental manner (24, 25). Previous studies have demonstrated that other NRG-ErbB family members, most notably NRG1 and ErbB4, undergo widespread alternative splicing, giving rise to multiple proteins with differential signaling properties and functions (14, 17, 18, 26). NRG3, like NRG1, is a sizable gene, spanning 1.2 Mb, but little information exists about the mature transcripts it produces. Our characterization of full-length NRG3 variants in the adult human brain reveals that NRG3 produces 15 distinct splice isoforms, which we designated into four classes. The mature transcripts all include an EGF-like bioactive domain, a TM domain, and a complete cytoplasmic tail. Class 1 variants are similar to the original clone identified in fetal brain (8) and class II similar to that identified by Carteron et al. (15), demonstrating that developmentally regulated species of NRG3 transcripts are transcribed in the adult human brain. In addition, we describe a family of NRG3 transcripts (class III) that contain a 75-bp extension to the 5′ leader exon of hFBNR3 (E2ext) (15). It is unclear what splicing mechanism accounts for this inclusion, but it is noteworthy that the extension is derived from intron 1 and results in the introduction of a premature stop codon in the EGF domain. Previous data have demonstrated that large introns permit more potential errors in intron splicing because they contain numerous false splice sites (so-called “pseudoexons”) (21, 27), and inclusion of pseudoexons from intronic elements has been shown to target

Fig. 2. Organization of NRG3 transcripts cloned from adult human brain (whole brain and hippocampus). Exons are shown in the order in which they occur in the transcript. The length of each exon is not proportional to the number of amino acids encoded. The nomenclature describing each exon is listed in the box (Bottom). The top row is a compendium of all exon nomenclatures (reference). The numbering is consistent with RefSeq gene annotations for NRG3 [University of California, Santa Cruz, Genome Browser or NCBI36/hg18 and GRCh37 Assembly (hg19)]. Isoforms are numbered and subdivided into classes based on common exon inclusion. Class categories were used to design primer probe sets for QPCR. Numerical annotation refers to “transcript.” Sequence annotation features for functional domains are derived from the Protein Knowledgebase (http://www.uniprot.org/uniprot/P56975). Nucleotide sequences for NRG3 variants have been deposited in GenBank database under accession numbers HM068873–HM068885.

Discussion

Previous work has demonstrated that a 13-kb region of NRG3 containing three SNPs in the 5′ domain (rs10748842, rs10883866, and rs6584400) acts as a susceptibility locus for delusion severity in patients with schizophrenia (4, 5). This region of association represents a conserved block of LD, proxied by rs10748842. In the present study, by using a family-based analysis of NRG3 SNPs, we identified that the common alleles at these polymorphisms are significantly overtransmitted to offspring with schizophrenia. Furthermore, we replicated prior association of these SNPs with quantitative trait measurements of delusion severity in patients, to the same common alleles in our sample.

The extension is derived from intron 1 and results in the introduction of a premature stop codon in the EGF domain.
transcripts for nonsense mediated decay (27), as is predicted for class III NRG3 transcripts. Conversely, it is unclear whether E2ext actually represents an authentic alternative exon, because its presence in transcripts containing exon 3 results in a complete ORF and predicted 55-58.84 kDa protein. Future studies are needed to determine the significance of these splicing events and the function of specific transcripts for NRG3 signaling.

Previous studies have identified genetic variants in the genome that influence gene expression (28, 29). We found that the schizophrenia risk–associated polymorphism, rs10748842, resides in a nonexonic UCE and strongly predicts expression ($P = 1.097 \times 10^{-12}$ to $1.445 \times 10^{-15}$) of specific, developmentally regulated NRG3 isoforms in the normal and developing human brain and in schizophrenia, whereby the ancestral (T) allele is associated with elevated expression. UCEs are sequences that are identical between species, are under negative selection, and include functional elements enriched in the homeodomain DNA-binding motif, and a core binding sequence for multiple TFs including Pax6. Studies of mice homozygous for the Pax6 mutation demonstrate defects in NRG3 expression and signaling accompanied by disruption of cortical patterning and cell migration (11), providing direct experimental evidence of transcriptional control of NRG3 by Pax6 and supporting the observation that NRG3 plays critical roles in neurodevelopment.

NRG3 is almost exclusively expressed in the developing and adult CNS (9, 15), and intronic UCEs, similar to the rs10748842 consensus motif, have been demonstrated to direct tissue-specific expression in their capacity as enhancer elements (30). These observations, combined with the strong association to brain NRG3 quantitative expression traits, suggest that a mechanism behind the clinical association of NRG3 with schizophrenia involves altered transcriptional regulation, which modifies in an isoform-limited and tissue-specific manner, the efficiency of NRG3 signaling. Indeed, the NRG3 isoforms that are elevated in association with rs10748842 and schizophrenia contain an N terminus that confers proteosome-mediated instability to the protein (15). This observation suggests that a pathophysiological shift in the balance of NRG3 isoforms toward a more unstable NRG3 signaling system occurs in relation to risk for schizophrenia which has potential consequences for NRG3’s role in neural development and plasticity. Interestingly, a similar biological phenomenon is observed for disease and genetic-related changes in NRG3’s receptor, ErbB4 (31), whereby increases in ErbB4 CYT1, an isoform highly susceptible to internalization, monoubiquitination, and degradation (32), have been reported in human brain (31).

In summary, schizophrenia is a complex, heritable psychiatric disorder and several genes in the NRG-ErbB pathway have been implicated in disease risk, suggesting a pathogenic network (8, 22, 31). Our results provide robust evidence that another member of this network, NRG3, is a schizophrenia susceptibility gene with polymorphisms that modify disease risk by influencing NRG3 alternative transcript expression in the developing and adult human brain.

Materials and Methods

Clinical Samples. Family-based and case-control samples were used for clinical genetic investigation of NRG3 polymorphisms. The samples were ascertained as part of the Clinical Brain Disorders/National Institute of Mental Health (NIMH) Sibling Study (CBDB/SS). DNA was available from 445 probands, 612 parents, and 488 unrelated controls. For family-based association analysis, 356 families with a single affected proband were available. A partially independent case-control analysis was used comprising 445 unrelated probands and 488 unrelated healthy controls. All individuals were white Americans of Western European ancestry. For further information on screening and diagnostic criteria see SI Materials and Methods.

Human Postmortem Brain Samples. Postmortem human brains from the Clinical Brain Disorders Branch were obtained at autopsy primarily from the Washington, DC, and Northern Virginia medical examiners’ offices, all with informed consent from the legal next of kin (protocol 90-M-0142 approved by the NIMH/National Institutes of Health Institutional Review Board). Additional postmortem fetal, infant, child, and adolescent brain tissue samples were provided by the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (www.bbdir.org) under contracts NO1-HD-4-3368 and NO1-HD-4-3383. The institutional review board of the University of Maryland at Baltimore and the State of Maryland approved the protocol, and the tissue was donated to the NIMH under the terms of a Material Transfer Agreement. Clinical characterization, diagnoses, and macro- and microscopic neuropathological examinations were performed on all CBDB cases using a standardized paradigm (31). The cases at the University of Maryland at Baltimore were handled in a similar fashion (http://medschool.umaryland.edu/BBBank/ProtocolMethods.html). Toxicological analysis was performed on every case. All control subjects were free of a history of psychiatric illness or significant alcohol or drug abuse.

A total of 245 normal control individuals [82 female, 163 male; 133 black, 105 white, four Hispanic, three Asian; mean age 33.3 ± 20.58 y (SD), range
0–85 yr; PMI, 29.92 ± 14.86 h; pH 6.49 ± 0.30 (SD); RIN 8.2 ± 0.86 (SD) and 113 individuals with schizophrenia (44 female, 69 male; 67 black, 42 white, three Hispanic, and one Asian Pacific; mean age, 52.6 ± 16.0 yr; PMI, 38.9 ± 23.1 h; pH 6.34 ± 0.25; RIN, 8.0 ± 0.93) were available for this study. Preferential cortical sample was derived from 42 normal subjects from elective termi-
nation (age range, gestational weeks 14–39, mean 18.28 ± 3.62; PMI, 2.48 ± 2.1 h; RIN, 8.72 ± 1.27; 18 female and 23 male). Diagnoses were determined using Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), criteria. Toxicological analysis was conducted on every subject. Total RNA was extracted using RNAeasy Lipid Tissue Mini Kit (Qiagen). RNA quality was assessed with high-resolution capillary electrophoresis (Agilent Technologies) as described previously (17).

Molecular Cloning of NRG3 cDNA: RT-PCR, cDNA Cloning and Sequencing. To isolate full-length cDNA encoding NRG3 transcripts, RT-PCR was performed using adult human brain cDNAs. Adult human brain cDNA libraries were generated using total RNA from the hippocampus (Clontech) and total brain (Ambion), respectively. Human cDNA libraries were constructed from 5 μg of total RNA and reverse-transcribed to cDNA in a total volume of 50 μL by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) primed with random oligo (dT)20 according to the manufacturer’s instructions. Two microliters of the first-strand cDNA was used in PCR amplification using Platinum Taq DNA Polymerase High Fidelity (Invitrogen), RT-PCR primers used for amplification of full-length NRG3 transcripts were designed specific to the unique 5′ exon of full-length NRG3, NM_001018488 (8) (Table S1). Forward primers for amplification of the full-length variant identified in human fetal brain (hNRG3DQ857894) (15) were designed to the unique first and second exons (E2 and E3) and the 5′ UTR. Reverse primers were designed specific to the termination exon (12) of NRG3 using Primer3 (http://frodo.wi.mit.edu). For further cloning, sequencing, and Western blot analysis, see SI Materials and Methods. The sequences reported in this article have been deposited in GenBank under the accession numbers HM068873–HM068885.

Quantitative Real-Time PCR. Expression levels of families of NRG3 transcripts identified through PCR cloning in human brain were measured using quantitative real-time RT-PCR (QPCR) in the DLPFC derived from normal individuals and individuals with schizophrenia. DLPFC derived from fetal specimens was measured by quantitative RT-PCR using an ABI Prism 7900HT sequence fast analysis system. Analysis of the effects of race was performed using ANOVA and post hoc analysis. Statistical Analyses. Correlations of mRNA levels with demographic variables were performed for all subjects (normal controls, patients with schizophrenia, and fetuses) with Spearman correlation. Correlations of mRNA levels with neuroleptic medication (lifet ime neuroleptic exposure, daily dose, and final neuroleptic dose) were investigated in individuals with schizophrenia. Primary planned comparisons between diagnostic groups were made with univariate analysis of covariance for each mRNA with diagnosis as the independent variable and age, RIN, and PMI as covariates. Effects of genetic variation on NRG3 isoform mRNA levels were examined using ANOVA with genotype and diagnosis as independent factors. Primary comparisons examined the effects of two clinically associated 5′ SNPs on class I–IV NRG3 expression in patients and controls. Analysis of the effects of race was restricted to black and white individuals as a result of the small sample size in other ethnic groups. Race was included as an independent factor in analyses, but no effects of race or race–diagnosis interactions were observed. All experiments were conducted blinded to diagnosis.

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6. Sundvall M, et al. (2008) Isoform-specific SNPs on class I NRG3 isoform expression in patients and controls. Analysis of the effects of race was restricted to black and white individuals as a result of the small sample size in other ethnic groups. Race was included as an independent factor in analyses, but no effects of race or race–diagnosis interactions were observed. All experiments were conducted blinded to diagnosis.
Supporting Information

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SI Materials and Methods

Clinical Samples. All probands met DSM-IV criteria for a broad diagnosis category consisting of schizophrenia; schizoaffective disorder; simple schizophrenia; psychosis not otherwise specified; delusional disorder; or schizotypal, schizoid, or paranoid personality disorder. Control subjects were ascertained from the NIMH normal volunteer office and required absence of diagnosis of a psychiatric disorder, extended to include first-degree relatives. All subjects were interviewed with the structured diagnostic evaluation for DSM-IV diagnoses (Structured Clinical Interview for DSM-IV Axis II Personality Disorders) and were examined for medical and psychiatric exclusions. Detailed description of recruitment is described in detail elsewhere (1). Inclusion criteria for all participants included: self-identification as American Caucasian of Western European ancestry, aged between 18 and 60 y, and IQ scores above 70 (for probands, premorbid IQ). All subjects gave written informed consent. Patients were also interviewed by a clinical psychiatrist or psychologist for evaluation of ongoing symptoms using the PANSS (2).

DNA Genotyping and Clinical Association Analysis. Genotype reproducibility was routinely assessed by genotyping all samples for selected SNPs and was generally greater than 99%. Overall genotyping failure rate was less than 1%. LD between SNPs was determined using the program LDmax/GOLD (3). Main effects analyses of single SNPs were conducted using unconditional logistic regression models and haplotype analysis was performed using the score statistic-based test implemented in the R package haplo.stats (4) controlling for sex and age in the case-control sample and using the family-based association test (http://www.biostat.harvard.edu/~fbat/fbat.htm) in families to test single SNPs (5) with permutation testing for significance assessment. Effects of delusions as a quantitative trait were investigated using the QFBAT test based on the unscaled subscore of the PANSS (2).

Molecular Cloning and Sequencing of NRG3 cDNA. PCR products were resolved on a 1% agarose gel in 1x TBE buffer. Gels were stained with ethidium bromide and the DNA bands were visualized using a Kodak EDAS 290 imaging setup with an orange band-pass filter, a Kodak DC290 camera, and a 302-nm UVA transilluminator. The PCR product was puriﬁed using the QIAquick PCR puriﬁcation kit (Qiagen).

To verify the identity of the amplified products, the puriﬁed PCR product was cloned into pCR2.1 TOPO vector (Invitrogen) and transformed into TOP10 chemically competent cells (Invitrogen) and cells were grown in LB-Amp agar plates (Invitrogen). Colonies were selected, grown at 37 °C, 225 rpm overnight. The plasmid miniprep DNAs were prepared from 1.5 mL of culture using Qiaprep Spin miniprep kit (Qiagen). The insert was sequenced using the BigDye terminator kit (Applied Biosystems) with M13 forward and reverse primers (M13F, 5′-GTAAACAGCACGGCCG-3′; M13R, 5′-CAGGAACACGCTATGAC-3′), as described in detail previously (6).

QPCR. All measurements were performed in triplicate for each mRNA and quantitative expression was calculated as an average of the triplicates. Experimental measurements with a greater than 20% variance from the mean of the triplicate samples based on CTs were omitted. Our primary data analysis is based on normalization of NRG3 mRNAs to the geometric mean of the quantity of three internal control genes β-actin, GADPH, and PGPD. Primers and probe were designed to be unique to specific NRG3 transcript families, class I–IV (Table S2). Probes were designed specifically to cross unique exon junctions present in each transcript. CTs for variants ranged from 23 to 30.

Western Blot Analysis of NRG3. Western blot analysis was performed by using two NRG3 antibodies: one to the N-terminal extracellular domain (aa 276–345; sc-67002; Santa Cruz Biotechnology) and one to the C-terminal domain (aa 685–696; ab77597; Abcam). Fifty micrograms of protein from fetal and adult human DLPFC was denatured in 4× NuPAGE LDS sample buffer (Invitrogen) and 0.75 μL of 1.0M DTT, at 95 °C for 5 min. Samples were separated by gel electrophoresis using NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. sc-67002 antibody dilution was 1:200 and ab77597 was 1:1,000. Following overnight incubation at 4 °C, membranes were washed three times with TBS with Tween 20 and incubated for 1 h with 1:2,000 goat antirabbit IgG-HRP (sc-2004; Santa Cruz Biotechnology) and donkey antigoat IgG-HRP (sc-2033; Santa Cruz Biotechnology). Membranes were washed five times (5 min) with TBS with Tween 20. HRP-immunoreactive protein bands were detected by enhanced chemiluminescence Western blotting analysis system (RPN2109; Amersham) and exposed to Kodak scientific imaging film. Prestained protein ladder plus (no. 65–0681; Crystalgen) were used for product size determination. Blots were stripped using Restore Western Blot Stripping Buffer (21062; Pierce Biotechnology) and probed with 1:10,000 anti–β-actin antibody (A5441; Sigma-Aldrich).

Fig. S1.  (A) Western blot analysis of NRG3 in the DLPFC using an N-terminal antibody (sc-67002) identifying a predominant approximately 75-kDa protein (and a weaker approximately 77-kDa band) expressed in adult and fetal human brain.  (B) Western blot analysis using a NRG3 C-terminal antibody (ab77597) confirms the presence of the 75/77-kDa NRG3 proteins and identifies additional bands in adult and fetal brain. NC, normal control; SZ, schizophrenia patient; F, fetal brain.

Fig. S2. Cloning of AK098823 in adult human and fetal brain.

Fig. S3. Allele-associated differences in expression of NRG3 class III in the adult DLPFC associated with rs10748842 (N = 315; T/T, n = 223; C/T, n = 87; C/C, n = 5; values are mean ± SEM).

Fig. S4. Age-related differences in class II and class IV NRG3 expression in the DLPFC during normal human development (N = 42; fetal, 14–39 wk; n = 245, 0–85 y; values are mean ± SEM).
Fig. S5. Quantitative expression of NRG3 transcripts class I–IV in the DLPFC of normal control individuals (n = 245, 0–85 y; values are mean ± SEM).

Table S1. Primers used for amplification of NRG3 full-length transcripts using PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Exon</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Sequence NM_001010848.3</td>
<td>1</td>
<td>CTCCTCCTACCCATCGATCTCT</td>
</tr>
<tr>
<td>E1F</td>
<td>12</td>
<td>AACCACAGAAGGATGAGT</td>
</tr>
<tr>
<td>E12R2</td>
<td>12</td>
<td>GCACCTTGATGCTTGA</td>
</tr>
<tr>
<td>DQ857894</td>
<td>5 UTR</td>
<td>CCCAGAGTTTTGTATCCTG</td>
</tr>
<tr>
<td>E3</td>
<td>3</td>
<td>TCTCCTGCTGAGGAGC</td>
</tr>
</tbody>
</table>

All primers were designed using Primer3 and have a melting temperature of approximately 60 °C and a 50% GC content. E1F is downstream of the final ATG start codon in exon 1. 5 ATGs are present in exon 1 of the gene.

Table S2. Oligonucleotide primer and probe sequences used for quantitative amplification of NRG3 transcript families identified in human brain

<table>
<thead>
<tr>
<th>NRG3 transcript class</th>
<th>Internal probe and primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I (E1–E4)</td>
<td>Forward 5’ cctcctacccatcctact 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ GTTGAAGTCTCGGATCG 3’</td>
</tr>
<tr>
<td></td>
<td>Internal probe 5’-FAM- CCCAAATTCATACGACGAC-MGB-3’</td>
</tr>
<tr>
<td>Class II (E2–E4)</td>
<td>Forward 5’ TGGTATACCTCCAACCCTTG 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ ACAAGACACTGCCCATTG 3’</td>
</tr>
<tr>
<td></td>
<td>Internal probe 5’-FAM- ACTACACAGATACGACGAC-MGB-3’</td>
</tr>
<tr>
<td>Class III (E2ext-E4)</td>
<td>Forward 5’ TCTTCAATTTACCTTTTG 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ ACAAGACACTGCCCATTG 3’</td>
</tr>
<tr>
<td></td>
<td>Internal probe 5’-FAM- AAGGCCAAGATACGACGAC-MGB-3’</td>
</tr>
<tr>
<td>Class IV and DQ857893 (E3-E4)</td>
<td>Forward 5’ TCCTCCTGCTGAGGAGC 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ GTTGAAGTCTCGGATCG 3’</td>
</tr>
<tr>
<td></td>
<td>Internal probe 5’-FAM- TACAAATTACGACGAC-MGB-3’</td>
</tr>
</tbody>
</table>