Molecular Psychiatry (2006), 1–11

www.nature.com/mp

ORIGINAL ARTICLE

Neuregulin 1 (8p12) and childhood-onset schizophrenia: susceptibility haplotypes for diagnosis and brain developmental trajectories

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Childhood-onset schizophrenia (COS), defined as onset of psychosis by the age of 12, is a rare and malignant form of the illness, which may have more salient genetic influence. Since the initial report of association between neuregulin 1 (NRG1) and schizophrenia in 2002, numerous independent replications have been reported. In the current study, we genotyped 56 markers (54 single-nucleotide polymorphisms (SNPs) and two microsatellites) spanning the NRG1 locus on 78 COS patients and their parents. We used family-based association analysis for both diagnostic (extended transmission disequilibrium test) and quantitative phenotypes (quantitative transmission disequilibrium test) and mixed-model regression. Most subjects had prospective anatomic brain magnetic resonance imaging (MRI) scans at 2-year intervals. Further, we genotyped a sample of 165 healthy controls in the MRI study to examine genetic risk effects on normal brain development. Individual markers showed overtransmission of alleles to affecteds ($P = 0.009$–0.05). Further, several novel four-marker haplotypes demonstrated significant transmission distortion. There was no evidence of epistasis with SNPs in erbB4. The risk allele (0) at 420M9-1395 was associated with poorer premorbid social functioning. Further, possession of the risk allele was associated with different trajectories of change in lobar volumes. In the COS group, risk allele carriers had greater total gray and white matter volume in childhood and a steeper rate of subsequent decline in volume into adolescence. By contrast, in healthy children, possession of the risk allele was associated with different trajectories in gray matter only and was confined to frontotemporal regions, reflecting epistatic or other illness-specific effects mediating NRG1 influence on brain development in COS. This replication further documents the role of NRG1 in the abnormal brain development in schizophrenia. This is the first demonstration of a disease-specific pattern of gene action in schizophrenia.
Molecular Psychiatry advance online publication, 10 October 2006; doi:10.1038/sj.mp.4001906

Keywords: endophenotype; neurodevelopment; linkage disequilibrium; child psychosis; TDT; QTDT

Introduction

Schizophrenia is a severe, debilitating brain disorder affecting approximately 1% of the population worldwide. Despite estimates of heritability as high as 85%,[1] consistent associations of particular risk genes with schizophrenia have only recently been reported.[2,3] Using a positional cloning approach, identification of a core risk haplotype in the 5′ region of neuregulin 1 (NRG1) on 8p12, which was associated with schizophrenia (relative risk = 2), was initially reported by Stefansson *et al*.[4] Since then, association between NRG1 and schizophrenia has been one of the most replicated associations in the schizophrenia genetics literature. However, the associated risk haplotypes across independent samples have not been consistent, and no clear susceptibility variant has yet been identified.

Childhood-onset schizophrenia (COS), defined as onset of psychosis by the age of 12, is a rare and severe form of the disorder that is clinically and neurobiologically continuous with the adult form of the illness.[5,6] Since 1989, these patients have been sought nationally at the National Institute of Mental Health (NIMH). Studies of first-degree relatives of these patients indicate higher familial rates of schizophrenia spectrum disorders,[7] and smooth pursuit eye movement abnormalities compared to families of later onset patients.[8] In addition, COS patients are characterized by more pronounced early delays in social, motor and language function.[9,10] A unique aspect of the COS study has been the acquisition of prospective anatomic brain magnetic

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Received 21 February 2006; revised 10 August 2006; accepted 21 August 2006

resonance imaging (MRI) data for patients and age-matched controls, thus enabling us to map global and regional brain trajectories for healthy and psychotic children.[11] COS patients show a striking loss of gray matter volume, which plateaus in adolescence.[12,13] Although rare, the higher rates of familial illness or biological markers of illness, extreme severity and 10% rate of chromosomal abnormalities seen in this sample[14] suggest that genetic influences may be more salient.[6]

NRG1 participates in glutamatergic signaling by regulating the *N*-methyl-D-aspartate (NMDA) receptor through the interaction of the NRG1 protein and its receptors. NRG1 plays a central role in neural development and is most likely involved in regulating synaptic plasticity, or how the brain responds or adapts to the environment. Animal studies have shown that NRG was broadly expressed in early embryonic development (embryonic day 6.5), with a gradual shift toward selective expression in the rostral brain.[15] Recent studies have demonstrated that NRG1 elicits unique effects on glutamatergic signaling that may vary between neuronal types and are activity- and time-dependent.[16]

The goal of the current study was to examine the association of haplotypes reported previously for the more typical adult-onset phenotype. Further, given the evidence for a functional and behaviorally relevant interaction between NRG1 and erbB4,[17,18] we genotyped two single-nucleotide polymorphisms (SNPs) in erbB4 to test for association with COS as well as potential epistatic effects. In addition to testing for association between polymorphisms in NRG1 and the diagnosis of COS, we explored the relationship between risk alleles and regional brain development throughout adolescence and early adulthood. Given the neurodevelopmental role of NRG1, one possibility is that NRG1 may continue to influence global or regional development during childhood. In addition to premorbid assessment and cognitive measures, we have collected volumetric MRI brain scans at approximately 2-year intervals from the first admission to our study. Using longitudinal analyses, we mapped the effects of allelic variation on the trajectory of change in lobar volumes in both COS patients and healthy children.

Subjects and methods

Patient recruitment and clinical assessment

DNA was available for 78 children and adolescents, mean age 14 ± 3 years, who participated in the COS study.[5] Seventy of these patients had at least one available parent, of which both parents were available for 59. Forty-six (59%) of the COS patients are male and, given the rarity of these patients (thus precluding selection), the ethnic background (based on parental report) is mixed: half the sample is Caucasian, 28% is African-American and the remaining is a mix of Hispanic (7%), Asian (5.5%) and 10% from other or mixed ethnicities. The NIMH Institutional Review Board approved the project and written consent was obtained from parents and assent from minor subjects.

Patients meeting Diagnostic and Statistical Manual of Mental Disorders (DSM)-III-R/DSM-IV[19] criteria for schizophrenia or psychosis not otherwise specified were recruited nationwide through an extensive screening process, including a review of over 1400 charts and in-person screening of over 230 subjects. The schizophrenic patients who participated were required to have a premorbid full-scale intelligence quotient (IQ) of 70 or above and an onset of psychotic symptoms by the age of 12. The diagnosis of COS was confirmed by two psychiatrists ($\kappa = 0.8$)[20] through an extensive evaluation that included clinical and structured interviews of the children and parents using portions of the Schedule for Affective Disorders and Schizophrenia for School-Age Children (K-SADS-E and K-SADS-PL)[21,22] and in-hospital observation during a 1- to 3-week medication-free period. Information on these subjects included cognitive and behavioral ratings of early development, history of medication response, neuropsychological test performance, smooth pursuit eye movements and MRI scans.

Age of onset of first psychotic symptoms was obtained through interview and patient records. Cognitive testing was completed using the Wechsler Intelligence Scale for Children – Revised (WISC-R) or Wechsler Intelligence Scale for Children – Third Edition (WISC-III). Premorbid development (defined as development up to 1 year before onset of psychosis) was evaluated based on clinical interview, neuropsychological testing, school records, standardized rating scales and parental recall. Because early-onset cases show more striking early developmental impairment,[5] ratings for early language, social and educational adjustment were completed using a modification of the ratings by Hollis.[9] In addition, the Premorbid Adjustment Scale (PAS)[23] and the Autism Screening Questionnaire, which has good discriminative validity with respect to the separation of pervasive developmental disorders (PDD) from non-PDD diagnoses at all IQ levels,[24] were completed through parental interviews and chart review.

One hundred sixty-five unrelated children and adolescents who had no personal or family history of psychiatric or neurological disorders, as determined by means of clinical examination and standardized interview, were recruited from the community as described in detail elsewhere.[25] Age appropriate IQ test were used: the WPPSI-III for children aged 4–6 years; the WISC-III for children aged 6–17 years; and the WAIS-III for children aged 18 years and above. Further demographic details of each of the groups are given in Table 3. Socio-economic status (SES) was calculated from the Hollingshead Scales.[26] Although there were no significant differences between the healthy controls and the COS group by age or gender, which are the strongest predictors of brain size,[25] there were significant differences by ethnicity (85%

of controls were Caucasian compared to 50% of COS probands $\chi^2 = 10.6$, $P = 0.03$), SES ($t = -7.1$, $P < 0.01$) and IQ (16.8, $P < 0.01$), which is not unusual in studies of a rare patient population. Approximately two-thirds of subjects in each group were scanned at least twice at the National Institutes of Health in Bethesda, MD, USA.

All patients and controls underwent MRI scans on the same GE 1.5-T Signa scanner (GE Medical Systems, Milwaukee). Scans were acquired on both groups over the same time period (1994–2005). After each major software/hardware upgrade, the reliability of the data before and after the upgrade was tested by scanning a set of subjects before and after the upgrade.[12,27] Quantification of MRI images was performed by means of a highly reliable fully automated process that determines gray and white matter volumes for the frontal, temporal, parietal and occipital lobes, as well as caudate and cerebellum volumes, as described elsewhere.[28] For this analysis, we had 59 COS subjects with both genotype and usable MRI data from at least one scan, with a total of 135 scans (75% had at least two scans; three individuals had as many as five scans), as well as 165 healthy individuals with 379 scans (65% had at least two scans; three individuals had as many as six scans).

Nucleic acid purification and PCR

Genomic DNA was extracted from immortalized lymphoblastoid cells using the QIAamp DNA Extraction Kit (Qiagen Inc., Valencia, CA, USA). SNPs in and around the *NRG1* gene were selected from previous publications, as well as dbSNP and HapMap databases and are detailed in Table 1. Given the iterative process of genotyping completed throughout this study, we designed *Taq*Man primers and probes for eight SNPs using Primer Express Software (Applied Biosystems www.appliedbiosystems.com), but most SNPs were ordered through Assays by Design (Applied Biosystems) and we used standard polymerase chain reaction (PCR) protocols. In addition, three SNPs were genotyped through direct sequencing.

Statistical analysis

Risk/genetic statistics. We tested for non-inheritance errors with Pedcheck and double-recombinants with the program MERLIN.[29] In all, only 10 (0.089%) of the 11 244 genotypes analyzed were omitted because of violation of Mendelian patterns of inheritance. Genotype drop-out rates due to PCR failures ranged from 0.5 to 5% for each individual marker. In addition, we included two duplicate DNAs on each PCR plate, and all genotypes agreed 100%. We measured linkage disequilibrium (LD) between markers with the D' and r^2 statistics from parental haplotypes (separately for Caucasians and African-Americans) by use of the program HaploView[30] and LD plot created with LocusView (T Petryshen, A Kirby, M Ainscow, unpublished software). None of the SNPs deviated from Hardy–Weinberg equilibrium. Phase known transmission disequilibrium test (TDT), where counts of allele transmissions from heterozygous parents at each SNP locus as well as multi-marker haplotypes were analyzed with the TDTPHASE program version 2.37.[31] We tested all two-, three- and four-marker haplotypes for association in a sliding window across the locus, dropping rare haplotypes below 3%. Tests for gene–gene interaction were completed with the multi-locus genotype-pedigree disequilibrium test (geno-PDT) option in PDTv5.1.[32] We carried out tests of association to quantitative traits using the program quantitative transmission disequilibrium test (QTDT), which performed variance-components testing of family-based samples for association and transmission disequilibrium.[33] The orthogonal model used is robust to population stratification. In order to protect against possible inaccuracies due to deviations from either normality or selection on the trait, empirical *P*-values derived from 10 000 permutations are reported.

MRI-anatomic data

Mixed-model regression was used to examine total and lobar volumetric developmental trajectories in relationship to risk allele status and diagnosis. Mixed-model regression was chosen over traditional methods, such as repeated measures analysis of variance, as it permits the inclusion of multiple measurements per person, missing data and irregular intervals between measurements, thereby increasing statistical power. F-tests or *t*-tests were used to determine whether cubic, quadratic or constant growth models best fit the data. For healthy controls, a cubic model was appropriate to model the trajectory of change in gray matter volumes, and a quadratic model for change in white matter. For the smaller COS group, a linear model was appropriate to model change in gray matter volumes and a quadratic model for white matter change. Fixed-effects parameter estimates were used to generate fitted values for graphing group trajectories.

The difference in gray and white matter total volumes was also determined using a mixed-effects model, entering group (using pairwise group contrasts) and age as fixed factors, and the individual as a random effect.

Results

The genotyping completed in this project was carried out in several stages. Initially, we genotyped two SNPs (SNP8NRG221533 and SNP8NRG243177) from the original report by Stefansson *et al.*,[4] and found a positive association with SNP8NRG221533 ($P = 0.04$), although it was with the opposite allele as the one associated with schizophrenia in that report, so we opted to genotype more markers. We genotyped additional markers from the Icelandic haplotype and

Table 1 Marker information and TDT results for single markers and haplotypes

Marker no.	*ID*	*Location UCSC May 2004 Freeze*	*Inter-marker distance*	*T/NT*	*TDTPHASE results*			
					Single locus	*2haps*	*3haps*	*4haps*
					Global P-*values*			
M1	rs2189145	31576593		22/21	0.890			
M2	rs4513929	31589021	12 428	24/20	0.546	0.687		
M3	SNP8NRG221132	31593282	4261	6/6	1.000	0.535	0.840	
M4	SNP8NRG221533	31593683	401	24/12 (T)	**0.043**	0.149	0.258	0.582
M5	rs10096573	31595198	1515	26/17	0.168	0.125	0.256	0.388
M6	rs4268090	31600430	5232	29/17	0.075	0.107	0.241	0.408
M7	rs4452759	31606992	6562	21/20	0.876	0.099	0.094	0.477
M8	rs4733263	31610016	3024	27/17	0.130	0.205	0.454	0.204
M9	rs4476964	31612210	2194	17/11	0.255	0.235	0.438	0.582
M10	SNP8NRG241930	31613877	1667	19/15	0.492	0.104	0.236	0.271
M11	SNP8NRG243177/rs6994992	31615123	1246	28/23	0.480	0.423	0.362	0.191
M12	rs7819063	31618950	3827	19/18	0.869	0.715	0.969	0.475
M13	rs4733267	31624181	5231	22/15	0.248	0.576	0.661	0.809
M14	rs11783236	31635896	11 715	30/25	0.500	0.377	0.236	0.053
M15	rs7000831	31637636	1740	14/8	0.198	0.115	0.151	0.170
M16	rs12677942	31659484	21 848	13/12	0.842	0.340	0.325	0.205
M17	478B14-848 (microsatellite)	31708358	48 874	21/15 (219)	0.198	0.339	0.463	**0.032**
M18	rs10503887	31752989	44 631	14/12	0.695	0.317	0.374	0.117
M19	rs1354335	31760521	7532	8/7	0.796	0.846	0.528	0.690
M20	rs2881272	31766011	5490	25/13 (A)	**0.050**	0.077	0.090	0.119
M21	420M9-1395 (microsatellite)	31784981	18 970	25/10 (274)	**0.015**	0.066	0.073	**0.007**
M22	rs1566778	31825136	40 155	30/22	0.266	**0.011**	**0.009**	**0.018**
M23	rs2062057	31826686	1550	13/12	0.842	0.378	**0.019**	**0.015**
M24	rs776385	31856167	29 481	28/17	0.099	0.372	0.119	**0.014**
M25	rs1503489	31896595	40 428	24/22	0.768	**0.038**	0.154	0.197
M26	rs800501	31902481	5886	11/10	0.827	0.414	0.099	0.212
M27	rs733230	31922124	19 643	14/13	0.847	0.509	0.214	0.079
M28	rs7002063	31923076	952	21/18	0.631	0.749	0.551	0.356
M29	rs383632	31925590	2514	7/6	0.781	0.802	0.620	0.176
M30	rs2200033	31935212	9622	23/13	0.093	0.262	0.423	0.383
M31	rs327417	31953192	17 980	15/4 (A)	**0.009**	**0.023**	**0.036**	0.147
M32	rs327325	31988398	35 206	26/15	0.084	0.138	**0.004**	**0.002**
M33	rs1599677	32022977	34 579	22/19	0.639	0.249	0.083	0.086
M34	rs7818326	32062914	39 937	26/16	0.121	0.185	0.118	**0.005**
M35	rs10503899	32067026	4112	25/17	0.216	0.281	0.057	0.136
M36	rs11776959	32068406	1380	26/20	0.376	**0.017**	**0.004**	**0.0004**
M37	rs7007662	32072287	3881	24/14	0.103	0.584	**0.030**	**0.012**
M38	rs1481756	32086787	14 500	27/20	0.306	0.287	0.550	**0.037**
M39	rs10808322	32090198	3411	22/19	0.639	0.629	0.546	0.589
M40	rs1383964	32123716	33 518	22/22	1.000	0.365	0.441	0.447
M41	rs4733306	32164650	40 934	18/15	0.601	0.953	0.650	0.527
M42	rs901561	32263004	98 354	24/16	0.204	0.560	0.542	0.730
M43	rs939077	32365080	102 076	24/22	0.768	0.698	0.525	0.962
M44	rs10093464	32470677	105 597	34/26	0.301	0.876	0.960	0.581
M45	rs3924999	32572900	102 223	19/19	1.000	0.500	0.344	0.764
M46	rs11780520	32592812	19 912	7/4	0.363	0.713	0.725	0.769
M47	rs2439272	32612634	19 822	25/23	0.773	1.000	0.997	0.742
M48	rs2954041	32642168	29 534	6/3	0.313	0.708	0.210	0.622
M49	rs764059	32650834	8666	15/10	0.316	0.418	0.843	0.551
M50	rs6988339	32665458	14 624	31/27	0.599	0.283	0.603	0.868
M51	rs3751929	32702209	36 751	13/12	0.842	0.848	0.337	0.603
M52	rs4262285	32702243	34	7/4	0.366	0.650	0.820	0.435
M53	rs9297196	32716957	14 714	23/21	0.763	0.868	0.631	0.852
M54	rs7007436	32721934	4977	28/28	1.000	0.866	0.923	0.315
M55	rs10503929	32733525	11 591	9/8	0.819	0.899	0.927	0.928
M56	rs6992642	32743929	10 404	26/24	0.777	0.635	0.531	0.674

Abbreviations: NT = not transmitted; T = transmitted; TDT, transmission disequilibrium test; UCSC, University of California, Santa Cruz.
Note: All *P*-values < 0.05 are in bold and alleles positively associated with COS in single-marker analyses are in parentheses.

selected SNPs spanning the entire gene selected from the International HapMap Project (http://www.hapmap.org/) focusing on the 5′ region of the gene where the initial risk haplotype was identified (see details in Table 1) in order to refine the region of association in our sample. Further, we attempted to genotype and replicate the positive SNP associations reported by Petryshen *et al.*[34] However, among the four 3′ SNPs that showed evidence of association in the Portuguese sample studied by Petryshen *et al.*,[34] rs6988339 and rs4262285 produced spurious results with the Taqman assay we received from ABI's Assays-By-Design, so we directly sequenced them (www.polymorphic.com). All of the genotypes derived from the sequencing followed patterns of Mendelian inheritance. Several of the SNPs had minor allele frequencies < 0.10 (rs2466058, rs3751929, rs4262285, rs16879878) so were not used for further analysis, including one novel SNP discovered 81 bp upstream of rs3751929. Ultimately, a total of 56 SNPs and microsatellite markers spanning the NRG1 locus were genotyped including four SNPs and two microsatellites contained in the Icelandic 'core haplotype' in the 5′ region described by Stefansson *et al.*[4] Figure 1 displays the LD map of the markers genotyped in this study derived from the 65 Caucasian parents in

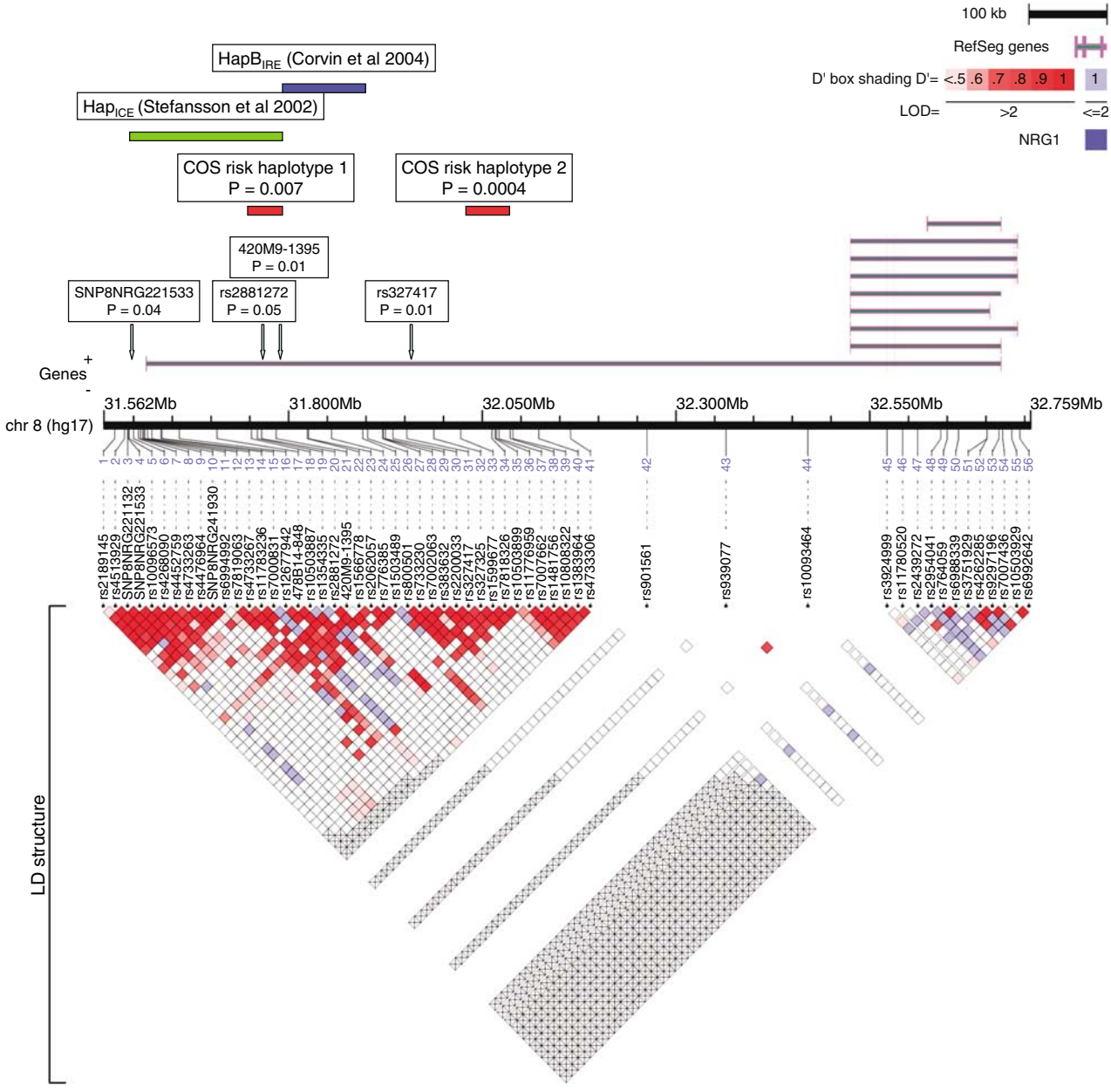

Figure 1 Schematic of LD (in Caucasians) at the NRG1 locus using markers genotyped in the current study, and significant single-marker (arrows) and haplotype (red bars) associations. Also included for reference are the locations of the risk haplotypes identified in the Icelandic (green bar) and Irish (blue bar) schizophrenia populations.

our sample. We also show locations of significantly associated markers and haplotypes.

In addition, given the evidence for a functional and behaviorally relevant interaction between NRG1 and erbB4,[17,18] we genotyped two SNPs in erbB4, rs3748962 and rs4673628. There was no evidence for association with either of these SNPs and COS by TDT, $P = 0.53$ and $P = 0.75$, respectively. Further, using multi-geno-PDT, there was no evidence for statistical interaction with either of the erbB4 SNPs and any of the NRG1 markers, all *P*-values > 0.05 (results not shown).

Several individual markers and haplotypes showed evidence of significant association with COS (see Table 1). SNP8NRG221533, which gave the single best-uncorrected association in the original report by Stefansson *et al.*,[4] also showed association in our sample ($P = 0.04$), although we observed overtransmission of the 'T' allele rather than the 'C' allele, as also reported for a Dutch schizophrenia sample.[35] We also found evidence for association between the core haplotype, comprised of SNP8NRG221533, 478B14-848 and 420M9-1395, global $P = 0.05$. However, the strongest evidence for association in our sample was observed with several novel four-marker haplotypes. The first encompassed markers M079-M080-M021-420M9-1395, spanning approximately 32 kb (global *P*-value = 0.007), and the most significant, M095-M096-M097-M098, spanning about 45 kb (global *P*-value = 0.0004) (see Figure 1). Details of over- and under-transmitted haplotypes for these haplotypes are shown in Table 2. The first risk haplotype we identified, which overlaps with the 3′ end of the Icelandic haplotypes, showed overtransmission of the most common haplotypes with 15 transmissions versus two non-transmissions, $P = 0.0008$. The next most common haplotypes was significantly under-transmitted, $P = 0.01$. There was a second set of haplotypes approximately 238 kb downstream of the first risk haplotypes. However, in this case, there were two haplotypes that were significantly over-transmitted and one haplotype that was under-transmitted.

In addition to TDT, we also carried out association analyses with quantitative endophenotypes that appeared clinically relevant in our sample. To minimize multiple testing issues, we limited analyses to the marker that showed the strongest and most consistent association with the diagnosis of schizophrenia, microsatellite 420M9-1395. All haplotypes that contained the 274-bp risk allele, which was part of the Icelandic risk haplotypes (coded 0 in the original

Table 2 Detailed results of significantly associated four-marker haplotypes

(a) COS risk haplotype 1 overlaps with Hap_{ICE}
Four-marker (M18–M21) haplotype approx. 32 kb (31.75–31.78)
LRS = 21.12, d.f. = 8, $P = 0.007$
Reference haplotype is T–C–T–276

Haplotype	*Frequency*	*T*	*Freq-T*	*NT*	*Freq-NT*	*RR*	*%T*	χ^2	P-*value*
G–C–T–274	**0.24**	**15**	**0.33**	**2**	**0.04**	**6.60**	**88.24**	**11.25**	**0.0008**
G–C–A–272	**0.22**	**5**	**0.11**	**19**	**0.42**	**0.45**	**20.83**	**6.70**	**0.010**
T–C–T–276	0.17	7	0.16	6	0.13	1.00	53.85	0.06	0.808
G–C–T–276	0.10	4	0.09	2	0.04	0.49	66.67	0.68	0.410
G–G–T–274	0.06	2	0.04	3	0.07	0.84	40	0.20	0.654
G–C–T–280	0.05	4	0.09	4	0.09	0.36	50	0.00	1.000
G–C–T–278	0.04	2	0.04	4	0.09	0.06	33.33	0.68	0.410
G–G–T–282	0.03	2	0.04	1	0.02	6.24	66.67	0.34	0.560
G–C–A–270	0.03	3	0.07	0	0.00	—	100	4.16	0.041

(b) COS risk haplotype 2 overlaps EST cluster with unknown function
Four-marker (M33–M36) haplotype approx. 45 kb (32.02–32.07)
LRS = 26.3498, d.f. = 7, $P = 0.0004$
Reference haplotype is G–A–G–G

Haplotype	*Frequency*	*T*	*Freq-T*	*NT*	*Freq-NT*	*RR*	*%T*	χ^2	P-*value*
G–A–G–G	0.28	11	0.21	12	0.23	1.00	47.83	0.04	0.847
A–G–A–A	**0.20**	**6**	**0.12**	**14**	**0.27**	**0.25**	**30.00**	**3.29**	**0.070**
A–A–A–A	**0.14**	**12**	**0.23**	**2**	**0.04**	**17.51**	**85.71**	**7.93**	**0.005**
A–G–A–G	0.11	5	0.10	7	0.13	0.33	41.67	0.29	0.592
G–A–A–A	0.10	5	0.10	6	0.12	0.65	45.45	0.09	0.763
A–A–A–G	0.08	4	0.08	8	0.15	0.69	33.33	1.17	0.279
G–A–G–A	**0.05**	**6**	**0.12**	**0**	**0.00**	**—**	**100.00**	**8.32**	**0.004**
G–A–A–G	0.03	3	0.06	2	0.04	2.93	60.00	0.20	0.654

Abbreviations: EST, expressed sequence tag; LRS, likelihood ratio statistic; NT, not transmitted; RR, relative risk compared to reference haplotype selected to be closest to 50% transmission ratio; T, transmitted.
Haplotypes in bold have $P < 0.05$.

report), were significantly associated with COS; therefore, we utilized the conceptualization that the 274-bp risk allele observed in the current sample functions as a tag of many potential risk haplotypes. Therefore, all other alleles were collapsed to create a dichotomous 'risk' and 'no-risk' genotype classification. Any individual with at least one 274-allele was considered 'risk'. Using QTDT, there was an association between transmission of the risk allele and early childhood social withdrawal ($P = 0.025$) and poor peer relationships ($P = 0.025$) assessed with the PAS (see Subjects and methods). Further there was a trend for association with a lower age of onset ($P = 0.07$). No other associations with IQ, negative or positive symptom scores, or severity were observed.

Table 3 provides detailed characteristics and the number of scans for COS patients and healthy controls by risk allele status. Within the COS and healthy control groups, there were no significant differences in age, gender, IQ, SES or ethnicity by risk allele status. Across groups, there was a significant main effect of risk allele status, with carriers having significantly larger gray and white matter volumes ($P = 0.001$ and 0.04, respectively). Within group, the effects of genotype on lobar volumes were confined to gray matter volumes in the COS patients in whom possession of a risk allele was associated with increased overall gray matter. However, in keeping with the presence of the significant main effect for risk allele status, there were trends among the healthy controls for a similar increase in gray matter volume among risk allele carriers (in the temporal and occipital lobes), suggesting that the COS patients show an exaggeration of the normative effects on lobar volumes of possession of the 'risk' allele (see Figure 2).

There were more striking effects of genotype on the trajectories of lobar change (Table 4). Across groups, there was a significant main effect of risk allele status, with carriers having significantly larger gray and white matter volumes. Within the COS patient group,

Table 3 Descriptive statistics and summary of ages and number of MRI brain scans in COS and healthy control subjects by NRG1 risk allele status

	Risk allele carriers		*No risk alleles*		P-*value**
COS total N = 59 with genotype and scan data					
Mean IQ (s.d.)	30	77.3 (18.6)	24	69.8 (18.0)	0.14
Gender (%male)	35	66%	24	46%	0.13
Ethnicity (%white)	35	71%	24	58%	0.30
Mean SES (s.d.)	34	59.26 (32.3)	21	70.14 (30.5)	0.22
Scans	N	*Mean age (s.d.)*	N	*Mean age (s.d.)*	
Time 1	35	13.9 (2.7)	24	15.1 (2.4)	0.10
Time 2	25	17.3 (2.2)	19	17.6 (3.1)	0.64
Time 3	17	19.6 (2.7)	6	18.4 (3.2)	0.33
Time 4	4	20.2 (0.9)	2	22.8	0.12
Time 5	3	23.6			NA
Total no. of scans	84		51		
Healthy controls, total N = 165 with genotype and scan data					
Mean IQ (s.d.)	118	112.5 (12.5)	29	112.8 (13.3)	0.92
Gender (%male)	131	58%	34	59%	0.93
Ethnicity (%white)	131	85%	34	79%	0.46
Mean SES (s.d.)	129	38.54 (18.2)	33	40.45 (16.5)	0.56
Scans	N	*Mean age (s.d.)*	N	*Mean age (s.d.)*	
Time 1	131	12.7 (6.8)	34	13.7 (6.5)	0.43
Time 2	86	13.4 (5.6)	21	15 (4.1)	0.21
Time 3	56	15.6 (4.8)	13	16.2 (3.9)	0.66
Time 4	24	18.1 (6.2)	2	17.7 (1.9)	0.93
Time 5	8	18.5 (6.1)	1	22.5	NA
Time 6	3	23.1			NA
Total no. of scans	308		71		

Abbreviations: COS, childhood-onset schizophrenia; IQ, intelligence quotient; MRI, magnetic resonance imaging; NA, not applicable; NRG1, neuregulin 1; SES, socio-economic status.
**P*-value from comparisons within diagnostic, across allele status groups: *t*-test for continuous variables such as age, IQ and SES or χ^2 for nominal variables such as gender and ethnicity.

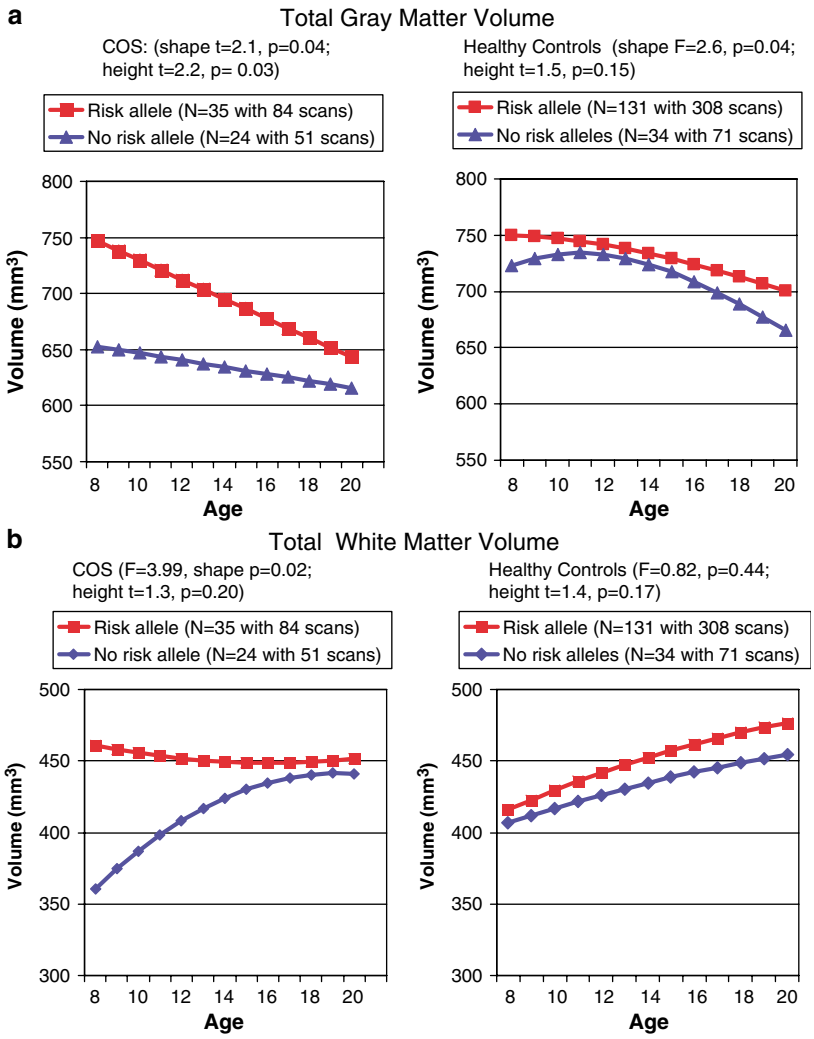

Figure 2 Effect of risk allele status at 420M9-1395 on brain MRI volume trajectories in COS and healthy controls for total (**a**) gray matter and (**b**) white matter.

possession of the risk allele was associated with a steeper rate of gray and white matter loss compared to those without a risk allele. Thus, while the COS risk allele carriers started with greater lobar volumes in childhood, by adolescence the volume difference between those with and without the risk allele had largely resolved. This pattern held throughout the frontal, temporal and parietal lobes. By contrast, in healthy individuals, possession of at least one risk allele was associated with different trajectories in gray matter only, and was confined to the frontotemporal regions. Direct comparisons of the trajectories across the diagnostic groups were not made as different models were used for each group (cubic for the healthy controls and linear for the COS sample, reflecting the differences in sample size).

Discussion

We report an independent replication with neuregulin, the first in a sample of patients with COS. It is notable that this relatively small, ethnically heterogeneous population could provide such a finding and supports other evidence that COS is genetically related to the more common adult-onset form of the disorder.[36–38] Further, the analysis of risk genotypes and neurodevelopmental trajectories throughout adolescence represents a unique endophenotype that could provide insight into the pathogenesis of specific genes in schizophrenia. Intriguingly, possession of an NRG1 risk allele had somewhat different effects on brain development trajectories of both gray and white matter in the COS patients as compared to healthy controls. Finally, the association with premorbid developmental delays/dysfunction supports a general influence on earlier brain development before the age range for our MRI measurements.

The observation of association with the opposite allele to the one initially reported by Stefansson and others for SNP8NRG221533 was disconcerting; however, a recent report from Bakker *et al*.[35] reported overtransmission of the T allele as well. In the

Table 4 Differences between carriers and non-carriers of the 'risk' allele for both the trajectory of lobar development (shape) and total volume (height)

Lobe	*COS*		*Healthy controls*	
	Shape	*Volume*	*Shape*	*Volume*
Gray matter				
Frontal	$T=2$, $P=0.05$	$T=1.4$, $P=0.16$	$F=3.1$ $P=0.03$	$T=1.4$, $P=0.16$
Parietal	$T=1.9$, $P=0.06$	$T=2.7$, $P=0.008$	$F=2.4$, $P=0.07$	$T=1.4$, $P=0.17$
Temporal	$T=2.5$, $P=0.02$	$T=2.5$, $P=0.02$	$F=3.7$, $P=0.01$	$T=1.6$, $P=0.10$
Occipital	$T=0.9$, $P=0.38$	$T=1.1$, $P=0.03$	$F=2.1$, $P=0.09$	$T=1.9$, $P=0.05$
White matter				
Frontal	$F=3.9$, $P=0.02$	$T=0.46$, $P=0.64$	$F=0.65$, $P=0.52$	$T=1.3$, $P=0.20$
Parietal	$F=3.0$, $P=0.05$	$T=1.7$, $P=0.10$	$F=0.86$, $P=0.42$	$T=1.3$, $P=0.20$
Temporal	$F=3.1$, $P=0.05$	$T=0.7$, $P=0.49$	$F=1.4$, $P=0.25$	$T=1.6$, $P=0.11$
Occipital	$F=0.53$ $P=0.59$	$T=1.0$, $P=0.32$	$F=0.81$, $P=0.44$	$T=2.5$, $P=0.01$

Abbreviation: COS, childhood-onset schizophrenia.
Where there was a significant difference in volume, the carriers of the 'risk' allele had a greater volume. The trajectories for gray or white matter by individual lobe resembled the trajectories shown in Figure 2 for total gray or white matter.

schizophrenia genetics literature, differing allele, SNP and haplotype associations across studies are common[39] and complicate the interpretation of these findings. Clearly, these statistical associations still require biological evidence to support them. Although our tests for interaction between NRG1 and erbB4 were negative, sample size limitations may have led to a false-negative finding given the recent report from a large case–control study in the UK.[18] Further, we genotyped only two SNPs in the erbB4 locus, which spans over 1 Mb in length, so clearly this was not a comprehensive study of this gene.

In the initial report of NRG1 association with schizophrenia, Stefansson *et al.*[4] reported that the 190 kb core haplotype represented a large block of LD. However, as denser SNP maps have become available, the extent of LD is not so clear, and there seems to be considerable variation across populations.[40] The first risk haplotype identified in this study overlaps with the original Icelandic haplotypes, with the same risk allele in the anchoring 3′ marker, 420M9-1395; however, the COS haplotype is smaller, covering a narrower region that is only ~32 kb, and spanning a more convincing region of LD. The second risk haplotype is ~238 kb downstream from the first haplotype, and encompasses a region where there are some expressed sequence tags (Hs.97362) of unknown function. This region deserves further investigation. Neither of these risk haplotypes is located in a region that is known to be involved in gene function, by either transcription or translation. Nonetheless, sequencing the regions for variants that may affect gene action may prove useful. Alternatively, it may be that no single variant confers risk for schizophrenia, but rather that a combination of variants, or haplotypes, together forms a background for susceptibility.

The neuregulins are at least four genes that together comprise a family of growth and differentiation factors for glia and neurons. NRG1, located on 8p12, is the best characterized of the neuregulin proteins and is spliced into many functional variants. At approximately 1.4 and 1.2 Mb, respectively, *neuregulin* is one of the largest genes to be sequenced in human and mouse genomes.[4] From this one gene, at least 15 different NRG isoforms are produced, and despite many differences, all isoforms share one characteristic epidermal-like growth factor domain that allows NRG proteins to bind with the tyrosine kinase receptor ErbB.[17] NRG complexes with ErbB outside the cell, which initiates the intracellular second messenger cascades that yield the varied effects of NRG on cell migration and development.[41] In culture studies, NRG stimulates the generation of neuronal precursors,[42] and radial glia transform into astrocytes only in the presence of NRG1 and its receptor.[43] Further, NRG1 is a likely modulator of neurotransmission *in vivo*, as Roysommuti *et al.*[44] demonstrated that NRG1 can potentiate glutamate neurotransmission at rat entorhinal-CA1 synapses.

The most striking finding in this study is the different trajectories of gray and white matter found among COS patients with or without the risk allele. Differences were most apparent in early childhood, perhaps in keeping with the phenotypic finding of more anomalous early development in COS among carriers of the risk allele. In both the COS patients and the healthy volunteers, possession of the risk allele was also associated with increased gray matter volumes, which was generally significant (at $P<0.05$) for the COS patients and at trend levels for the healthy volunteers. This is in keeping with an exaggeration among the COS patients of the effects of a 'risk' allele in typically developing children. The finding of increased gray matter volumes among

carriers of a 'risk allele' belies any simplistic link between a deleterious gene and smaller volumes. This pattern of accelerated volume loss in patients with COS who carry the risk allele is in keeping with the pervasive deleterious effects on brain development of a dysregulation in a gene that is critical to the growth of glia and neurons in a vulnerable population. The *NRG1* gene effect was pervasive in patients with COS, appearing in the frontal, temporal and parietal lobes, a pattern that stands in contrast to gene effects seen with SNPs in the GAD1 locus, which are limited to the frontal lobes.[36]

Limitations

NRG1 is a hugely complicated gene, with many transcripts and functions in various cell types spread throughout the body. Further investigation of this gene region, and the extremely large intron with apparent unknown function, particularly in relation to schizophrenia pathogenesis, is ongoing at several centers. Moreover, the cellular mediators of anatomic brain MRI measures are unknown, and thus hypotheses about gene function in this study remains speculative. Given the small sample size and number of marker tested in the current study, one must always consider the possibility of Type I error. Nevertheless, taken together with the ever-growing evidence for positive association between polymorphisms in NRG1 and schizophrenia, the prospect of 'spurious' association seems less likely. While we are fully aware that this sample size is small given today's standards, this sample is truly unique with regards to the phenotypic severity, extreme early onset of illness and collection of longitudinal brain scans during a critically informative time during brain development. Taken together, we believe there is much to be learned from careful examination of this sample with regards to genetic susceptibility to schizophrenia.

While this study was not designed as a case–control association study, several differences between the case and control groups raise possible questions about differences in allelic effects on brain development. In other studies, we have found that variation in intelligence is associated with differing developmental trajectories in localized frontal and temporal cortical regions, but has less marked effects on overall brain volumes across development.[45] Thus, it is unlikely that difference in IQ between the COS and healthy control groups contributes to the differing developmental trajectories for total gray and white matter we report here. Additionally, within the healthy control cohort used in the current study, there were no significant differences between Caucasians and non-Caucasians in the shape of gray or white matter developmental trajectories. The similarity in brain development of Caucasians and non-Caucasians makes it unlikely that the genotype-related differences we note are attributable to the differing racial composition of the COS and healthy control groups. Although the control subjects in this study had a higher overall frequency of the risk allele than the COS group (47 vs 32%, respectively), direct comparison is not appropriate given the differences in ethnic heterogeneity between the two groups: 84% of the controls were Caucasian, while only 50% of the COS patients were Caucasian. Of note, however, is that the frequency of this allele in our control group is consistent with other published studies examining this polymorphism.[46,47] Also noted in the literature is that this allele is rare, if not totally absent, from some Asian populations, so variations in allele frequency, as we observed here, are not unexpected. Further, given the rareness and difficulty of recruiting COS patients, the observed allele frequency may be a spurious finding reflective of the small sample, which might likely change if we were able to collect larger numbers. Finally, the brain trajectories suggest that maximal effects occur in very early childhood and future studies will need to evaluate this gene during fetal and infant development.

Summary

The present findings add to the replications of NRG1 as a risk gene for schizophrenia, and supports continuity between COS and adult-onset schizophrenia. In addition, the endophenotype association with developmental delays in infancy and toddler years, long before onset of psychosis, supports other evidence for the role of neuregulin in early brain development.

Acknowledgments

We would like to acknowledge and thank all of the patients and their families that have participated in our study.

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