Strong evidence that GNB1L is associated with schizophrenia

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Received September 28, 2007; Revised and Accepted November 9, 2007

Evidence that a gene or genes on chromosome 22 is involved in susceptibility to schizophrenia comes from two sources: the increased incidence of schizophrenia in individuals with 22q11 deletion syndrome (22q11DS) and genetic linkage studies. In mice, hemizygous deletion of either Tbx1 or Gnb1l can cause deficits in pre-pulse inhibition, a sensory motor gating defect which is associated with schizophrenia. We tested the hypothesis that variation at this locus confers risk of schizophrenia and related disorders in a series of case–control association studies. First, we found evidence for a male-specific genotypic association (P = 0.00017) in 662 schizophrenia cases and 1416 controls from the UK. Moreover, we replicated this finding in two independent case–control samples (additional 746 cases and 1330 controls) (meta analysis P = 1.8 x 10⁻⁵) and also observed significant evidence for genotypic association in an independent sample of 480 schizophrenia parent-proband trios from Bulgaria with markers at this locus, which was again strongest in the male probands (P = 0.004). Genotyping the most significant SNPs in a sample of 83 subjects with 22q11DS with and without psychosis again revealed a significant allelic association with psychosis in males with 22q11DS (P = 0.01). Finally, using allele specific expression analysis, we have shown that the markers associated with psychosis are also correlated with alterations in GNB1L expression, raising the hypothesis that the risk to develop psychosis at this locus could be mediated in a dose sensitive manner via gene expression. However, other explanations are possible, and further analyses will be required to clarify the correct functional mechanism.

INTRODUCTION

Schizophrenia is a severe psychiatric syndrome characterized by varying combinations of psychotic symptoms, such as delusions and hallucinations, together with defects of motivation, affective response and cognitive functioning. It has a life-time morbid risk of ~1%. Twin studies typically result in estimates of heritability of around 80% (1), with the main contributions thought to result from multiple genes of small to moderate effect sizes (2,3). Evidence that a gene or genes on chromosome 22 is involved in susceptibility to schizophrenia comes from two sources: the increased incidence of schizophrenia in individuals with deletions of chromosome 22q11 and genetic linkage studies. Deletion of a region, typically ~3 Mb, on chromosome 22q11 results in a group of related syndromes including Velo-Cardio-Facial syndrome (VCFS), DiGeorge syndrome and conotruncal anomaly face syndrome (4) which are collectively referred to as 22q11 deletion syndrome (22q11DS). 22q11DS has a complex and variable phenotype.
which, in addition to craniofacial, immunological and cardiovascular defects (4), includes behavioral abnormalities and psychiatric disorders (5). In particular, high rates of psychosis (schizophrenia, schizoaffective disorder and bipolar disorder) have been reported in adults (6–9). The majority of cases fulfill diagnostic criteria for schizophrenia which has an estimated prevalence in adults with 22q11DS of 25% (10). This makes 22q11DS one of the few known heritable causes of schizophrenia, albeit one that only accounts for a small proportion of the population risk (11,12). The existence of one or more susceptibility loci for schizophrenia on chromosome 22q is also suggested by numerous linkage studies, with 22q being one of only two regions supported by the two major meta-analyses of schizophrenia linkage studies (13,14). The observations of association between 22q11DS and schizophrenia, and linkage to 22q, has led to the hypothesis that one or more genes within the deleted region contains variation that confers risk in the population without a deletion. Recently, Hamshere et al. (15) reported genome-wide suggestive evidence for linkage to 22q11 in families ascertained on probands with schizoaffective disorder suggesting that a locus in this region might be particularly strongly associated with psychotic disorders with features of both psychosis and mood disorder.

Several genes within the 22q11DS region have so far been implicated in schizophrenia by genetic studies (16–20), but for none is the evidence from follow-up studies robust (18,21–27), and psychosis has been reported in 22q11DS cases with atypical deletions that do not involve PRODH (28). This, allied to the possibility that the very high risk of psychosis in 22q11DS relates to haplo-insufficiency in multiple genes in the region, means that 22q11 remains an important focus of investigation in schizophrenia.

Two genes in the region that have recently been suggested as potentially involved in neuropsychiatric disorders are TBX1 and GNB1L. These two adjacent genes show temporal co-expression during mouse embryonic development (29) and in brain during murine adolescence (29). The transcription factor TBX1 (30) is a member of the T-Box protein family, which is characterized by an evolutionarily highly conserved, palindromic DNA-binding domain (T-Box). GNB1L encodes a G-protein beta-subunit-like polypeptide (31,32), which contains six WD40 repeats but which lacks homology to known proteins (31). WD40 repeats are known to facilitate the formation of heterotrimeric or multiprotein complexes but the function of GNB1L is currently unknown.

Chromosome engineering experiments in mice have shown that haploinsufficiency of Tbx1 is sufficient to induce most of the physical defects of the 22q11DS phenotype (33–35). The relevance of the findings to the human disorder is supported by the presence of TBX1 point mutations in patients with 22q11DS phenotype but without detectable microdeletion (36). Moreover, Paylor et al. (37) demonstrated that hemizygous deletion of either Tbx1 or Gnb1l can cause deficits in pre-pulse inhibition, a sensory motor gating defect which is associated with schizophrenia as well as other severe psychiatric disorders (38). Mutations in TBX1 have recently been shown to be present in individuals with unspecific cognitive deficits (39). The involvement of TBX1 in the generation of psychiatric features of the 22q11DS is also supported by a functional TBX1 deletion observed in a non-deleted 22q11DS family, where one family member was additionally affected by Asperger syndrome, an Autism spectrum disorder also associated with reduced pre-pulse inhibition (37).

In view of these findings, we hypothesized that genetic variation in TBX1 and/or GNB1L might be associated with schizophrenia and related psychotic disorders. We tested this hypothesis by conducting a systematic association study of SNPs capturing most of the common population variation across the two genes in a schizophrenia case–control sample from the UK. We report evidence for a male-specific genotypic association due to excess homozygosity in three case–control samples. Moreover, the associated markers also showed a significant male-specific association with psychosis in individuals with 22q11DS. We excluded copy number variation (CNV) as a cause of excess homozygosity by scanning the region for CNVs using a Multiplex Amplicon Quantification (MAQ) assay. Finally, using allelic expression analysis, we found evidence that the markers associated with psychosis are also correlated with the presence of cis-acting influences on alterations in GNB1L expression. This suggests the hypothesis that the risk of developing psychosis at this locus could be mediated in a dose sensitive manner via gene expression. However, there are other possible explanations, and further analyses of the locus will be required to clarify the correct functional mechanism.

RESULTS

Genetic association analysis: UK schizophrenia case–control sample

In the UK schizophrenia case–control sample, of 19 markers (Fig. 1), we identified nominally significantly allelic association for TBX1 F140F (\(P = 0.027\)) and rs12165908 (\(P = 0.037\)) (Supplementary Material, Table S1). Neither met our criteria for experiment-wise significance. We observed evidence for genotypic association at several of the markers (Supplementary Material, Table S1), with rs5746832 (\(P = 0.0041\)) and rs2269726 (\(P = 0.01\)) meeting a nominal threshold of \(P < 0.01\) (Table 1). We also observed nominally significant evidence (\(P < 0.05\)) at rs737869 and rs8139091 (Supplementary Material, Table S1). Analysis of the genotype distributions revealed that all four genotypically associated SNPs as well as two additional markers significantly deviated from Hardy–Weinberg equilibrium (HWE) in the patients (\(P = 0.00013–0.03\)) but not the controls (min \(P = 0.14\)) (Table 1 and Supplementary Material, Table S1).

Post hoc analysis revealed that the four genotypic associations observed occurred exclusively in males with P(male) rs5746832 = 0.00075 and P(male) rs2269726 = 0.0012 (Table 1). Similar gender-specific patterns were seen for disturbance of HWE (Table 1 and Supplementary Material, Table S1). Assuming conservatively that allelic, genotypic, whole sample, male, and female tests represent five independent comparisons, the Nyholt correction factor is the original Nyholt threshold (0.0042) divided by 5 which is approximately 0.0008. This threshold was matched by rs5746832 while the uncorrected Nyholt threshold was met by the male-specific analysis for rs2269726.
Inspection of the genotype counts in male cases in comparison to male controls and also in comparison to that expected under HWE for both rs5746832 and rs2269726 revealed an excess of both classes of homozygote. Between male cases and controls (Table 2), the association with increased homozygosity at rs5746832 was significant at \( P = 0.0004 \) [OR = \( 1.54 \) (1.21–1.97)] and for rs2269726, at \( P = 0.0004 \) [OR = \( 1.56 \) (1.22–2)]. Allowing for an additional genotypic test, the Nyholt threshold of 0.0007 (0.0042/6) is surpassed by both markers. No significant genotypic association or departures from HWE were observed in the females for either rs5746832 (\( P = 0.77 \)) or rs2269726 (\( P = 0.93 \)) (Table 2).

Our observation of a gender-specific effect at this locus was supported by a sex–genotype interaction analysis where rs5746832 met the criteria recently defined by Patsopoulos et al. (40) whereas rs2269726 fell just short (\( P = 0.04 \) and \( P = 0.08 \), respectively). However, the excess of homozygotes in male cases compared with female cases with a similar effect size to the comparison of male cases and controls also suggests a sex effect at this locus [rs5746832, OR = \( 1.40 \) (1.01–1.95); rs2269726; OR = \( 1.35 \) (0.96–1.90)]. No difference was observed between male and female controls (\( P = 0.27 \), OR = 0.96; \( P = 0.11 \), OR = 0.86).

Given that the deviation from HWE was observed selectively in males and for multiple markers, it seemed to us unlikely that this effect was attributable to genotyping error. Moreover, the high call rate (e.g. rs5746832, 98% in male cases) suggests it is not attributable to a high drop out rate of one genotype. Nevertheless, as a further control for the possibility that our results might be due to genotyping error, we re-genotyped four of the markers showing departure from HWE (including the two markers showing the strongest effect; rs5746832 and rs2269726) in 90 individuals using alternative genotyping techniques. This revealed 100% concordance rates for re-genotyped markers. We also genotyped a subset of our sample (613 cases and 687 controls) for two additional SNPs (rs2073762 and rs5748427) which in the HapMap CEU sample are highly correlated (\( r^2 = 0.96 \)) with rs5746832 and rs2269726, respectively. Both proxy SNPs showed a pattern of intermarker LD similar to that seen in the HapMap in cases and controls (\( r^2 = 0.96 \)) and again, deviated from HWE in males in the expected way. Such a result could potentially be caused by the presence of duplicate or closely related individuals in our case–control sample. We therefore used PLINK (41) to estimate the IBS distances at all possible pairs of individuals across 143 random SNPs that had been previously genotyped in this sample. Pairs with an IBS distance of 1.0 are likely to be identical. No individuals used in this study were either duplicates or closely related individuals.

### Genetic association analysis: follow-up samples

Genotypic associations due to an excess of homozygosity in male cases were also observed in the UK SASP sample (\( N = 241 \); 90 male, 151 female) with similar effect sizes to those observed in the other samples [rs5746832, \( P = 0.05 \); OR = \( 1.71 \) (1–2.94); rs2269726, \( P = 0.01 \); OR = \( 1.33 \) (1.12–1.57)] (Table 2). Again, no effect was observed in females, despite the fact that there were more females than males in this sample. We should note however that for this test, the control group is the same as that used for the primary case–control analysis, and therefore these results cannot be

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**Table 1. Analysis of rs5746832 and rs2269726 in UK schizophrenia cases and controls**

<table>
<thead>
<tr>
<th>Full sample SNP (A1:A2)</th>
<th>Cases (11:12:22 (freq))</th>
<th>HWE</th>
<th>Controls (11:12:22 (freq))</th>
<th>HWE</th>
<th>Genotypic</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs5746832 (A:G)</td>
<td>143 (0.22):271 (0.42):237 (0.36)</td>
<td>0.00013</td>
<td>239 (0.18):659 (0.49):438 (0.33)</td>
<td>0.74</td>
<td>0.0041</td>
</tr>
<tr>
<td>rs2269726 (C:T)</td>
<td>88 (0.15):237 (0.39):278 (0.46)</td>
<td>0.0018</td>
<td>152 (0.11):617 (0.46):569 (0.43)</td>
<td>0.43</td>
<td>0.011</td>
</tr>
<tr>
<td>Males only</td>
<td>rs5746832 (A:G)</td>
<td>95 (0.22):170 (0.39):172 (0.39)</td>
<td>0.000038</td>
<td>121 (0.17):352 (0.5):225 (0.32)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>rs2269726 (C:T)</td>
<td>56 (0.14):150 (0.37):200 (0.49)</td>
<td>0.0018</td>
<td>75 (0.11):330 (0.48):285 (0.41)</td>
<td>0.15</td>
</tr>
<tr>
<td>Females only</td>
<td>rs5746832 (A:G)</td>
<td>48 (0.22):101 (0.47):65 (0.31)</td>
<td>0.46</td>
<td>118 (0.18):307 (0.48):213 (0.33)</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>rs2269726 (C:T)</td>
<td>32 (0.16):87 (0.44):78 (0.40)</td>
<td>0.36</td>
<td>77 (0.12):287 (0.44):284 (0.44)</td>
<td>0.73</td>
</tr>
</tbody>
</table>
considered independent. However, analyses of the genotype distributions under HWE in this sample do constitute independent tests and are therefore arguably more informative (in the absence of HWE disruption in any non-affected group we have examined). As expected, for both markers, male cases with SASS deviated from HWE (P < 0.05) and the allele and genotype frequencies did not significantly differ to those of the first UK sample. As an additional test, we combined the male SASS and schizophrenia cases and tested them against the original UK controls. This enhanced the evidence for association with homozygosity compared to that obtained with the schizophrenia cases alone [rs5746832, P = 7.04 × 10^{-5}; OR = 1.6 (1.27–2.02); rs2269726, P = 9.4 × 10^{-5}; OR = 1.6 (1.27–2.04)]. In addition, SNP rs2269726 has been genotyped in a large independent UK population sample and the data has been deposited by WTSI and published online from the British 1958 Birth Cohort DNA Collection (http://www.b58gene.ssgul.ac.uk/allele.php?.snp=ill2269726). The genotype frequencies at rs2269726 in this independent sample (Table 2) do not deviate from those expected under HWE (P = 0.55 overall, P = 0.92 males only). Comparison of the SASS genotypes at this locus with those population controls (fully independent of our own controls) again supported our observation of a significant excess of homozygotes in the male SASS cases (P = 0.05).

The German sample of 513 schizophrenic cases (334 males, 179 females) and 1330 controls (609 male, 721 female) was genotyped for rs5746832 and rs2269726 at the University of Munich blind to the results from the Cardiff samples. Male cases (Table 2) again revealed evidence for increased homozygosity with similar effect sizes to those observed in the UK at both loci [rs5746832; P = 0.05; OR = 1.31 (1.1–1.71); rs2269726; P = 0.05; OR = 1.31 (1.1–1.71)]. As before, no effect was seen in females (Table 2). Allele and genotype distributions were similar in the UK and Germany for cases and controls (Heterogeneity P = 0.26 for both rs5746832 and rs2269726) as were LD relationships between the markers (UK controls D’ = 0.96, r^2 = 0.65; German controls D’ = 0.97, r^2 = 0.64). Combined analysis of all cases and control samples by inverse variance meta analysis again resulted in 0.96, r^2 = 0.65; German controls D’ = 0.97, r^2 = 0.64). Combined analysis of all cases and control samples by inverse variance meta analysis again resulted in
enhanced evidence for association with homozygosity and psychosis in males (rs5746832; \( P = 4.66 \times 10^{-5} \); OR = 1.44; rs2269726; \( P = 1.8 \times 10^{-5} \); OR = 1.46).

In an independent sample of schizophrenia trios from Bulgaria neither rs5746832 nor rs2269726 yielded evidence for either genotypic association in males (Supplementary Material, Table S2). However, each of these markers showed highly significant differences in the frequencies of alleles between the parents of the probands and the UK controls. For rs5746832, this even surpassed the equivalent of genome wide significance; \( \chi^{2} = 2.9, P = 1.8 \times 10^{-9} \) compared with UK controls, \( P = 1.9 \times 10^{-7} \) compared with German controls, \( P = 2.9 \times 10^{-10} \) combined controls. Highly significant differences were also observed between cases from Bulgaria and from each of the UK and Germany (data not shown). Consequently, the data from the Bulgarian sample were not analysed with the UK and German samples by meta-analysis. Since such allelic differences may markedly influence power to detect indirect (and direct) association, we re-evaluated the Bulgarian trio sample using the full panel of 19 markers. As for all analyses thus far, we found male-specific evidence for genotypic association due to increased homozygosity in the schizophrenic probands (Table 3, full data given in Supplementary Material, Table S2), despite similar numbers of male and female probands. No marker showed evidence of Mendelian errors. It is appropriate to correct this analysis for the number of markers since we have no hypothesis concerning a specific marker, although correction for gender and model are not required since all analyses are predicated on a specific hypothesis. \( TBX1 \) F104F \( P = 0.0051 \) met the Nyholt threshold for the number of independent markers tested while rs12165908 \( P = 0.022 \) fell just short. Marker F104F was re-genotyped in 394 trios using an alternative genotyping technique and 100% concordance was observed.

### Table 3. Analysis of \( TBX1 \) F104F and rs12165908 in Bulgarian trios

<table>
<thead>
<tr>
<th>SNP (A1:A2)</th>
<th>Genotypic association Proband counts (freq)</th>
<th>Pseudo-control counts (freq)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Full sample</td>
<td></td>
</tr>
<tr>
<td>F104F (C:T)</td>
<td>0.0051</td>
<td>39 (0.08):160 (0.35):263 (0.57)</td>
</tr>
<tr>
<td>rs12165908 (C:G)</td>
<td>0.022</td>
<td>32 (0.07):152 (0.33):279 (0.60)</td>
</tr>
<tr>
<td>Males only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F104F (C:T)</td>
<td>0.0043</td>
<td>25 (0.10):87 (0.36):132 (0.54)</td>
</tr>
<tr>
<td>rs12165908 (C:G)</td>
<td>0.008</td>
<td>21 (0.09):80 (0.33):141 (0.58)</td>
</tr>
<tr>
<td>Females only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F104F (C:T)</td>
<td>0.20</td>
<td>14 (0.06):73 (0.33):131 (0.60)</td>
</tr>
<tr>
<td>rs12165908 (C:G)</td>
<td>0.43</td>
<td>11 (0.05):72 (0.33):138 (0.62)</td>
</tr>
</tbody>
</table>

Given the unstable nature of 22q11, the presence of smaller pathogenic micro-deletions in the \( TBX1/GNB1L \) region provides a potential explanation for the finding of excess homozygosity at this locus. To test for this, five MAQ amplicons spanning the \( TBX1/GNB1L \) locus, including two MAQ amplicons specifically located between our two SNPs showing the greatest excess of homozygosity (rs5746832 and rs2269726), were analysed in 248 cases (175 male, 73 female) and 264 controls (181 male, 83 female) selected from our UK schizophrenia association sample and ten 22q11 deletion positive controls. Significant deviation from HWE was observed at rs5746232 in this subset of cases \( P = 0.002 \) but not the controls \( P = 0.76 \). All MAQ amplicons identified the hemizygous deletion in the positive controls but none showed evidence for a microdeletion in the schizophrenia cases and controls (Supplementary Material, Table S3).

### Genetic association analysis in individuals with 22q11 deletion syndrome

Given hemizygosity at the locus for this sample, we could not specify excess homozygosity as a model. We therefore undertook an allele-based analysis. Since this sample was collected in the UK, we expected association in del22q11 males to those markers that were associated the UK and German samples. Once again, and despite a smaller sample of males, both rs5746832 and rs2269726 showed significant association with psychosis selectively in males (both \( P = 0.018 \)) (Table 4).

### Mutation screening

Direct sequencing of the coding sequence of both \( TBX1 \) and \( GNB1L \) identified only one non-synonymous variant in \( TBX1 \) (rs4819522) and two in \( GNB1L \) (rs2073770 and rs5748449). Genotyping in our UK case-control sample revealed no significant association with psychosis (Supplementary Material, Table S4).

### Allele-specific expression analysis: \( GNB1L \)

We sought to determine whether the expression of \( TBX1 \) or \( GNB1L \) is influenced by cis-acting polymorphisms by performing allelic expression analysis on RNA extracted post mortem from DLPFC. As subjects were of northern European origin (predominantly UK and Sweden), allelic expression was based on rs5746832 (one of the two genotypic associated SNPs in the UK and German samples) that occurs in the 3′-UTR of \( GNB1L \) transcript ENST00000329517. Allelic expression of the 43 individuals who were heterozygous for rs5746832 revealed evidence for differential allelic expression of \( GNB1L \) transcripts in some individuals as indicated by marked deviation from the 1:1 ratio observed in
genomic DNA (Fig. 2). This suggests the presence of polymorphisms with cis-acting effects on expression of \textit{GNB1L}. Moreover, although there are individual differences, allele ‘A’ was generally under-expressed compared with allele ‘G’ \((P = 0.0006)\). To confirm this finding, we selected 15 samples and performed allele-specific expression analysis using a second SNP (rs2073762) that was in complete LD \((D' = 1, r^2 = 1)\) with rs5746832 and was also located in the 3’-UTR of \textit{GNB1L}. Evidence for differential allelic expression was also obtained using rs2073762 (data not shown), the results being highly correlated with those from rs5746832 \((r = 0.78, P = 0.001)\).

We also investigated whether rs2269726, the other marker for which homozygosity was consistently associated in the case–control samples, was associated with differential \textit{GNB1L} expression. Because rs2269726 is intronic, its effects cannot be tested independently of the allelic expression test-SNP (rs5746832). However, both SNPs are in high LD \((D' = 1)\), therefore by conditioning on rs2269726, we are effectively testing the relative effects of the three haplotypes at the two loci. Moreover, since only heterozygotes for rs5746832 are informative for the assay, each informative individual must carry one copy of the (rs5746832–rs2269726) G–T haplotype (G always being in phase with T). Analysis of the 30 individuals (13 male, 17 female) who were heterozygous with phased diplotypes (A–C/G–T) revealed significant differential allelic expression \((P = 6.3 \times 10^{-7})\) (Fig. 3). In general, A–C haplotypes were relatively under-expressed, with the mean ratio of mRNA expressed from A–C haplotypes compared with G-T being 0.86:1. Analysis of the remaining 13 individuals (8 male, 5 female) who were heterozygous for the diplotypes ‘A–T’/G–T’ revealed equal expression from each haplotype \([\text{mean ratio} = 1.02:1 (0.78–1.26), P = 0.62]\). The mean expression ratio in the ‘A–T’/G–T’ diplotypes was also significantly different from those carrying the ‘A–C’/G–T’ diplotype \((1.02:0.86, P = 0.005)\). Thus, with respect to \textit{GNB1L} expression, the ‘A–T’ and ‘G–T’ haplotypes are functionally similar, but distinct from the ‘A–C’ haplotype which is relatively under-expressed. The expression ratios were similar in males and females, indicating no gender-specific influences (data not shown).

The rs5746832–rs2269726 haplotypes that correlate with a relative increase (‘A–T’ and ‘G–T’) and decrease (‘A–C’) in \textit{GNB1L} expression can be perfectly tagged by rs2269726 alleles ‘T’ and ‘C’ respectively. In the context of our case–control findings of excess homozygosity for rs2269726, our association data indicate that in male cases, risk is associated with both a relative over-expression and under-expression of \textit{GNB1L}.

**Allele-specific expression analysis: TBX1**

Given the genomic proximity of \textit{TBX1} and \textit{GNB1L}, we postulated that the two genes might exhibit coordinated expression, and that the true effects on risk might be mediated by either direct or indirect effects of the associated variants on \textit{TBX1}. We therefore investigated the possibility that the associated polymorphisms might additionally influence \textit{TBX1} expression. To test this, we first examined \textit{TBX1} for evidence of cis-acting polymorphic effects on expression using rs2301558, a synonymous variant that occurs in all three known \textit{TBX1} transcripts (ENST00000359500, ENST00000329705 and ENST00000323710. U000329517). We observed marked deviation from the ratio equivalent to 1:1 expression (Supplementary Material, Figure S1) indicating the presence of polymorphisms with cis-acting effects on expression of \textit{TBX1}.

If the genetic variants associated with increased risk of psychosis mediate their effects through \textit{TBX1} expression instead of, or in addition to, effects on \textit{GNB1L}, we reasoned that the extent of allelic expression we observed at \textit{TBX1} should be modified by genotype at rs2269726. However, analysis of the \textit{TBX1} allelic expression data stratified by rs2269726 revealed highly significant evidence for allelic expression changes at \textit{TBX1} irrespective of genotype status at rs2269726 \((rs2269726; \text{heterozygotes } P = 3 \times 10^{-5}, \text{ homozygotes } P = 3 \times 10^{-3})\); Supplementary Material, Figure S2a) with no significant differences between the homozygous and heterozygous groups \((P = 0.9)\). Moreover, when analysed with respect to phased haplotypes, the T allele at \textit{TBX1} was similarly relatively over-expressed regardless of whether it is in phase with the T or the C allele at rs2269726 (Supplementary Material, Figure S2b). Similarly, the converse experiment failed to show that \textit{GNB1L} expression (measured at rs5746832) was influenced by the alleles at rs2301558 (Supplementary Material, Figure S3). These data suggest that the marker is associated with both schizophrenia and

| Table 4. Association analysis in unrelated patients with confirmed deletions at 22q11 |
|----------------------------------|-----------|---------------|-----------|---------------|-----------|---------------|
|                                  | rs5746832 allele counts (freq) |                | rs2269726 allele counts (freq) |                |
|                                  | G         | A             | Fishers P | T         | C             | Fishers P |
| Full sample                      |           |               |           |           |               |
| Psychosis                        | 13 (0.59) | 9 (0.41)      | 0.32      | 13 (0.59) | 9 (0.41)      | 0.8       |
| Non-psychosis                    | 27 (0.44) | 34 (0.56)     |           | 34 (0.56) | 27 (0.44)     |           |
| Male                              |           |               |           |           |               |
| Psychosis                        | 8 (0.80)  | 2 (0.20)      | 0.018     | 9 (0.90)  | 1 (0.10)      | 0.018     |
| Non-psychosis                    | 5 (0.29)  | 12 (0.71)     |           | 7 (0.41)  | 10 (0.59)     |           |
| Female                           |           |               |           |           |               |
| Psychosis                        | 5 (0.42)  | 7 (0.58)      | 0.74      | 4 (0.33)  | 8 (0.67)      | 0.1       |
| Non-psychosis                    | 22 (0.50) | 22 (0.50)     |           | 27 (0.61) | 17 (0.39)     |           |
cases/probands (P and acting variation. revealed no evidence for a genotypic association (rs2073770) in the UK case/controls and the Bulgarian trios synonymous variant reported to be subject to selection (42). However, genotyping the non-

TBX1 In this study, we investigated whether genetic variation in expression.

DISCUSSION

In this study, we investigated whether genetic variation in TBX1 and/or GNB1L might be associated with schizophrenia and related psychotic disorders. Genotyping 19 SNPs spanning the TBX1/GNB1L locus in a sample of schizophrenia cases and matched controls from the UK identified significant evidence for genotypic association at a number of markers, with rs5746832 and rs2269726 generating the strongest signals. For all associated SNPs, we observed an excess of homozygotes in the cases which caused a significant deviation from the genotype frequencies expected under HWE. While this signal was apparent in the full sample, post hoc analysis revealed that it was much stronger in the male patients.

We subsequently replicated these findings in an independent schizophrenia case–control sample from Germany and in a sample of cases with mixed features of schizophrenia and mood disorder. We also observed significant genotypic association with SNPs spanning TBX1/GNB1L in a Bulgarian schizophrenia trio sample, where again the signal was present only in males. In our experience, significant differences in allele frequency between the UK and Bulgarian populations are not common, but it is interesting that analysis of the phase II Hapmap data suggest that the GNB1L locus is subject to recent selection (42). However, genotyping the non-synonymous variant reported to be subject to selection (rs2073770) in the UK case/controls and the Bulgarian trios revealed no evidence for a genotypic association (P = 0.74 and P = 0.98, respectively) or deviation from HWE in the cases/probands (P = 0.23 and P = 0.71, respectively).

Given that large deletions at 22q11 confer an increased risk of psychosis (6–9), our observation of a significant excess of homozygote genotypes at the TBX1/GNB1L locus could potentially have been explained by the presence of smaller microdeletions. However, in a subsample of cases that showed significant deviation from HWE at both rs5746832 and rs2269726, we did not identify any evidence for copy number variation, even using amplicons located between the SNPs showing the greatest evidence for increased homozygosity (rs5846832 and rs2269726).

In the absence of deletions to explain the findings, the association in the general male schizophrenia population points to the presence of susceptibility variants for schizophrenia at the TBX1/GNB1L locus. In keeping with this hypothesis, we observed a gender-specific genotypic association between variants on the non-deleted chromosome in males with 22q11DS.

Although the evidence for association is strong, direct sequencing of all TBX1 and GNB1L exons failed to reveal non-synonymous variants that could to explain our significant association data. It is well recognized that both TBX1 and GNB1L are sensitive to changes in gene dosage (37), we therefore set out to identify whether cis-acting changes in gene expression could provide a mechanism for our observed genotypic association. Using SNPs in TBX1 and GNB1L, we demonstrated that the expression of each gene is under the influence of cis-acting variation, but that variation at the SNPs associated with schizophrenia correlated only with GNB1L expression. Thus, the ‘T’ allele at rs2269726 is associated an average of 20% increased expression relative to that of ‘C’. However, as can be seen from the observed expression ratios, several of the samples that were homozygous ‘TT’ also showed allelic expression distortion (Fig. 3) suggesting this SNP does not in itself account for all the cis-acting variation in GNB1L expression.

Our observation of increased homozygosity in schizophrenia at loci associated with altered allelic expression of GNB1L is consistent with the hypothesis that increased risk
of psychosis results from abnormal gene expression at GNB1L. Given that we observe an excess of both the TT and CC homozygotes (OR = 1.28 and 1.36, respectively), our findings suggest that risk to psychosis reflects dosage sensitivity, with risk being related to over- or under-expression of GNB1L relative to heterozygotes. According to this model, we expected that risk for psychosis in males with 22q11DS would be related to low expression of GNB1L. In fact, we observed the opposite; alleles that are correlated with increased GNB1L expression were associated with psychosis in 22q11DS males, an observation that is apparently incompatible with a simple dosage sensitivity model. One possible, albeit speculative, explanation of our data is that hemizygosity for low expression GNB1L alleles results in levels of expression that are sufficiently low to induce compensatory mechanisms whereas hemizygosity for high expression variants does not. Alternatively, our results could represent a gene dosage imbalance between GNB1L and an interacting gene also located within the 22q11 deletion (43). The exact function of GNB1L is unknown. However, it clearly contains a WD40 repeat domain which is known to mediate protein–protein interactions (31). It therefore follows that other WD40 repeat containing genes at 22q11, such as HIRA, would be obvious candidates to explore such a mechanism.

Although our data consistently point to significant association with psychosis only in males, the precise functional mechanisms underpinning our genetic data are unclear. Our allele-specific expression analysis did not reveal gender-specific effects on gene expression at either GNB1L or GNB1L, suggesting that either males and females differ in sensitivity to dosage of the risk gene, or that the genetic mechanism conferring risk to psychosis occurs via mechanisms that do not involve effects on expression. For example, from in silico analysis, we cannot unambiguously determine whether the SNP we used to examine allelic expression at GNB1L (rs5746832) occurs in only a single transcript or in all transcripts. We therefore do not know whether our allelic expression data reflect expression at all or just specific transcripts at GNB1L, it therefore follows that the alleles associated with psychosis in 22q11DS might actually be associated with reduced expression of specific isoforms of GNB1L. It is also possible that the genetic associations reflect other mechanisms by which genetic variation can impact on gene function such as effects on translation, mRNA stability, splicing of transcripts and interference with miRNA binding sites, at either the GNB1L locus or another in LD with the associated SNPs.

In conclusion, we have identified significant evidence for a gender-specific association between both schizophrenia and schizoaffective spectrum phenotype and genotypes at the GNB1L locus. Our data also show a relationship between the associated markers and cis-acting changes in GNB1L expression, suggesting that abnormal gene expression at GNB1L should be considered as a possible mechanism that mediates an increased risk to develop psychosis. While this is a plausible explanation for our data, we have not excluded other possible explanations and therefore future work will be required to explain the findings in terms of primary disease mechanisms.

MATERIALS AND METHODS

Patient samples

All individuals provided written consent to participate in genetic association studies. Ethics committee approval was obtained from the local ethics committees in the UK, Bulgaria and Germany.

UK case–control sample. All subjects were unrelated, white, resident in the British Isles and provided written informed consent to participate in genetic studies. Protocols and procedures were approved by relevant ethical review panels including the UK West Midlands Multi-centre Research Ethics Committee (Birmingham, UK) and UK Wales Multi-centre Research Ethics Committee (Cardiff, Wales). Diagnoses were made by the consensus lifetime best estimate method (44) on the basis of all available information including a semi-structured interview [Schedules for Clinical Assessment in Neuropsychiatry (SCAN)] (45) and review of psychiatric case records. An OPCRT checklist was completed (46). Formative team reliability meetings took place weekly throughout recruitment.

UK schizophrenia cases. All 662 cases met DSM-IV criteria for schizophrenia and consisted of 446 (68%) males and 216 (32%) females. The mean age at interview was 44 years (SD ± 15). The average age at onset was 24 years (SD ± 7 years) and a family history of psychiatric illness was present in 27% of the patients. One hundred and forty one of the schizophrenia cases were ascertained for an affected sib-pair linkage study (47,48).

UK schizoaffective spectrum phenotype (SASP) cases. Schizoaffective spectrum phenotype (SASP) cases (N = 233; 76 male, 157 female) consisted of 204 patients who satisfied DSMIV criteria for bipolar I disorder but who had had psychiatric features in at least 50% of episodes (62 male, 142 female), together with 29 cases (14 male, 15 female) that met DSMIV criteria for schizoaffective disorder. Key clinical variables relating to psychosis were rated using the Bipolar Affective Disorder Dimensional Scale (BADDs) (49)—in this scale, a score in the range 1–100 on the Psychosis (P) dimension shows the best estimate of the proportion of total episodes of illness in which psychotic features occurred. Inter-rater reliability was formally assessed using 20 cases and resulted in mean kappa statistics of 0.85 for DSMIV diagnoses and mean intra-class correlation coefficients for the BADDs P dimension of 0.86.

UK controls. One thousand four hundred and sixteen white controls [738, 51.6% male; mean age 42.4 years (SD 11.1 years)] resident in the British Isles were collected from two sources: (a) The British Blood Transfusion Service (N = 1307). This sample was not specifically screened for psychiatric illness but individuals were not taking regular prescribed medications. In the UK, blood donors are not remunerated even for expenses and are not over-represented for indigents or the socially disadvantaged in whom the rate of psychosis might possibly rise above a threshold that
would influence power (50,51). (b) Family practitioner clinic (N = 109)—individuals were recruited from amongst those attending for non-psychiatric reasons. This sample was screened to exclude a personal history of mood disorder or schizophrenia.

1958 birth cohort control sample. The genotypes of rs2269726 for 1421 individuals (males, females) from a large population based control sample composed of all persons born in Britain during 1 week in 1958 (http://www.b58cgene.sgunl.ac.uk/). Data are publicly available, being deposited by WTSI and published online from the British 1958 Birth Cohort DNA Collection.

German cases. Five hundred and thirteen individuals with schizophrenia (334 males and 179 females) were ascertained from the Munich area in Germany. Case participants had a DSM-IV diagnosis of schizophrenia. Detailed medical and psychiatric histories were collected, including a clinical interview using the SCID (52,53). All diagnoses were double-rated by a senior researcher. Exclusion criteria included a history of head injury or neurological diseases. Mean age was 38 years and mean age at onset was 24.6 years.

German controls. Unrelated Caucasian volunteers were randomly selected from the general population of Munich, Germany, and contacted by mail. Subjects were intensively screened to exclude subjects with any brain disorder, or psychotic disorders, or who had first-degree relatives with psychiatric disorders, culminating in a comprehensive interview including the SCID I and SCID II (52,53) to validate the absence of any lifetime psychotic disorder, a neurological examination to exclude CNS impairment, and the Family History Assessment Module (54) to exclude psychotic disorders among their first-degree relatives. These procedures resulted in 1330 control subjects (609 males and 721 females) with a mean age of 49 (SD 16) years.

Bulgarian schizophrenia trio sample. The trio sample consisted of 480 patients with schizophrenia and their parents. Diagnoses were made according to DSMIV criteria by trained psychiatrists using methods similar to those employed in the UK sample as described previously (55). The mean age of the probands at ascertainment was 33 years (SD ± 8 years), the mean age of their fathers 61 years (SD ± 9.0 years) and the mean of their mothers 58 years (SD ± 9 years). The sample contained 248 male (52%) and 232 female (48%) probands.

22q11 deletion syndrome sample. The 22q11 deletion syndrome sample consisted of 83 individuals (27 Male, 56 female) who carried hemizygous deletions at 22q11. All individuals were white and born in the British Isles. The presence of the hemizygous deletion at 22q11 was confirmed by fluorescence in situ hybridization with the N25 probe (Oncor Inc., Gaithersburg, MD, USA). Psychiatric assessment of all individuals was performed by trained psychiatrists using methods similar to those employed in the UK schizophrenia sample (10) identified 22 individuals (10 male, 12 female) that had experienced episodes of psychosis (11 schizophrenia, 6 schizoaffective disorder, 5 psychotic disorder).

Selection of SNPs

We selected markers from an approximately 115 kb interval spanning TBX1 and GNB1L (chr22:18105100–18219000, UCSC March 2006 freeze, NCBI Build 36.1). Marker selection was based on Phase II HapMap (http://www.hapmap.org) as well as variants previously reported by Gong et al. (56). For markers not included in the HapMap, the 30 CEPH parent-offspring trios that constitute the HapMap CEU sample were genotyped and the data then merged with the HapMap data. The Tagger function of Haploview v3.32 (57) was used to select 19 tag SNPs that captured >80% of HapMap alleles with MAF >0.05 by pairwise tagging (mean $r^2 = 0.94$, 88% of alleles captured with $r^2 > 0.8$).

Genotyping

Markers were either genotyped using the Amplifluor™ SNPs HT Genotyping System FAM-SR (Serologicals, Norcross, GA, USA), the Sequenom MassARRAY™, the Sequenom iPLEX™ systems or SNaPshot™ (Applied Biosystems, Foster City, USA) according to manufacturers’ manuals. All assays were optimized in the 30 reference CEU parent-offspring trios (http://www.hapmap.org). All plates for genotyping contained a mixture of cases, controls (or parents), blanks and CEU samples. Genotypes were called in duplicate blind to sample identity, affected status, and blind to the other rater. Genotypes of CEU samples were compared to those available on the HapMap to provide a measure of genotyping accuracy. In our laboratory, genotyping assays are only considered suitable for analysis if (a) during optimization, our own data from CEU individuals are the same as those in the HapMap and (b) all subsequent duplicate genotypes from the CEU samples are also consistent with the HapMap data. This was achieved for all the 16 markers for which there are HapMap data, while for the remaining three markers the genotypes of all duplicate samples and negative controls were 100% consistent. Mendelian transmission was checked in the trios. Further quality control measures are described in the results section.

Allelic expression studies

Brain samples. Allelic expression analysis was performed on post-mortem brain tissue (either frontal, temporal or parietal cortex) from 149 unrelated, anonymized Caucasians (86M, 63F; mean age = 58, SD = 19). Of these, 86 had received no psychiatric or neurological diagnosis at the time of death, 22 had a diagnosis of Alzheimer’s disease, 12 a diagnosis of schizophrenia, 14 a diagnosis of bipolar disorder and 15 a diagnosis of major depression. These samples were obtained from three sources (The MRC London Neurodegenerative Diseases Brain Bank, UK; The Stanley Medical Research Institute Brain Bank, Bethesda, MD, USA; The Karolinska Institute, Stockholm, Sweden). For all samples, genomic DNA was extracted using standard phenol-chloroform procedures, and total RNA extracted using the RNAwiz™ isolation reagent.
Allelic expression assay. Genomic DNA from all subjects was initially genotyped in order to identify heterozygotes for the expressed SNPs rs2301558 and rs5746832. Duplicate cDNA samples from each heterozygous subject were then assayed twice, each time as two separate RT reactions, alongside the corresponding genomic DNA samples. Samples were amplified using primers based on single exonic sequence, capable of amplifying either cDNA or genomic DNA. RNA samples did not yield detectable levels of product in the absence of an RT step. The same analytic conditions were used for genomic DNA and cDNA so that we could use, for each assay, the average of the ratios obtained from genomic DNA (representing a perfect 1:1 ratio of the two alleles) to correct allelic ratios obtained from cDNA for any inequalities in allelic representation specific to that assay (58). Allele ratios for genomic DNA and cDNA are therefore reported as the mean of the ratios for that sample after correction by the average genomic ratio for the corresponding assays. Allelic representation was measured by primer extension and SNaPshot chemistry (Applied Biosystems), as described previously (58).

Statistical analysis

Disease association tests and LD. We used a Cochran-Armitage trend test (1 df) and a $\chi^2$ -test (2 df) respectively for allelic and genotypic tests of single locus association in the case–control samples. Goodness of fit tests for HWE were performed in the case, control, and parent populations separately. The transmission disequilibrium test (TDT) (59) was used to analyse the data from the parent–proband sample. To investigate genotypic association in the trios, we used the case–pseudocontrol approach (60) where genotypes transmitted to probands are compared with the pseudo-genotypes constructed from each non-transmitted alleles. Tests for allelic association in the 22q11 deletion syndrome sample were made using Fisher’s Exact test.

Estimation of the experiment-wide significance threshold. Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD) on pair-wise LD correlations between the 19 SNPs was carried out in the UK control sample and also on fully genotyped founders in the parent-offspring trio panel to estimate an experiment-wide significance threshold corresponding to a Type I error rate of 0.05. Using the software provided by Nyholt (61) (http://genepi.qimr.edu.au/general/daleN/SNPSpD/), 12 effective independent marker loci were estimated for both the parent-offspring trios and the UK schizophrenia case–control sample corresponding to experiment-wise significance thresholds of 0.0042. This should be considered as an approximation since this does not allow for the non-independent tests of genotype, allele and gender, where we rely for robustness on the results from multiple replication samples. In order to be conservative, all tests are two-tailed in the replication samples, although 1-tailed tests would also be justifiable.

MAQ assay. The Multiplex Amplicon Quantification (MAQ) technique (62) was used to analyse copy number at the $TBX1/GNB1L$ locus. A multiplex panel was designed for the simultaneous amplification of 15 PCR amplicons, 5 of which spanned the $TBX1/GNB1L$ locus (locations according to UCSC March 2006 freeze: 18150315–18150458, 18151204–18151448, 18155636–18155838, 18174322–18174614, 18221713–18221888) while the other 10 were located in genomic positions outside known CNVs and served as internal references. All MAQ assays were run on ABI3100 sequencers and analysed using the MAQ software (MAQs) (www.vibgeneticservicefacility.be/MAQ.htm) using protocols previously described (62). Each plate contained positive controls (22q11DS), 248 test cases and 264 test controls.

Mutation screening. The sample for mutation screening consisted of 14 unrelated Caucasian subjects from the UK meeting DSM-IV criteria for schizophrenia, each of whom had at least 1 affected sib. PCR products were designed to span the coding sequence of $TBX1$ and $GNB1L$. Each amplimer was screened for sequence variation by Denaturing High Performance Liquid Chromatography using a sensitive protocol we have described elsewhere (63). PCR products from individuals showing chromatograms suggestive of heteroduplex formation were sequenced in both directions using Big-Dye terminator chemistry and an ABI3100 sequencer according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We also thank co-workers at the Department of Psychiatry, LMU Munich for their clinical characterization of the German subjects and the laboratory handling of the samples. We are grateful to the MRC London Neurodegenerative Diseases Brain Bank (UK) and the Department of Clinical Neuroscience at the Karolinska Institute (Stockholm, Sweden) for donating brain tissue. Post-mortem brain tissue was also donated by The Stanley Medical Research Institute’s brain collection, courtesy of Drs Michael B. Knable, E. Fuller Torrey, Maree J. Webster, Serge Weis and Robert H. Yolken. MALDI-TOF genotyping of the German replication sample was conducted at the Genetics Research Centre GmbH which is a joint initiative between GlaxoSmithKline and the Dept. of Psychiatry, LMU Munich.

Conflict of Interest statement. The authors and/or their immediate family have nothing to disclose in terms of the source of funding and any affiliations with or involvement in any companies, trade associations, unions, litigants or other groups with a direct financial interest in the subject matter or materials discussed in the manuscript.
FUNDING
This work was supported by grants from the Wellcome Trust and the Medical Research Council (UK). Recruitment of the patients from Munich was partially supported by Glaxo-SmithKline. The collection of families in Bulgaria was funded by the Janssen Research Foundation, Belgium. We acknowledge use of genotype data from the British 1958 Birth Cohort DNA collection, funded by the Medical Research Council grant G0000934 and the Wellcome Trust grant 068545/Z/02.

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