Disease-associated intronic variants in the *ErbB4* gene are related to altered *ErbB4* splice-variant expression in the brain in schizophrenia

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The neuregulin 1 (*NRG1*) receptor, *ErbB4*, has been identified as a potential risk gene for schizophrenia. HER4/ErbB4 is a receptor tyrosine kinase whose transcript undergoes alternative splicing in the brain. Exon 16 encodes isoforms containing a metalloprotease cleavable extracellular domain (JM-a), exon 15 for a cleavage resistant form (JM-b) and exon 26 for a cytoplasmic domain (CYT-1) with a phosphotidylinositol-3 kinase (PI3K) binding site. Disease-associated variants in the *ErbB4* gene are intronic and implicate altered splicing of the gene. We examined *ErbB4* splice-variant gene expression in the hippocampus and dorsolateral prefrontal cortex (DLPFC) in schizophrenia using qPCR and investigated whether expression levels are associated with previously reported genomic risk variants in *ErbB4* in a large cohort of human brains. In the DLPFC, we confirmed previous observations, in a separate cohort, that mRNA for *ErbB4* splice isoforms containing exon 16 (JM-a) and exon 26 (CYT-1) are significantly elevated in patients with schizophrenia. A main effect of genotype was observed in the DLPFC and hippocampus at a single risk SNP located in intron 12 (rs4673628) on isoforms containing exon 16 (JM-a). We also found that three intronic risk SNPs (rs7598440, rs707284, rs839523) and a core-risk haplotype surrounding exon 3 are strongly associated with elevated expression of splice variants containing exon 26 (CYT-1). These findings suggest that dysregulation of splice-variant specific expression of *ErbB4* in the brain underlies the genetic association of the gene with schizophrenia and that the *NRG1*/*ErbB4* signaling pathway may be an important genetic network involved in the pathogenesis of the disease.

INTRODUCTION

Schizophrenia is a genetically complex psychiatric disorder. Evidence of association in several genes related to risk for schizophrenia has led to the identification of key protein networks and pathways potentially involved in the cell biology and ultimately the pathogenesis of the disorder. The neuregulin 1 (*NRG1*) gene located on 8p12.21 is one of the most promising candidates in schizophrenia genetics (1). The *NRG1* locus shows linkage to the disorder, and genetic association has been found between schizophrenia and various non-coding polymorphisms and haplotypes primarily at the 5′ end of the gene (1). Alterations in *NRG1* expression (2,3) and

NRG1-mediated signaling (4) have been identified as putative molecular mechanisms mediating the influence of *NRG1* upon schizophrenia risk.

The recent identification of the *NRG1* receptor, *ErbB4* as a candidate risk gene for the disease (5–7), has prompted the notion that other molecules in the *NRG1* signaling pathway may be involved in the disorder. Molecular genetic studies in separate populations have identified specific DNA variants in the *ErbB4* gene that are directly linked with risk for the disease. Norton *et al*. (5), performed a mutation screen of the *ErbB4* gene, including 5.5 kb of exonic, and 6 kb of flanking intronic sequence. They identified 15 sequence variants and provided evidence of modest association with
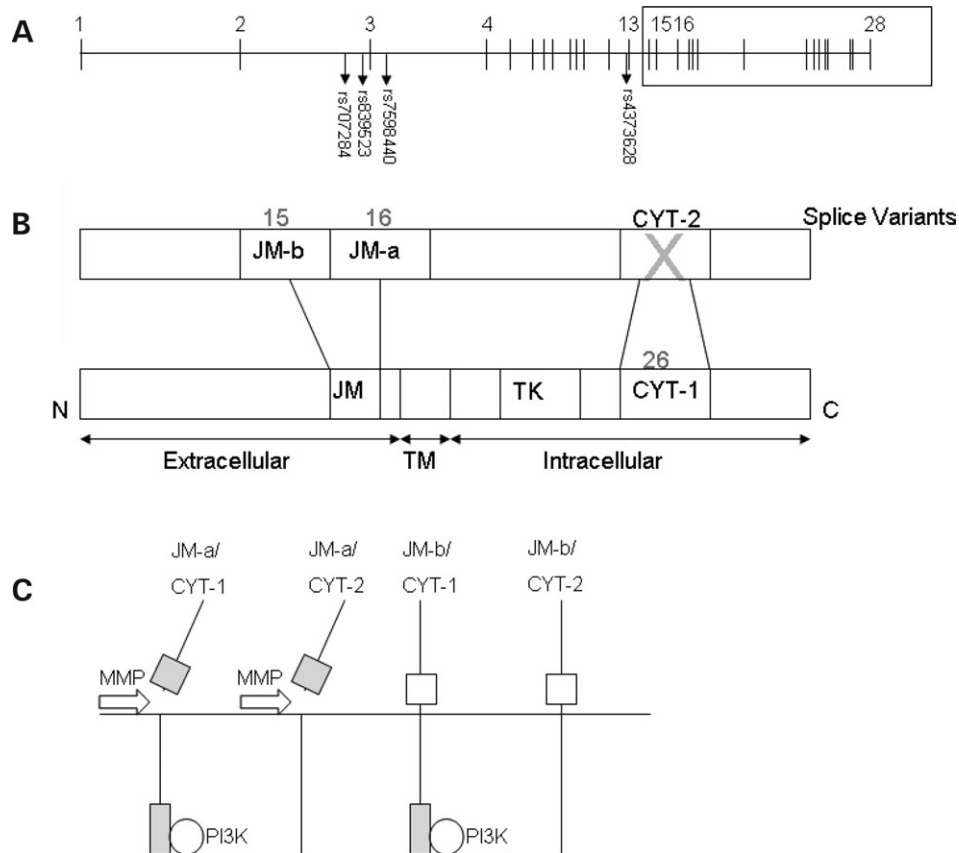
Figure 1. (**A**) Simplified schematic showing the exon structure of the 1.16 Mb *ErbB4* gene and the position of the risk SNPs examined in the study. Box indicates region enlarged in (B). (**B**) Schematic showing alternative ErbB4 splice variants. JM-a and JM-b are generated through inclusion of exon 16 or 15 and, CYT-1 and CYT-2 through inclusion or deletion of exon 26, respectively. TK, tyrosine kinase domain, TM, transmembrane domain. Spliced exons are numbered. (**C**) ErbB4 juxtamembrane and cytoplasmic isoforms. Four possible combinations of receptor exist. The ErbB4 JM isoforms are susceptible (JM-a) or resistant (JM-b) to cleavage by metalloproteases (MMP). The cytoplasmic isoforms differ in their coupling to the PI3K pathway. Both CYT isoforms couple to the Shc-MAPK pathway [adapted from (10)]. All drawings not to scale.

schizophrenia of a single non-coding SNP (rs4673628, IVS 12–14 C>T) located in intron 12 and a significant but complex genetic interaction between this SNP and the original NRG1 'Icelandic' risk haplotype (8) in a large mixed UK, Irish population. Individuals heterozygous for the *ErbB4* SNP (rs4673628) exhibited significantly elevated disease risk as the load of the *NRG1* haplotype increased. A separate study in the individuals of Ashkenazi Jewish decent confirmed a strong association of *ErbB4* with schizophrenia albeit to a different region of the gene. Allele, genotype and haplotype frequency analysis identified three SNPs (rs7598440, rs707284, rs839523) and a 46 kb core-risk haplotype comprised of these SNPs, surrounding exon 3 of the gene, which were strongly associated with the disease (6). Separate follow-up studies, in three independent family samples reported association with two 3 marker SNP haplotypes in the *ErbB4* gene and schizophrenia status, one haplotype which overlaps with the region reported by Silberberg *et al.* (6) and another in the 3′ end of the gene (7). Together, these studies provide strong preliminary evidence that *ErbB4* is a susceptible gene for schizophrenia.

ErbB4 is a member of the ErbB subfamily of type I receptor tyrosine kinases that regulate cell growth, proliferation and differentiation. The gene spans 1.15 Mb (megabases) on chromosome 2q34 and consists of 28 exons. An interesting feature of *ErbB4* is the presence of isoforms, created through alternative splicing of the *ErbB4* transcript (9,10). The JM-ErbB4 isoforms differ within their extracellular juxtamembrane domain with the inclusion of exon 16 (JM-a) or exon 15 (JM-b), which renders them susceptible or resistant, respectively, to processing by a metalloprotease/presenilin-dependent γ-secretase proteolytic pathway (Fig. 1; 11–13), a pathway known to regulate processing of other transmembrane proteins such as Notch and the amyloid precursor protein (APP; 14). The second pair of isoforms differ in their cytoplasmic tail region with the inclusion (CYT-1) or deletion (CYT-2) of 16 amino acids (exon 26). Both CYT-isoforms couple with the Shc-MAPK pathway, while CYT-1 has in addition a unique docking site that links it to the phosphotidy-linositol 3-kinase (PI3K) pathway (9,10,15; Fig. 1). The occurrence of these ecto- and cytoplasmic domain changes depicts that four structurally and functionally different isoforms of *ErbB4* exist (JM-a/CYT-1, JM-a/CYT-2, JM-b/CYT1 and JM-b/CYT2; Fig. 1A and B).

At the biological level, *ErbB4* is of interest to the pathophysiology of schizophrenia for several reasons. First, *ErbB4* is

highly expressed in neurons of the adult and developing human brain (16–18). Second, *NRG1*-mediated *ErbB4* signaling is implicated in neurobiological processes postulated to be disturbed in the disorder, including neurogenesis, synaptic plasticity, neuronal migration, axonal pathfinding, synapse formation and the regulation of GABAA and NMDA receptor-mediated neurotransmission (19–25). Third, mutant mice heterozygous for the *ErbB4* gene exhibit behavioral phenotypes similar to *NRG1* mutants and established rodent models of schizophrenia (8). And finally, alterations in *NRG1*-mediated *ErbB4* activation (4) and *ErbB4* splice isoform gene expression (6) have been reported in the brains of patients with the disease.

To date, no coding mutations have been identified in the *ErbB4* gene and the schizophrenia-associated risk SNPs are non-coding intronic variants (5–7). An increasing amount of evidence indicates that intronic genetic variations can have deleterious implications for gene splicing (26). Therefore, the disease-associated SNPs identified in *ErbB4* may be functional in terms of splicing regulation or alternatively they may monitor functional elements within the gene associated with schizophrenia risk. Evidence supporting the fact that altered expression, splicing or function of the *ErbB4* gene may underlie its association with schizophrenia comes from recent data demonstrating altered expression of JM-a and CYT-1 variant *ErbB4* splice isoforms in the dorsolateral prefrontal cortex (DLFPC) of individuals with the disease (6). In light of these earlier associations of *ErbB4* intronic SNPs and schizophrenia and evidence of abnormal levels of ErbB4 splice-variant mRNAs in brain tissue from schizophrenic patients combined with our previous observations of altered transcriptional regulation of the *NRG1* gene underlying genetic risk at *NRG1* (3), we hypothesized that genetic variations in *ErbB4* associated with schizophrenia would impact on the expression of splice variants of the *ErbB4* gene.

We examined mRNA abundance for *ErbB4* splice isoform variants JM-a, JM-b, CYT-1, CYT-2 and total *ErbB4* in a large cohort of human post-mortem brain samples including hippocampus and DLPFC, regions prominently implicated in schizophrenia and abnormalities of *NRG1/ErbB4* signaling in the disease (2–4,6). Examination of the effects of genetic variation on *ErbB4* splice isoform expression patterns comprised three intronic SNPs, which form a 46 kb risk haplotype, previously reported to show a strong association with schizophrenia [rs7598440, rs839523, rs707284 (6)], and a single intronic risk SNP (rs4673628) (5). Our findings demonstrate that genetic variants in the *ErbB4* gene potentially increase the risk for schizophrenia by influencing levels of alternative splice variants of the gene in the human brain.

RESULTS

CYT-1 and JM-a *ErbB4* splice-variant mRNAs are increased in the DLPFC in schizophrenia

In the DLFPC, mean mRNA expression for CYT-1 variant isoforms was increased by 38% in schizophrenic patients when compared with controls [Fig. 2A; $F(1,76) = 5.21$; $P = 0.025$] and JM-a variants were increased by 65% [Fig. 2B; $F(2,75) = 5.00$; $P = 0.028$] in the patient tissue.
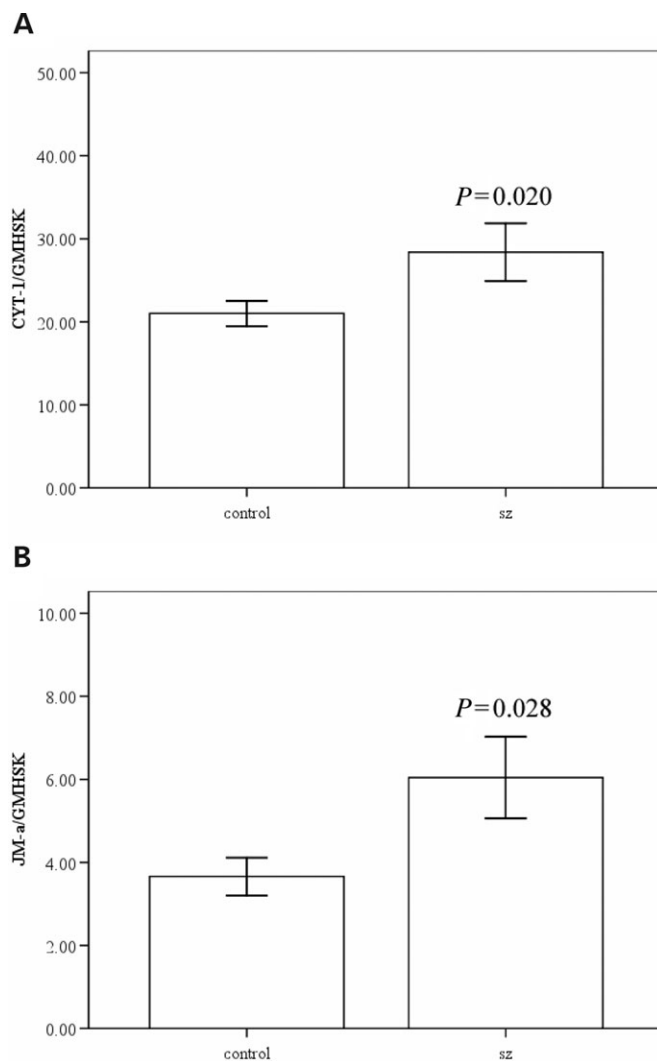

Figure 2. *ErbB4* splice-variant isoforms CYT-1 (**A**) and JM-a (**B**) are increased in the DLPFC of patients with schizophrenia. Quantitative RT-PCR analysis, normalized to the geometric mean of three housekeeping genes (GMHSK) (PBGD, SDH and cyclophilin). $N = 45$ normal control subjects and 32 patients with schizophrenia (sz). Mean ± SEM, ANOVA.

No significant differences were observed between diagnostic groups for splice-variant isoforms JM-b or CYT-2 [JM-b, $F(2,75) = 0.10$; $P = 0.74$; CYT-2, $F(2,75) = 0.71$; $P = 0.42$] in the DLPFC. Pan, *ErbB4* mRNA expression was also not significantly different between the groups [$F(2,75) = 1.4$; $P = 0.20$].

In the hippocampus, no alterations in the expression levels of any *ErbB4* splice-variant or pan *ErbB4* was observed between the diagnostic comparisons [all $F(2,75) < 0.50$; $P > 0.50$; Fig. 3]. No significant differences were observed between the diagnostic groups in either the DLPFC or hippocampus for the individual housekeeping genes (PBGD, SDH or GUSB), or the geometric mean of these genes used for normalization [DLPFC, $F(1, 76) = 1.2$; $P = 0.36$; hippocampus, $F(2,75) = 0.006$; $P = 0.93$, data not shown]. No correlations with neuroleptic medication status and CYT-1 or JM-a variant levels were observed in either the DLPFC or hippocampus.
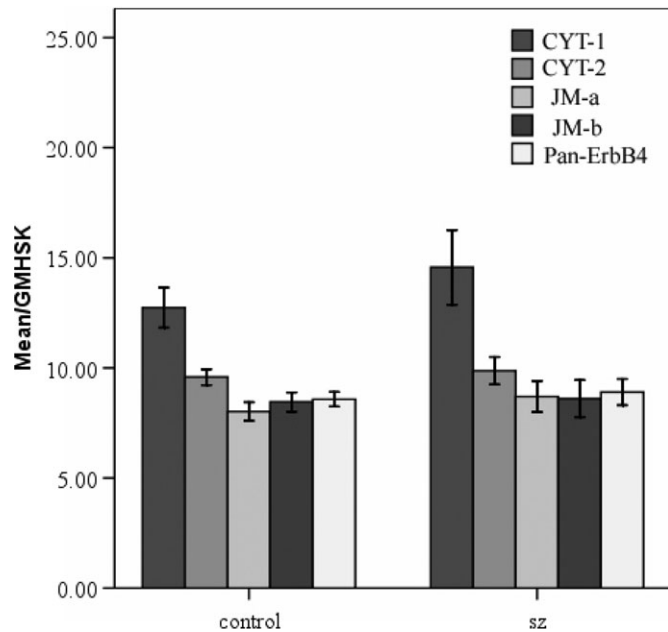
Figure 3. *ErbB4* splice isoforms are unaltered in the hippocampus in schizophrenic patients compared to normal controls. Quantitative RT-PCR analysis of CYT-1, CYT-2, JM-a, JM-b and 'pan' ErbB4 isoform variant expression normalized to the GMHSK of PBGD, SDH and GUSB. $N = 45$ normal control subjects and 32 patients with schizophrenia (sz). Mean ± SEM.

Effects of disease-associated SNPs on *ErbB4* splice-variant expression levels

Genetic variation at rs4673628 is associated with the expression of JM-a variant isoforms of *ErbB4*. Effects of genetic variation on expression levels of *ErbB4* isoforms were examined in the entire cohort of 75 controls and 40 schizophrenic patients. For investigation of JM-a variant isoforms, some individuals were missing from the analysis either due to missing qRT-PCR data or genotyping failure.

In the hippocampus, a main effect of genotype at rs4673628 was observed on the expression levels of JM-a variant isoforms $[F(5,91) = 4.64; P = 0.01; Fig. 4A]$. *Post hoc* comparisons revealed that individuals homozygous for the G allele exhibited lower levels when compared with individuals heterozygous (*post hoc* LSD test, $P = 0.03$) or homozygous for the A allele (*post hoc* LSD test, hippocampus, $P = 0.007; Fig. 4A$). No diagnosis × genotype interaction was observed and the effect was apparent in both schizophrenic patients and normal controls (Fig. 4B).

In the DLPFC, a trend for a main effect of rs4673628 on JM-a variant isoform expression was observed $[F(5,91) = 2.5; P = 0.06; Fig. 4C]$. A significant diagnosis × genotype was also observed in this region $[F(5,91) = 3.1; P = 0.04; Fig. 4D]$. *Post hoc* analysis revealed that the effect of rs4673628 was significant only in the patients but paralleled that seen in the hippocampus, whereby individuals homozygous for the G allele exhibited lower levels of JM-a variant expression when compared with those heterozygous (*post hoc* LSD, $P = 0.03$) or homozygous (*post hoc* LSD, $P = 0.03$) for the A allele (Fig. 4D). No other SNPs examined in this study were associated with JM-a variant isoform

expression and rs4673628 was not associated with any other *ErbB4* splice isoform. No effects of race or race × genotype interactions were observed in either the DLFPC or hippocampus.

Genetic variation at rs7598440, rs839523, rs707284 and a schizophrenia risk haplotype is associated with the expression of CYT-1 variant isoforms of *ErbB4*

In the hippocampus we found a main effect of genotype at each of the three single risk SNPs (rs7598440, rs839523, rs707284) and a 46 kb risk haplotype comprised of these three markers on abundance of CYT-1 variant isoforms. A main effect of rs7598440 was observed in the whole cohort $[F(5,96) = 5.1; P = 0.009]$ whereby individuals homozygous for the risk A allele exhibited elevated levels of CYT-1 variant isoform mRNA (50%>) compared with individuals heterozygous or homozygous for the G (non-risk) allele (Fig. 5). A main effect was also observed in the entire cohort for rs839523 $[F(5,96) = 3.5; P = 0.03; Fig. 5]$ and rs707284 $[F(5,91) = 5.5; P = 0.006; Fig. 5]$ with individuals homozygous for the risk G allele exhibiting elevated levels of CYT-1 variant isoform *ErbB4* mRNA (rs839523 > 30%, rs707284 > 30%). As the differences due to genotype are seen only in individuals homozygous for the risk alleles, these data suggest a potential 'recessive' effect at each of the individual SNPs on CYT-1 variant mRNA expression. No diagnosis × genotype interactions were observed, however the effects of allelic variation at each of the SNPs on splice isoform abundance appeared more pronounced in patients (Fig. 5, right hand column).

In the DLPFC, we also found a main effect of rs7598440 $[F(5,101) = 3.5; P = 0.03]$ on the abundance of CYT-1 variant isoforms, whereby again individuals homozygous for the risk A allele exhibited elevated levels (Fig. 6). We also observed a main effect of rs839523 $[F(5,100) = 9.2; P < 0.001]$. However, unlike the hippocampus, this effect was driven by individuals homozygous for the risk allele G having lower abundance of CYT-1 isoform variants. Similar findings were observed for rs707284 $[F(5,94) = 95.2; P < 0.007]$ whereby a diagnosis × genotype interaction was also observed $(P = 0.02; Fig. 6)$. In accordance with our observations in the matched sub-cohort, an effect of diagnosis was observed in the entire sample in the DLPFC as part of the ANOVA readout [ANOVA $F(5,100) = 8.5; P < 0.004$] whereby individuals with schizophrenia exhibited elevated levels of CYT-1 variant mRNA. No other SNPs examined in the study predicted CYT-1 splice isoform expression and rs7598440, rs839523 and rs707284 did not predict the expression of any other *ErbB4* splice isoform mRNA. No effects of race or race × genotype interactions were observed for CYT-1 variant mRNA levels in either the DLFPC or hippocampus. The negative control SNP (rs3748960) showed no association with any *ErbB4* splice isoform in either the controls or the schizophrenic patients $(F < 1.4; P > 0.8)$.

Haplotype analysis

Results of LD analysis between three markers (rs7598440, rs839523, rs707284) in African American and Caucasian
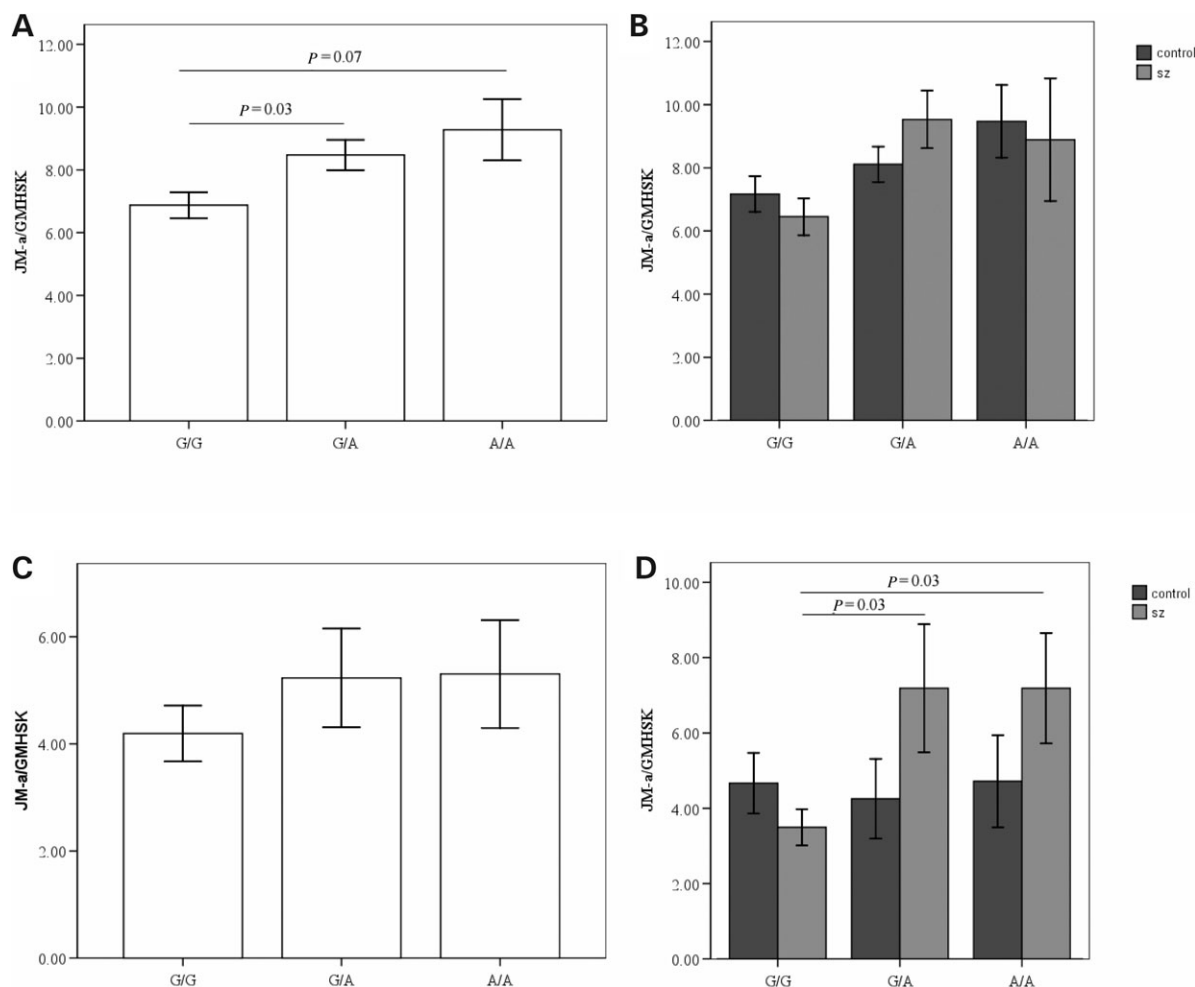
Figure 4. Association between rs4673628 and JM-a variant isoform mRNA levels in the hippocampus and DLPFC. (**A**) In the hippocampus, in the whole cohort a main effect of genotype was observed (ANOVA, $P = 0.01$). *Post hoc* comparisons revealed that individuals carrying the A allele (heterozygous or homozygous) exhibited higher levels of expression compared to G allele homozygotes; G/G, $N = 37$; G/A, $N = 37$; A/A, $N = 22$. (**B**) Data from the hippocampus at SNP rs4673628 parsed by diagnosis. No genotype × diagnosis interaction was observed. Mean ± SEM. (**C**) In the whole cohort a trend for a main effect of genotype at SNP rs4673628 was observed in the DLPFC (ANOVA, $P = 0.06$; G/G, $N = 37$; G/A, $N = 37$; A/A, $N = 22$). A significant diagnosis × genotype was observed in DLPFC ($P = 0.04$). (**D**) *Post hoc* analysis in the DLPFC revealed that the effect of rs4673628 on JM-a variant isoform expression was apparent in the patient group. Schizophrenic individuals homozygous (*post hoc* LSD, $P = 0.03$) or heterozygous (*post hoc* LSD, $P = 0.03$) for the A allele exhibited elevated levels of JM-a splice isoforms compared to schizophrenic individuals homozygous for the G allele. Mean ± SEM.

individuals and for controls and patients with schizophrenia separately, can be found in Supplementary Material, Table S1. The three markers (rs7598440, rs839523, rs707284) were in significant, but moderate, LD in all groups. Four common haplotypes were identified and the frequency of these can be seen in Supplementary Material, Table S2.

To test whether the schizophrenia risk haplotype reported previously (6), spanning 46 kb and consisting of the three single SNPs described above (rs7598440, rs839523 and rs707284), is associated with CYT-1 variant isoform abundance we used the program SNPHAP to assign haplotypes and a diplotype (haplotype pair) to each individual. Given the potential 'recessive' effect of the individual risk alleles on CYT-1 variant splice isoform levels, we predicted that individuals homozygous for the risk haplotype (AGG) would exhibit the greatest difference in expression. We thus compared AGG risk homozygotes (diplotype AGG/AGG), AGG carriers (diplotype AGG/non-risk) and non-risk carrying individuals. Consistent with our prediction, ANOVA revealed a main effect of the diplotype in the entire sample in the hippocampus [$F(3,91) = 6.1$; $P = 0.003$]. As suggested by the effects of the single SNPs, individuals homozygous for the risk haplotype (AGG/AGG) exhibited the highest levels of CYT-1 variant isoform mRNA, whereby a 61% higher abundance was observed compared with individuals carrying only one-risk haplotype or individuals with no-risk haplotype (*post hoc* LSD $P < 0.001$; Fig. 7A). Examination of the effects of the risk haplotype based on diagnostic group, showed that the effect was primarily apparent in the schizophrenic patients where a 107% increase in CYT-1 variant mRNA was seen in AGG homozygotes compared with AGG carriers or non-carriers (*post hoc* LSD; $P < 0.001$; Fig. 7B). This finding was reflected by a significant diagnosis by genotype interaction [$F(3,91) = 6.4$; $P = 0.002$].
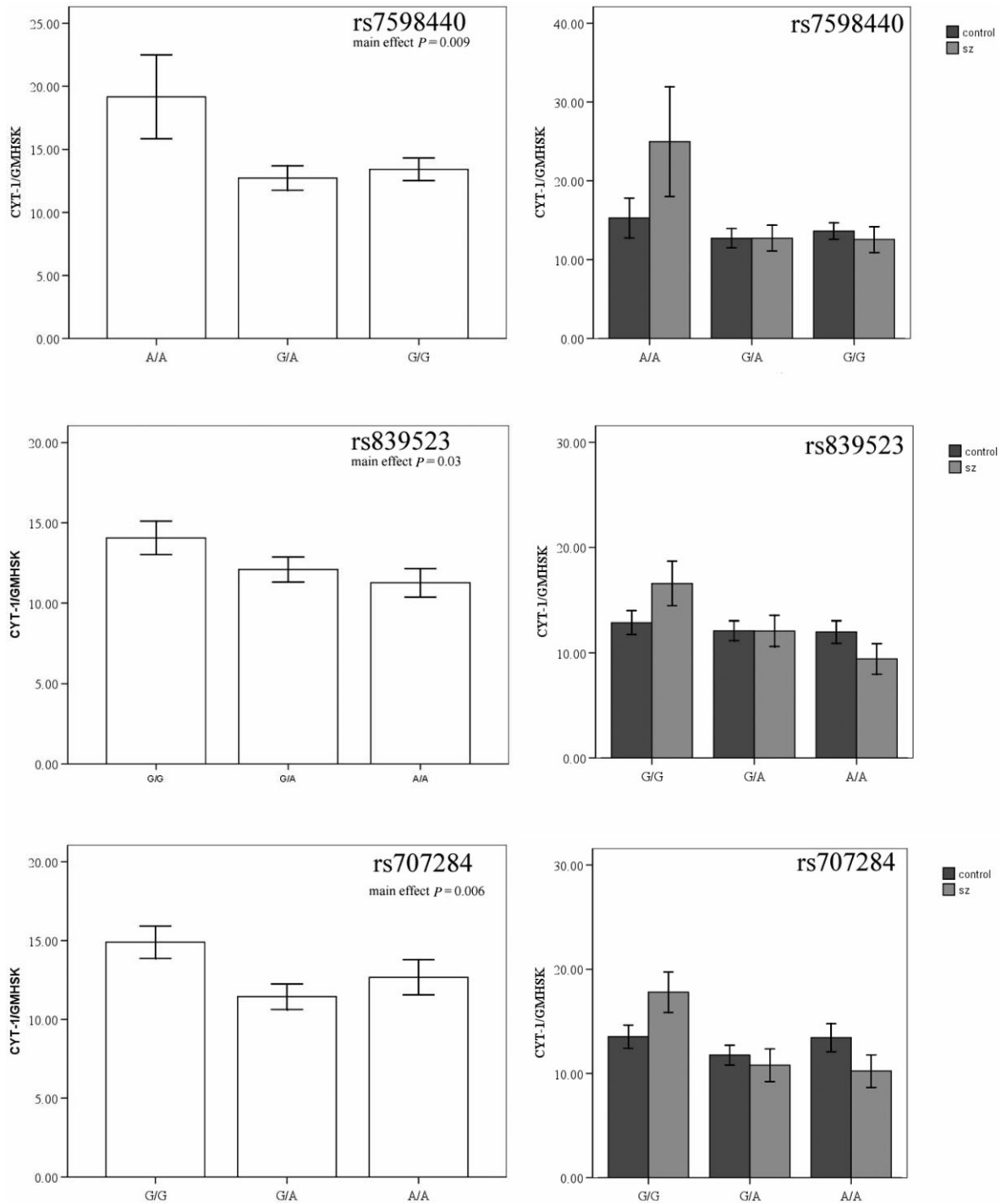
Figure 5. Association between schizophrenia risk variants (rs7598440, rs839523 and rs707284) and CYT-1 splice isoform levels in the hippocampus. Main effects of genotype on CYT-1 variant expression were observed for all SNPs in the whole group (left column). Data parsed by diagnosis (right column) Mean ± SEM. rs7598440: A/A, $N = 13$; G/A, $N = 48$; G/G, $N = 40$; rs83952: G/G, $N = 32$; G/A, $N = 50$; A/A, $N = 19$; rs707284: G/G, $N = 31$; G/A, $N = 44$; A/A, $N = 20$.

Comparable genetic effects were observed in the whole cohort in the DLFPC, where a main effect of the diplotype was found $[F(3,97) = 4.1; \ P = 0.02]$ on CYT-1 variant isoform mRNA levels (Fig. 7C). Individuals homozygous for the risk haplotype (AGG/AGG) exhibited elevated expression.

Again, the effect was pronounced in the schizophrenic patients, reflected by a significant diagnosis × genotype interaction $[F(3,97) = 5.3; \ P = 0.006; \ \text{Fig. 7D}]$. The AGG risk haplotype showed no association with any other *ErbB4* splice isoform in either the DLPFC or hippocampus ($P > 0.5$ for all isoforms).
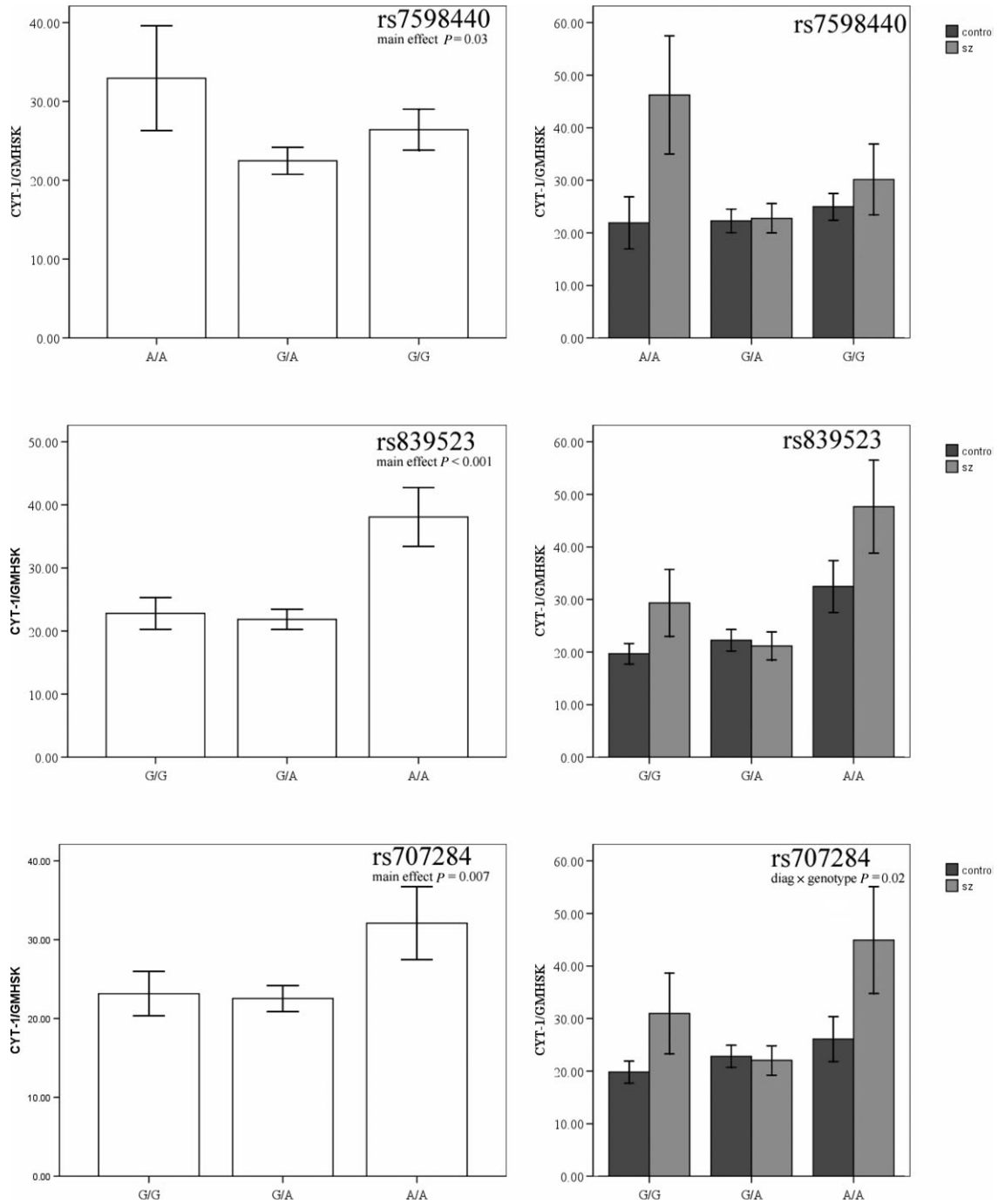
Figure 6. Association between schizophrenia risk variants (rs7598440, rs839523, rs707284) and CYT-1 splice isoform levels in the DLPFC. Left column, whole group; right column, data parsed by diagnosis. Mean $\pm$ SEM rs7598440: A/A, $N = 11$; G/A, $N = 53$; G/G, $N = 42$; rs83952: G/G, $N = 35$; G/A, $N = 52$; A/A, $N = 18$; rs707284: G/G, $N = 30$; G/A, $N = 48$; A/A, $N = 21$.

DISCUSSION

Molecular genetic, transgenic animal and postmortem human brain studies implicate *NRG1* and its receptor *ErbB4* in the etiology of schizophrenia (1,4–8). We have previously demonstrated that a molecular mechanism contributing to the genetic association of the *NRG1* gene with schizophrenia involves altered transcriptional regulation of a novel variant of the gene (3). Given the growing evidence implicating the *NRG1/ErbB4* network as a functionally and behaviorally relevant pathway in schizophrenia, we turned our investigation to the examination of *ErbB4* transcript variants. In this study, we demonstrate that expression of JM-a and CYT-1 *ERbB4* variant isoforms is upregulated in the DLPFC in schizophrenia but unchanged in the hippocampus in the disease. We report association of JM-a variant isoforms in
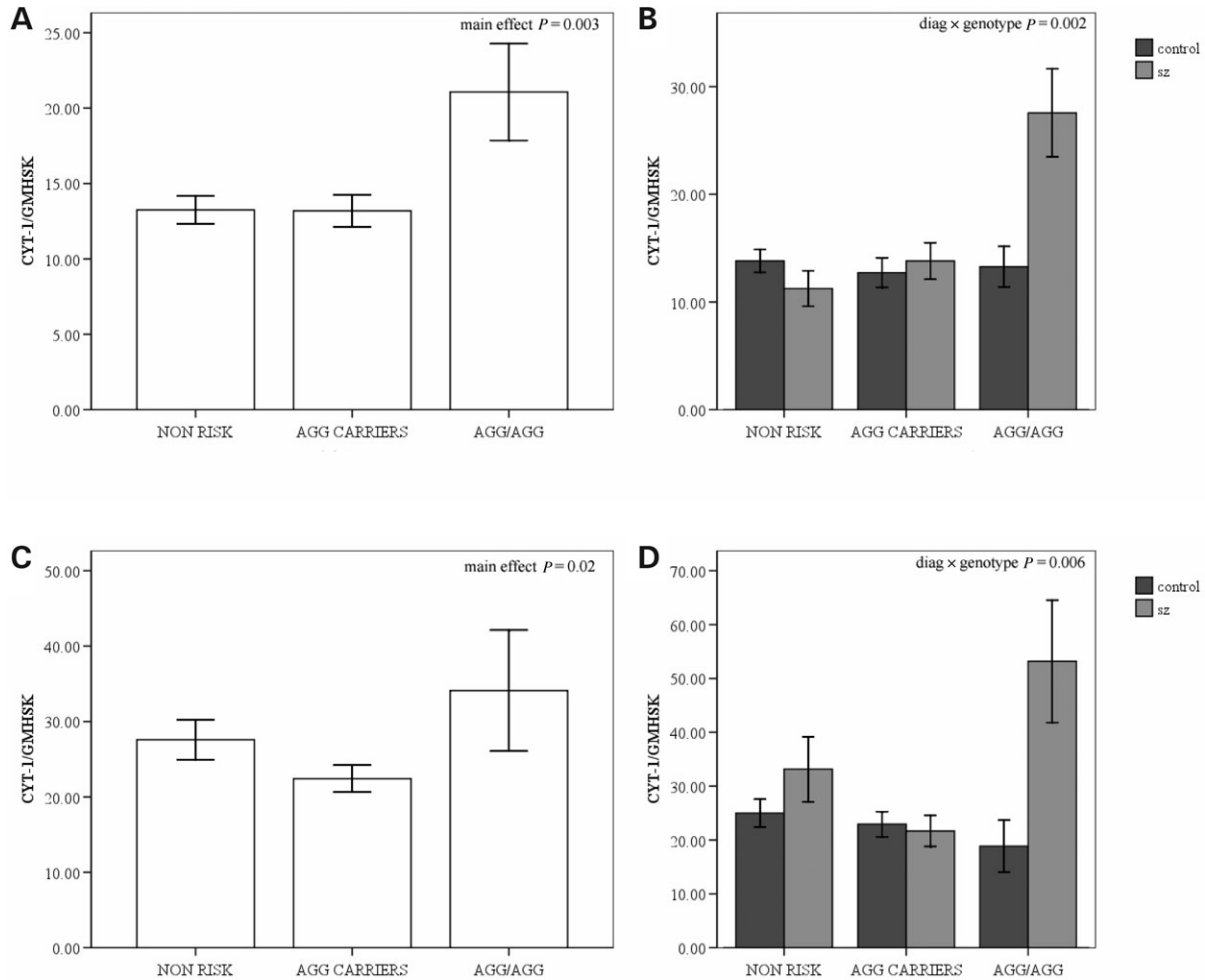
Figure 7. Association between diplotypes comprised of a 46 kb risk haplotype (AAG: rs7598440, rs839523, rs707284) and CYT-1 variant isoform levels in the hippocampus (**A** and **B**) and DLPFC (**C** and **D**). Individuals were divided according to diplotype into three groups, non-risk hap carriers (all others); risk hap carrier (AAG/hap2, 3 or 4) and risk hap homozygotes (AAG/AAG). A main effect of diplotype was observed in the whole cohort on CYT-1 variant isoform levels in the hippocampus (A; ANOVA, $P = 0.003$; Non-risk, $N = 40$; AAG carriers, $N = 42$; AAG/AAG, $N = 12$) and DLPFC (C; ANOVA, $P = 0.02$; Non-risk, $N = 43$; AAG carriers, $N = 47$; AAG/AAG, $N = 10$). Individuals homozygous for the risk haplotype had increased levels compared to individuals carrying one copy of the haplotype or none (A and C). (B and D) Effect of diplotype on CYT-1 variant levels, parsed by diagnosis. A diagnosis × genotype interaction was observed in both brain areas. Eleven individuals were not included in the diplotype analysis due to either failure of genotyping at one or more of the SNPs or low probability ($<93\%$) of diplotype assignment according to SNPHAP.

the DLPFC and in the hippocampus with a single-risk SNP located in intron 12 of the *ErbB4* gene and of CYT-1 variant isoforms with three single-risk SNPs comprising a 46 kb schizophrenia risk haplotype. Our results provide the first evidence of association between disease linked-variation in ErbB4 and differential expression of *ErbB4* splice-variant mRNAs in the human brain and suggest a molecular mechanism for the clinical associations.

In the DLFPC in schizophrenia, we observed a large increase (40–60%) in the expression of JM-a and CYT-1 variant isoform mRNAs and no alterations in any other splice isoform, suggesting that an *ErbB4* receptor that contains both JM-a and CYT-1 domains is preferentially upregulated in the disease. These findings are remarkably consistent with recent observations in a separate brain collection showing comparable increased expression of JM-a and CYT-1 mRNA expression in the DLPFC in schizophrenia (6). Together, these findings

suggest that altered *ErbB4* splice-variant expression patterns are robustly detected in the DLFPC in schizophrenia. At present it is unclear whether changes in splice-variant expression levels of *ErbB4* in the DLPFC are primary or secondary to abnormalities of *NRG1* regulation reported in this area (2) or to other molecular changes related to the disease or possibly to its treatment. The observation that *ErbB4* splice patterns are unaltered in the hippocampus in schizophrenia, may argue against the idea that changes in *ErbB4* expression are downstream of the changes in *NRG1*, as altered *NRG1* expression has been reported in the hippocampus in these individuals in the absence of changes in *ErbB4* expression (3). The lack of findings in the hippocampus also argues against a general effect of illness state, related for example, to treatment or illness chronicity.

When we examined the effects of genetic variation at a single-risk SNP (rs4673628) reported to show modest

association with schizophrenia and statistical epistasis with the *NRG1* 'Icelandic' risk haplotype (5,8), a main effect of genotype was observed in the hippocampus and a strong trend in DLPFC on the expression levels of JM-a variant isoforms. *Post hoc* tests in the hippocampus showed that the A allele was associated with increased abundance of JM-a *ErbB4* variants compared with individuals homozygous for the G allele. In the DLFPC, this effect was observed preferentially in schizophrenic patients, as reflected by a significant diagnosis × genotype interaction. The observation that the main effect of genotype was statistically stronger in the hippocampus, compared to the DLPFC may be due to the fact that the expression levels of JM-a variant isoforms are increased in the DLPFC in the patient group, both in our sample and in the Stanley Brain Series reported by Silberberg *et al*. (6), independent of genotype. This robust region-specific effect of illness state may obscure a more specific effect of genotype within the DLFPC. Furthermore, absence of disease-associated change in the hippocampus suggests that levels of JM-a variant isoform expression are more immediately genotype-dependent.

As genetic association at rs4673628 which was based on an interaction of heterozygosity at the SNP and *NRG1* risk haplotype load did not identify a specific allelic bias (5), it is impossible to determine the directionality of the effect of risk on JM-a variant isoform expression. The authors report that the nature of the association was reflected by an excess of heterozygosity (5). Therefore, our data demonstrate only that elevated levels of JM-a variant isoform mRNAs are associated with genetic variation at this SNP.

The direct functional consequences of rs4673628 in terms of regulation of JM-a variant isoform abundance or splicing of the *ErbB4* gene are unclear and complicated by the physical location of the SNP in relation to the juxtamembrane splice region. SNP rs4673628 resides within intron 12 of the *ErbB4* gene, 13 kb from the splice donor site for the JM-region of *ErbB4*. One possibility is that rs4673628 represents an enhancer element downstream of the *ErbB4* promoter that affects splicing/expression of the JM domain. Downstream intronic enhancer elements that act to modify expression have been identified in a number of other genes (27,28). Alternatively, and more plausibly, rs4673628 may be in LD with a causative variant further downstream and closer to the JM-splice site. One further possibility is that the SNP predicts expression of an, as yet, unidentified variant of *ErbB4* that contains the exon 16 JM-a domain, suggested by the observation that the SNP resides only 15 bp from the start of exon 13 and is therefore optimally located to be central to a splice acceptor site which may regulate splicing of nearby exons. Ideally, we would have liked to test this prediction by analysis of splicosome recognition sequences within this region of the gene, but unfortunately computational suites for this analysis are not available. We therefore performed an analysis of putative genomic regulatory elements using MatInspector software (http://www.genomatix.de/matinspector.html), to determine if the SNP resides in a predicted protein binding region, i.e. an enhancer element. Interestingly, rs4673628 is within a putative binding site for growth factor independent-1 zinc finger protein (GFi-1B). Carrying the G allele results in abolition of this

recognition site. These observations provide *in silico* support for the potential functional relevance of rs4673628.

Interestingly, we report that rs4673628 did not predict the abundance of CYT-1 or CYT-2 variant isoform mRNAs in either the DLFPC or hippocampus. Our observations therefore dictate that genetic risk at this SNP is related to a 'population' of exon 16 (JM-a) containing variants which can contain either a CYT-1 or CYT-2 COOH-terminal. These findings suggest that altered signaling via JM-a/CYT-1 and JMa/CYT2, may be equally associated with genetic variation at rs4673628, implicating both the MAPK and PI3K pathways in genetic risk for schizophrenia. In a similar vein, we report that rs7598440, rs839523 and rs707284 and the risk haplotype did not predict the abundance of *ErbB4* isoforms containing either of the juxtamembrane domains (JM-a or JM-b). Again, suggesting that genetic risk in this region of the gene is related to a 'population' of CYT-1 containing *ErbB4* variants, which can contain either JM-a or JM-b juxtamembrane domains. Taken together, these data suggest that the two areas of risk identified so far by genetic association are associated with distinct, and separate abnormalities of *ErbB4* gene regulation in the brain in schizophrenia.

In contrast to the intron 12 risk-SNP, single SNP analysis of three putative risk variants surrounding exon 3 revealed a main effect of genotype in the hippocampus on CYT-1 variant isoform abundance. The risk alleles at each of the SNPs (AGG, respectively) predicted higher levels in the whole sample in the hippocampus with individuals homozygous for the risk alleles having the highest with no evidence of an allele dose-dependent effect. The finding that only individuals homozygous for the risk allele exhibited elevated levels of CYT-1 variant isoform mRNA suggests that the effect at each of these SNPs is potentially 'recessive' in terms of the expression levels. This molecular phenotype is consistent with genetic association data showing the highest odds ratio (OR) related to homozygosity at the risk alleles [OR: 3.54−5.14 (6)].

In the DLPFC, while genotype effects were again found for each of these SNPs on expression of CYT-1 variant transcripts the associations were not straightforward. At rs7598440, comparable effects of genetic variation to those found in hippocampus were observed in the whole cohort, again with individuals homozygous for the risk (A) allele having elevated abundance. In contrast, the predicted effects of rs839523 and rs707284 on CYT-1 variant isoform abundance were reversed in the DLPFC when compared with the hippocampus. Individuals carrying the risk allele (G) showed lower abundance of CYT-1 variant isoform mRNA in the DLPFC, while individuals homozygous for the protective allele demonstrated elevated levels. The explanation for this apparent differential effect of genotype in the DLPFC versus the hippocampus at rs839523 and rs707284 is at present unclear, but genotype effects in DLPFC may once again be confounded by the schizophrenia group increase in CYT-1 variant isoform expression. Clearly, because genotypes are characteristics of individual brains, the directionality of the relationships in hippocampus and in DLPFC would be expected to be the same if these SNPs had a strong and penetrant effect at the molecular level of the splicing machinery. The fact that there is a consistent effect of these SNPs on expression, but

that the directionality varies across regions, argues that the SNPs interact with other brain region-specific factors that impact differentially on gene processing in these regions. Though highly speculative, it is conceivable that the splicing signals could vary depending on the unique developmental history and molecular biology of these distinct brain regions and circuits.

In our sample the three risk SNPs (rs7598440, rs839523 and rs707284) were in LD in both Caucasian and African American populations similar to that reported previously in individuals of Ashkenazi Jewish decent (6), albeit weaker in the African American population. We report the association of CYT-1 variant isoform levels with a 46 kb risk haplotype (AGG) comprised of rs7598440, rs839523 and rs707284, in both the DLPFC and hippocampus. Based on the apparent 'recessive' effect of each of these alleles in the hippocampus, we hypothesized that homozygosity at the risk haplotype would show the greatest effect on abundance. Consistent with our prediction, the effect of the risk haplotype was driven entirely by homozygous individuals (AGG/AGG diplotype) having elevated levels of CYT-1 variant mRNA. In addition, we observed that the effect of the risk haplotype in the DLPFC and hippocampus appeared to be driven primarily by the schizophrenic group. The reason behind this effect of the haplotype being more apparent in the schizophrenia patients is unknown, but may reflect that these genetic variants are not directly linked to regulation of transcript levels independently and that variation in more than one gene imparts risk to developing the disorder. Several possibilities exist to explain the diagnosis by genotype interactions all of which are speculative and require replication. One possibility is that the SNPs measured are monitoring different haplotypes in individuals with schizophrenia when compared with healthy individuals and that the haplotypes unique to patients with schizophrenia contain a functional disease-associated variant. Likewise, it is plausible that the effect of these SNPs on *ErbB4* expression may be modified by other disease genes, disease variants or environmental factors associated with the disease.

It is interesting to note that the apparent discrepancy between allele effects of SNPs, rs839523 and rs707284 on CYT-1 variants in the hippocampus compared with the DLPFC was not reflected in the risk haplotype analysis. This phenomenon could be explained by the fact that only one of the SNPs in this region of the gene may be functionally relevant to schizophrenia risk and the others are monitoring the variant through LD. Interestingly, of the haplotypes identified in our population, the risk allele at rs7598440 (the SNP showing comparable directionality of association with CYT-1 variant abundance in both brain areas) is present only on the background of the risk haplotype, while the risk alleles at rs839523 and rs707284 (GG) are present on the background of another haplotype, designated here as HAP2 (rs7598440, rs839523 and rs707284; GGG; Supplementary Material, Table S2). As HAP2 was not associated with CYT-1 variants, this may suggest that rs7598440 is driving the risk haplotype association. Additional support for this notion is the observation that the same allele at rs7598440 is associated with schizophrenia risk as part of an overlapping haplotype in a separate population (7).

In our sample, the risk haplotype was more common in the Caucasian sample compared with the African American sample; however, similar effects of genetic variation on ErbB4 splice patterns were observed in both groups. In the original report (6), in the Ashkenazi Jewish population, the risk haplotype was present in 33% of control individuals and 52% of schizophrenic patients. In our Caucasian sample we found no evidence to suggest that the frequency of the risk haplotype was higher in our patient population compared with control. However, our sample is too small to meaningfully rule out association with clinical phenotype. Of note, we did observe that the frequency of the risk haplotype was increased two-fold in the African American patients (31.5%) compared with African American controls (16%). These observations warrant further investigation of clinical association of risk SNPs in ErbB4 in African American individuals.

The biological consequences of altered *ErbB4* splicing patterns in the brain in schizophrenia, either related to the disease or genetic risk, are at present unclear. Furthermore, confirmation of our findings at the protein level utilizing splice-variant specific antibodies will be required. The JM-a domain (exon 16) of *ErbB4* gives rise to a cleavable receptor which transmits signals to the cytoplasm and nucleus (10,13,29–31) and the CYT-1 domain (exon 26) serves as a PI3K docking site (10). Binding of NRG1 to JM-a variant isoforms results in cleavage and shedding of an 120 kDa ectodomain, catalyzed by the metalloprotease tumor necrosis factor-A converting enzyme (TACE) and resulting in the production of an 80 kDa membrane-bound cytoplasmic domain (9,29,30,32). Subsequent intramembrane cleavage by presenilin-dependent γ-secretase results in the release of an ErbB4 intracellular domain (4ICD; 11–13). Studies in cancer indicate that proteolytic processing of *ErbB4* in response to ligand binding results in a dramatic downregulation of *ErbB4* proteins containing JM-a at the cell surface (10,29,30). This mechanism has been suggested to represent a method for downregulation of ErbB4 receptor activity (9,29,30) and is consistent with the overexpression of JM-a containing variants in schizophrenia translating into reduced *ErbB4* receptor signaling in the disease. However, cleavage events also result in the generation of a membrane bound (M80) fragment that retains tyrosine kinase activity and signaling capacity (32). As the second cleavage by γ-secretase generates a soluble 80 kDa fragment that represents only a small fraction (<10%) of the 80 kDa pool (11), overexpression of JM-a containing *ErbB4* receptors may conversely result in 'overactive' membrane-bound *ErbB4* receptors in schizophrenia. This suggestion is consistent with the recent observations using a postmortem stimulation paradigm in schizophrenic brain tissue, whereby *NRG1*-induced activation of *ErbB4* tyrosine phosphorylation levels was found to be significantly elevated in DLFPC in patients (4). Notably, this event was linked to the suppression of NMDA receptor activity, consistent with the 'NMDA hypofunction' hypothesis of schizophrenia (33,34).

Finally, the inclusion of exon 26 (CYT-1) in the *ErbB4* protein links the receptor to the PI3K pathway. PI3K are members of a unique and conserved family of intracellular lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositol upon stimulation of growth factor receptor

tyrosine kinases. This event leads to the activation of many intracellular signaling pathways, including the AKT pathway, which regulates functions as diverse as cell metabolism, survival migration, polarity and vesicle trafficking (35) and has itself been identified as a potential risk gene for the disease (36–39). In neurons, the PI3K pathway is associated with regulating dendritic outgrowth (40) and *NRG1*-induced neuronal and glia migration (41,42). Our observation of increased expression of *ErbB4* variants which activate the PI3K pathway may suggest 'overactivation' of PI3K signaling in schizophrenia. At the transcript level these observations are consistent with protein studies in the human brain in schizophrenia, whereby NRG1-induced stimulation of *ErbB4* in the schizophrenic brain is accompanied by over-activation of AKT (4). It is also noteworthy, that PI3K activation results in the recruitment and activation of other signaling molecules, including Rac GTPase, which plays critical roles in neuronal growth, differentiation, migration and intracellular vesicular trafficking by regulation of the actin cytoskeleton (43). These observations suggest that a number of downstream signaling pathways may be affected in schizophrenia as a consequence of aberrant *NRG1/ErbB4* signaling.

In summary, we provide a direct link between disease-associated genetic variation and *ErbB4* splice-variant mRNA expression levels in the human brain. We demonstrate that the molecular correlates of genetic risk in *ErbB4* appear to preferentially involve altered expression of *ErbB4* variants containing JM-a or CYT-1. We propose that altered regulation of *ErbB4* splice-variant expression in schizophrenia leads to altered *NRG1/ErbB4* signaling in the disease and aberrant regulation of the PI3K pathway.

MATERIALS AND METHODS

Human brain tissue collection

Postmortem brain tissue was collected at the Clinical Brain Disorders Branch, NIMH, with informed consent from the legal next of kin. Seventy-five normal controls [22 females/53 males, 46 African American/24 American Caucasian/4 Hispanic and 1 Asian individual; mean age $41.5 \pm$ (SD) 15.2 years; postmortem interval (PMI) 30.2 ± 14.1 h; pH 6.59 ± 0.32]; and 40 schizophrenic patients [16 females/24 males, 20 African Americans/20 Caucasians; mean age 48.5 ± 17.7 years; PMI, 35.1 ± 17.6 h; pH 6.49 ± 0.24] were available for this study (as described previously; (3)]. Diagnoses were determined by independent reviews of clinical records and family interviews by two psychiatrists using DSM-IV criteria. Inpatient and outpatient clinical records were reviewed for every subject. Macro- and microscopic neuropathological examinations and toxicology screening were performed on all cases prior to inclusion in the study. Brain sections through several cortical regions and the cerebellar vermis were examined microscopically including the use of Bielschowsky's silver stain. Cases with cerebrovascular disease (infarcts or hemorrhages), subdural hematoma, neuritic pathology or other significant pathological features were excluded from further study.

The entire cohort was used for the analysis of effects of genetic variation on *ErbB4* isoform expression. The different genotype groups in this cohort did not differ on any of the measured variables that potentially affect gene expression in human postmortem brain [i.e. age, PMI, pH and RNA Integrity Number (RIN)]. As previously reported (3), we selected a sub-cohort of 47 controls (14 females/33 males, 26 African Americans/16 Caucasians/4 Hispanic individuals and 1 Asian; mean age 44 ± 14.1 years; PMI, 32.48 ± 13.1; pH 6.5 ± 0.23) and 32 schizophrenic individuals (11 females/21 males, 16 African Americans/15 Caucasians/1 Hispanic individuals; mean age 47 ± 18.7 years; PMI 35.9 ± 16.05; pH 6.51 ± 0.24), which were group-matched specifically for these potential confounding variables. This cohort was used for diagnostic comparisons of *ErbB4* splice-variant levels. In addition, both groups were matched for RIN (controls RIN 5.5 ± 1.1; schizophrenics RIN 5.4 ± 0.9).

ErbB4 isoform-specific primer and Taqman probe design

Primer and probe designs for JM-a, JM-b, CYT-1 and CYT-2 variant isoforms of *ErbB4* were as described previously (44). Briefly, the Taqman probes were designed to differentiate *ErbB4* isoforms through hybridization to isoform-specific exons, 16 or 15 JM-a, JM-b, respectively, and exon 26 for CYT-1. The Taqman probes/primer sets used for total *ErbB4* (Pan) and endogenous control genes were porphobilinogen deaminase (PBGD), GUSB (Beta-glucuronidase) and SDHA (succinate dehydrogenase complex, A) were purchased from Applied Biosystems (Foster City, CA, USA), Assays-on-Demand, Cat. No. hCG2012284; GenBank accession nos Hs_00609297, Hs_99999908 and Hs01549169, respectively.

Reverse transcription, RNA quality measurement and quantitative real-time PCR

Tissue from the hippocampus and DLPFC was pulverized and stored at $-80°$C. Total RNA was extracted from 300 mg of tissue using TRIZOL Reagent (Life Technologies Inc., Grand Island, NY, USA). The yield of total RNA was determined by absorbance at 260 nm. RNA quality was assessed with a high-resolution capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Approximately 700 ng of RNA was applied to an RNA 6000 Nano Lab Chip without prior heating. RIN, obtained from the entire Agilent electrophoretic trace using the RIN software algorithm was used for the assessment of RNA quality (scale 1–10, with 1 being the lowest and 10 being the highest RNA quality). Individuals with a RIN below 5 were excluded from the cohort. Total RNA (3 μg) was used in 50 μl of reverse transcriptase reaction to synthesize cDNA, using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. To control for potential variability between reverse transcriptase reactions, a total of three sequential reactions were performed (3 μg total RNA each) and the products pooled.

ErbB4 mRNA splice isoform expression levels were measured by qRT-PCR using an ABI Prism 7900 sequence detection system with 384-well format (Applied Biosystems) as described previously (3). Briefly, each 20 μl reaction contained 900 nM of each primer, 250 nM of probe and Taqman

Universal PCR Mastermix (Applied Biosystems) containing Hot Goldstar DNA Polymerase, dNTPs with dUTP, uracil-*N*-glycosylase, passive reference and 200 ng of cDNA template. PCR cycle parameters were 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s and 59°C or 60°C for 1 min. PCR data were acquired from the Sequence Detector Software (SDS version 2.0, Applied Biosystems) and quantified by a standard curve method. In each experiment, the R^2 value of the curve was more than 0.99 and controls comprising no-template cDNA resulted in no detectable signal. SDS software plotted real-time fluorescence intensity and selected the threshold within the exponential phase of the amplicon profiles. The software plotted a standard curve of the cycles at threshold (Ct) versus quantity of RNA. For each target isoform, in each brain region, all samples were measured with constant reaction conditions and their Ct values were in the linear range of the standard curve. All measurements were performed in triplicates for each mRNA and expression level calculated as an average of the triplicates. Experimental measurements with a >2% variance from the mean of the triplicate samples based on CTs were omitted. Our primary data analysis is based on normalization of ErbB4 mRNAs to the geometric mean of the quantity of three internal control genes (45).

ErbB4 genotype determination

We genotyped four intronic risk SNPs in the *ErbB4* gene [rs7598440, rs839523, rs707284 and rs4673628; (5,6)]. A single SNP at the most 3′ end of NRG1 was selected from the dbSNP database as a negative control genotype (rs3748960), based on the criteria that this SNP has not previously been associated with schizophrenia. Genotyping was performed using the Taqman 5′-exonuclease allelic discrimination assay (details available on request). Genotype reproducibility was routinely assessed by re-genotyping all samples for selected SNPs and was generally >99%. Overall genotyping failure rate was <1%. LD between SNPs was determined using the program LDMAX/GOLD. The program SNPHAP written by David Clayton (version 1.0, http://www.gene.cimr.cam.ac.uk/clayton/software/) was used to calculate haplotype frequencies and to assign diplotypes to individuals.

Statistical analyses

Comparisons between diagnostic groups were made using univariate ANCOVA for each mRNA with diagnosis as the independent variable. Correlations of mRNA levels with potential confounding demographic variables (age, pH, PMI and RIN) were performed for all subjects using Spearman's correlations. pH was observed to correlate with levels of *ErbB4* splice-variant expression and therefore was included as a covariate in all diagnostic comparisons. Correlations of mRNA levels with neuroleptic medication (lifetime neuroleptic exposure, daily dose and final neuroleptic dose) were investigated in the schizophrenic cohort. Effects of genetic variation on *ErbB4* mRNA expression were examined using ANOVA with genotype and diagnosis as independent factors. Where there was a significant genotype × diagnosis interaction, individual group *post hoc* tests were examined. Effects of

race were examined by including race as a third factor in the ANOVA. Examination of all three genotype groups was conducted for SNPs when the minor allele frequency was >10%. Examination of the effects of genetic variation on *ErbB4* splice-variant expression included four SNPs and a risk haplotype, with each SNP being tested individually for associations with JM-a, JM-b, CYT-1 and CYT-2 variant isoform expression and pan *ErbB4* mRNA levels in patients and controls. All experiments were conducted blind to diagnosis and genotype.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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