Converging evidence that sequence variations in the novel candidate gene MAP2K7 (MKK7) are functionally associated with schizophrenia

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Schizophrenia is a debilitating psychiatric disease with a strong genetic contribution, potentially linked to altered glutamatergic function in brain regions such as the prefrontal cortex (PFC). Here, we report converging evidence to support a functional candidate gene for schizophrenia. In post-mortem PFC from patients with schizophrenia, we detected decreased expression of MKK7/MAP2K7—a kinase activated by glutamatergic activity. While mice lacking one copy of the Map2k7 gene were overtly normal in a variety of behavioural tests, these mice showed a schizophrenia-like cognitive phenotype of impaired working memory. Additional support for MAP2K7 as a candidate gene came from a genetic association study. A substantial effect size (odds ratios: ~1.9) was observed for a common variant in a cohort of case and control samples collected in the Glasgow area and also in a replication cohort of samples of Northern European descent (most significant P-value: 3 x 10^{-4}). While some caution is warranted until these association data are further replicated, these results are the first to implicate the candidate gene MAP2K7 in genetic risk for schizophrenia. Complete sequencing of all MAP2K7 exons did not reveal any non-synonymous mutations. However, the MAP2K7 haplotype appeared to have functional effects, in that it influenced the level of expression of MAP2K7 mRNA in human PFC. Taken together, the results imply that reduced function of the MAP2K7-c-Jun N-terminal kinase (JNK) signalling cascade may underlie some of the neurochemical changes and core symptoms in schizophrenia.

INTRODUCTION

Schizophrenia is a devastating mental illness which affects 1% of the world population, characterized by positive symptoms (such as hallucinations and delusions), negative symptoms, (such as social withdrawal, avolition, anhedonia and self-neglect) and cognitive deficits (including impairments in executive function and attention). While the aetiology of schizophrenia remains elusive, there is known to be a strong genetic component. The current theories emphasize the contribution of large numbers of common genetic variants of small effect, combined with rare variants of larger effect. Intensive research has revealed a number of candidate genes that may be involved in schizophrenia; yet, the degree of genetic asso-

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A few specific neurobiological features have been identified in the central nervous system (CNS) of patients with schizophrenia. Compared with control subjects, schizophrenic patients exhibit a lower metabolic activity in the prefrontal cortex (PFC) when performing a cognitive task (7–9). This metabolic ‘hypofrontality’ is generally accompanied by parallel alterations in metabolic activity in the temporal cortex and hippocampus. In addition, post-mortem studies have revealed a selective loss of parvalbumin-containing GABAergic interneurons in the PFC and hippocampus from patients with schizophrenia (10–12). This regionally specific pathology focuses attention on the PFC as a major site of dysfunction in schizophrenia and is consistent with evidence that many of the cognitive tasks where patients show deficits are dependent on PFC function (13,14). In addition, the severity of negative symptoms and cognitive deficits has been shown to correlate with the degree of metabolic hypofrontality in the PFC (7,8,15,16).

The neurobiological substrates for hypofrontality are unclear. However, antagonists at the N-methyl-D-aspartate (NMDA) class of glutamate receptor such as phencyclidine, which can induce a range of symptoms virtually indistinguishable from schizophrenia in humans (17,18), are able to reproduce metabolic hypofrontality, along with reduced PFC parvalbumin expression in rats (19). This suggests that dysfunctional NMDA receptor signalling may be linked to the generation of hypofrontality. Intriguingly, changes in cortical metabolic activity in imaging studies are now being linked to altered synaptic plasticity (20,21), and the premise that hypofrontality in schizophrenia might represent impaired synaptic plasticity is consistent with emerging concepts of the disease (22–24). This would be consistent with the prominent role of NMDA receptors in many forms of cortical and hippocampal synaptic plasticity. Various signalling pathways link NMDA receptor stimulation to long-term plasticity (25) and hence are potentially involved in activity-dependent metabolic changes in cortical regions. However, a number of these are linked to other CNS diseases and are hence unlikely to cause schizophrenia: for example, altered Ras-Erk activity causes the cardio-facio-cutaneous syndromes (26). Our attention was drawn to the c-Jun N-terminal kinase (JNK) pathway, which mediates aspects of cortical and hippocampal synaptic plasticity (27,28). Although JNK activation is less well-characterized than that of extracellular signal-regulated kinase (ERK) in relation to glutamatergic stimulation, impaired JNK function has been specifically linked to deficits in synaptic plasticity (29–31). The kinase involved in the activation of JNK by NMDA receptor stimulation is MKK7 (derived from the MAP2K7 gene) (32). Here, using a combination of genetic, genomic and behavioural approaches, we test the hypothesis that dysfunction of MKK7/MAP2K7 signalling might underlie aspects of schizophrenia.

**RESULTS**

**MAP2K7 expression in the PFC**

The levels of MAP2K7 mRNA were assessed, via real-time quantitative reverse transcriptase–PCR (qRT–PCR), in post-mortem PFC tissue (Brodmann areas 9 and 10) from schizophrenic patients, compared with equivalent tissue from control subjects. The subjects were matched for age, gender and post-mortem delay, and data were normalized to housekeeping gene (GAPDH) expression. We observed a profound decrease in MAP2K7 expression in the PFC from schizophrenia patients (~30%) (Fig. 1A). The down-regulation of MAP2K7 expression was independent of the reference (housekeeping) gene used to normalize the data (Supplementary Material, Fig. S1). For comparison with previous reports, we also monitored the levels of parvalbumin (PVALB) mRNA—one of the most robustly altered genes in post-mortem tissue from schizophrenia patients. As expected, we observed a decrease in parvalbumin mRNA expression (Fig. 1B), although a number of other mRNAs did not show altered expression in...
tissue from patients when compared with controls (data not shown). The data suggest that MAP2K7 expression is decreased in the PFC in patients with schizophrenia.

Phenotypic analysis of Map2k7 heterozygote mice

To investigate whether decreased MAP2K7 expression may be sufficient to cause any schizophrenia-related changes in behaviour, we employed mice haploinsufficient for the Map2k7 gene. Mice heterozygous for a functional deletion of the Map2k7 gene (Map2k7HZ mice) were initially tested on elements of the SHIRPA protocol (33) to screen for any overt behavioural abnormalities. Map2k7HZ mice appeared normal and performed at normal levels on all the SHIRPA tasks, including the rotorod test (Fig. 2A). In the open field test, mice habituated to the test environment in a similar manner to wild-type control animals and did not differ in locomotor activity, once habituated to the environment (Fig. 2B). However, when assessed using a working memory task designed to parallel tests where patients with schizophrenia show deficits (34), mice showed an impaired ability to perform the task, with reduced numbers of correct responses, and an increased level of perseverative responding (tendency to persist in a response when it is no longer correct) (Fig. 2C).

Is MAP2K7 a candidate risk gene for schizophrenia?

These functional data suggested that MAP2K7 signalling is impaired in the PFC in schizophrenia and that dysfunctional MAP2K7 signalling is sufficient to cause schizophrenia-like phenotype. We therefore looked for supportive evidence that MAP2K7 might be a contributory factor in causing schizophrenia, by testing the hypothesis that sequence variations in the MAP2K7 gene may show association with schizophrenia. Two sets of blood sample DNA were analysed. Initially, a small ethnically homogeneous cohort (Cohort 1) consisting of Caucasian subjects in the Glasgow/West of Scotland area, comprising around 200 cases and 200 controls, was used to test the hypothesis. Little population structure is expected in this cohort, but we tested them for stratification as described in Supplementary Material. No detectable population structure was observed (Supplementary Material, Fig. S2). A second set of samples, the University College London schizophrenia case-control sample (Cohort 2) comprising around 300 cases and 300 controls, was used to test for replication.
Population stratification has been excluded by extensive testing in this cohort (35,36). Two MAP2K7 single-nucleotide polymorphisms (SNPs) were genotyped: rs3679 in the 3′-untranslated region (UTR) and rs4804833 immediately upstream of an alternatively spliced exon present in MAP2K7 γ isoform transcripts (Fig. 3A).

Significant associations with schizophrenia were observed for both SNPs in the Glasgow samples (Table 1). Significant
effects of both genotype and allele were noted. These significant associations were replicated in the separate London samples with similar odds ratios (Table 1). When the two sets of samples were combined by meta-analysis, the level of significance for the association of both SNPs was enhanced (Table 1). The association remained significant after correcting for multiple comparisons. Odds ratios in both cohorts indicated that the risk genotype almost doubled disease risk (Table 1).

We note that the \( MAP2K7 \) gene may have escaped detection in the recent GWAS of schizophrenia because it is relatively poorly represented on the arrays used (e.g. no SNPs on the Affymetrix 500K, 5.0 or 6.0 genotyping platforms). The fact that association of the \( MAP2K7 \) gene with schizophrenia has not been detected by GWAS approaches suggests that the association signal may not be captured by SNPs in \( MAP2K7 \) or neighbouring genes. To test this, in the same two cohorts of samples, we genotyped two SNPs in the \( LRRC8E \) gene (which is represented on the Affymetrix 500K platform and lies only 2 kb upstream from the \( MAP2K7 \) gene on chromosome 19). The positions of these SNPs are shown in Supplementary Material, Fig. S3. While existing SNP databases suggest modest linkage disequilibrium between these \( LRRC8E \) SNPs and the \( MAP2K7 \) SNPs, our own data, in a substantially larger sample, show only limited linkage disequilibrium between SNPs in the two genes (Table 2; Supplementary Material, Fig. S4). No significant association with schizophrenia was detected for the \( LRRC8E \) SNPs (Supplementary Material, Table S1), strongly suggesting that the association signal is specific for \( MAP2K7 \).

Age of onset data were available for the patients in the London cohort of samples. We therefore tested whether there was any evidence for the association of rs4804833 with age of onset of schizophrenia. We found that patients with early onset (16 years old or lower) were more likely to be homozygous for the rare allele than those with later onset (Table 3).

### MAP2K7 resequencing

These results implicate \( MAP2K7 \) dysfunction in increasing the risk of schizophrenia. To assess whether the SNPs could be proxy markers for coding mutations in the \( MAP2K7 \) gene, all exons (incorporating all the alternatively spliced exons and the 5' and 3' UTRs) were fully sequenced in patients homozygous for both risk alleles (rs4804833-A and rs3679-T; \( n = 20 \)) and healthy controls homozygous for the common haplotype (GC; \( n = 3 \)). No further mutations were identified and indeed sequencing of the intronic regions, with the exception of the large first intron, showed very little allelic heterogeneity across the entire gene (Supplementary Material, Fig. S5), regardless of the risk/non-risk carrier status. In fact, of the 23 3'UTR SNPs listed at dbSNP, only 4 were found to be polymorphic in a few cases.

Sequencing confirmed the genotype data for the analysed samples with the three healthy control samples having the ancestral G allele of intronic SNP rs4804833 and ancestral C allele of rs3679 3'UTR SNP, while all samples from schizophrenic patients had an A allele at rs4804833 and T allele at rs3679. None of the dbSNP listed exonic SNPs were found to be polymorphic in these samples. Overall, this region of chromosome 19 showed remarkably little heterogeneity, in-keeping with the HapMap recombination data, suggesting that \( MAP2K7 \) is in one haplotype block (Supplementary Material, Fig. S4). This was evident in our data from the lack of variation seen in the coding, intronic and 3'UTR SNPs that are listed at dbSNP.

### Table 1. Results of SNP analysis for \( MAP2K7 \) in samples from schizophrenia patients and controls

<table>
<thead>
<tr>
<th>Position</th>
<th>SNP</th>
<th>Cohort</th>
<th>Control genotype</th>
<th>Case genotype</th>
<th>MAF Cont.</th>
<th>MAF cases</th>
<th>P-value (Fisher)</th>
<th>Trend P-value (Armitage exact)</th>
<th>Odds ratio: 11 versus 12 or 22; (95% confidence interval)</th>
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</thead>
<tbody>
<tr>
<td>19:797635 rs4804833</td>
<td>1</td>
<td>21</td>
<td>90</td>
<td>78</td>
<td>38</td>
<td>90</td>
<td>62</td>
<td>0.35</td>
<td>0.44</td>
</tr>
<tr>
<td>19:797635 rs4804833</td>
<td>1</td>
<td>16</td>
<td>92</td>
<td>82</td>
<td>32</td>
<td>85</td>
<td>73</td>
<td>0.33</td>
<td>0.39</td>
</tr>
<tr>
<td>19:797635 rs4804833</td>
<td>2</td>
<td>30</td>
<td>148</td>
<td>121</td>
<td>52</td>
<td>142</td>
<td>97</td>
<td>0.35</td>
<td>0.42</td>
</tr>
<tr>
<td>19:797635 rs4804833</td>
<td>2</td>
<td>28</td>
<td>128</td>
<td>143</td>
<td>43</td>
<td>134</td>
<td>115</td>
<td>0.31</td>
<td>0.38</td>
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<tr>
<td>19:797635 rs4804833</td>
<td>1 + 2*</td>
<td>51</td>
<td>238</td>
<td>199</td>
<td>90</td>
<td>232</td>
<td>159</td>
<td>0.35</td>
<td>0.43</td>
</tr>
<tr>
<td>19:797635 rs4804833</td>
<td>1 + 2*</td>
<td>44</td>
<td>220</td>
<td>225</td>
<td>75</td>
<td>219</td>
<td>188</td>
<td>0.31</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Results are shown for both the Glasgow cohort (Cohort 1) and the London cohort (University College London schizophrenia case–control sample—Cohort 2) of samples. For rs4804833, 1 = A, 2 = G; for rs3679, 1 = T, 2 = C. Results were analysed using two complementary statistical tests—Fisher’s exact test and the Armitage trend test.

The combined analysis of the two sets was done by meta-analysis using both fixed and random effects using the metan routine in Stata 11 (Statacorp, College Station, TX, USA). As there was virtually no heterogeneity between the two cohorts (\( I^2 = 0.00 \) in all three analyses), the random- and fixed-effects analyses were identical.
respect to age of onset of symptoms (in years old). Patients homozygous for the rare rs4804833 allele, with prevalent in the early age of onset group. The table shows the proportion of

<table>
<thead>
<tr>
<th>Age of onset</th>
<th>rs4804833</th>
<th>rs3745382</th>
<th>rs533822</th>
<th>rs3679</th>
<th>rs4804833</th>
<th>rs3745382</th>
<th>rs533822</th>
<th>rs3679</th>
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<tr>
<td>21–25</td>
<td>11</td>
<td>70</td>
<td>0.112</td>
<td>0.012</td>
<td>11</td>
<td>70</td>
<td>0.112</td>
<td>0.012</td>
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<tr>
<td>16–20</td>
<td>16</td>
<td>96</td>
<td>0.805</td>
<td>0.805</td>
<td>16</td>
<td>96</td>
<td>0.805</td>
<td>0.805</td>
</tr>
<tr>
<td>≥26</td>
<td>7</td>
<td>83</td>
<td>0.797</td>
<td>0.900</td>
<td>7</td>
<td>83</td>
<td>0.797</td>
<td>0.900</td>
</tr>
</tbody>
</table>

Table 2. Linkage disequilibrium data

Table 3. Homozygosity for MAP2K7 risk allele, with respect to age of onset of schizophrenia

<table>
<thead>
<tr>
<th>Age of onset</th>
<th>rs4804833</th>
<th>No. of cases, AA</th>
<th>No. of cases, AG/GG</th>
<th>% AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;16</td>
<td>7</td>
<td>12</td>
<td></td>
<td>36.8</td>
</tr>
<tr>
<td>16–20</td>
<td>16</td>
<td>96</td>
<td></td>
<td>14.3</td>
</tr>
<tr>
<td>21–25</td>
<td>11</td>
<td>70</td>
<td></td>
<td>13.6</td>
</tr>
<tr>
<td>≥26</td>
<td>11</td>
<td>83</td>
<td></td>
<td>11.7</td>
</tr>
</tbody>
</table>

Patients homozygous for the MAP2K7 risk allele are disproportionately prevalent in the early age of onset group. The table shows the proportion of patients in London cohort homozygous for the rare rs4804833 allele, with respect to age of onset of symptoms (in years old).

MAP2K7 alleles are functionally relevant

We then investigated whether the SNPs themselves were acting as cis-regulatory variants affecting the expression of MAP2K7 transcripts. qRT–PCR and quantitative single-base extension (SBE) were used to analyse differential expression of MAP2K7 transcripts (37) from RNA extracted from the PFC of schizophrenia patients and healthy controls. The rs4804833 polymorphism is immediately upstream of alternatively spliced exon b, which is present in the γ isoforms (37). It is noteworthy that the short intronic region containing the rs4804833 polymorphism is highly conserved between species (Fig. 3A), implying some important functional relevance. qRT–PCR showed that subjects homozygous for the rs3679 risk genotype showed an increase in the expression of these MAP2K7 γ isoforms (Fig. 3B, left panel). A similar increased expression in rs3679 homozygotes was observed for transcripts containing exon 1 and exon b spliced together (Fig. 3B, right panel). While there was a trend towards similarly elevated expression of the major MAP2K7 transcripts, this did not reach significance (data not shown).

To provide independent confirmation of this effect, SBE analysis of cDNA from heterozygous subjects was used to determine whether there were allele-specific MAP2K7 mRNA expression differences using the rs3679 polymorphism as a copy-specific tag. This tag is present in the majority of MAP2K7 transcripts (Fig. 3A). If the genotype has no effect on expression, then a 1:1 ratio of the two different alleles is expected, as is the case in genomic DNA where each allele is present on one chromosome. However, if either allele is acting as a cis-regulatory factor then the expression of transcripts from the two different chromosomes will be different and therefore the allelic ratio will deviate from 1:1 (38).

We considered that the apparent discrepancy related to the increased expression of MAP2K7 transcripts derived from the risk allele, and the decreased expression observed in PFC from patients with schizophrenia, probably related to the well-characterized ability of signalling pathways in the CNS to compensate for elevated activity by decreasing the expression of key pathway components. As a corroboration of this idea, we treated cultured embryonic mouse cortical neurons with sorbitol, a prototypic activator of MAP2K7 pathways (39–41). We observed the expected increased phosphorylation of MAP2K7 at early time points following sorbitol treatment (Supplementary Material, Fig. S6A). Twenty-four hours after sorbitol treatment, the levels of MAP2K7 were reduced by ~50% (Supplementary Material, Fig. S6B), confirming the down-regulatory response of the MAP2K7 gene to an increased level of activation.

DISCUSSION

A large number of neurochemical changes have been reported in post-mortem PFC tissue from patients with schizophrenia. Of those that have been robustly observed, altered glutamate receptor expression, and deficits in GABAergic markers including parvalbumin, are among the most prominent (11,12,42). In the present study, qRT–PCR analysis of PFC tissue from patients revealed a reduction in the expression of the Map2k7 gene. Conversely, the levels of expression of a number of genes unrelated to this pathway were not changed relative to control tissue (data not shown). This evidence suggests a dysregulation of the signalling mediated by this kinase in the PFC in schizophrenia.

Influence of altered Map2k7 expression on behaviour

Dysfunctional networks involving the PFC and hippocampus have been linked to working memory deficits in schizophrenia. Mice haploinsufficient for Map2k7 were therefore tested on a...
variety of behavioural tasks. These mice appeared normal on a variety of tasks and showed no obvious abnormalities, despite the fact that complete deletion of this gene is embryonic lethal (43,44). In a working memory task which involves the PFC (34), the Map2k7HZ mice showed reduced correct responses and increased perseveration. This pattern of dysfunction is reminiscent of the deficits observed in patients with schizophrenia (45–47). In particular, perseverative responses in cognitive tests are a feature of the disease (48,49). Indeed, deficits in this working memory task have been detected in mice with genetic disruptions of the Disc1 gene (50,51). Importantly, in the Map2k7HZ mice, the working memory deficits were not confounded by alterations in locomotor activity, as can be the case in the behavioural analysis of genetically manipulated mice. In the open field test, Map2k7HZ mice displayed a similar habituation to the environment and similar locomotor activity to wild-type littermate control mice. Thus, the effects observed in the working memory task are likely to be attributable to cognitive processes rather than resulting from motor dysfunction. While there are always caveats required when extrapolating rodent behaviour to human, the data are consistent with MAP2K7 dysfunction potentially contributing to the PFC relevant cognitive aspects of the disease.

Investigation of genetic association

We tested whether sequence variations in the MAP2K7 gene might be more prevalent in patients when compared with ethnically matched controls. We found a clear association between MAP2K7 alleles and schizophrenia in each of the two separate cohorts of samples, suggesting that the finding in the first set of samples does not represent the familiar ’winner’s curse’. The similar levels of significance, and similar odds-ratios, in the two separate cohorts satisfy the key criteria for replication of genetic association (52). In general, previous studies have not detected genetic evidence for the involvement of the MAP2K7 locus at 19p13.2 in schizophrenia. Lack of previous detection of this functional candidate gene may be due to its relatively poorly representation in the chips used for the recent GWAS of schizophrenia (e.g. no SNPs on the Affymetrix 500K genotyping platform). In addition, the HapMap genotyping data (release #28) for rs3679 do not pass Haploview quality control checks, rendering the information and use of these data unreliable. However, two studies found nearby markers to show association or linkage with schizophrenia in early genome-wide scans (53,54), while an unconfirmed report suggested that chromosomal rearrangement in this region may represent a high penetrance site for schizophrenia (55). A recent report describes duplication of a 9 Mb region including the MAP2K7 gene in a patient with schizophrenia (56). As with all initial studies reporting significant association of candidate genes with a complex disease, our findings will require replication. This is especially true because the gene has not been detected by GWAS. Replication is necessary to rule out the possibility of type I errors. However, our functional and genetic data provide intriguing evidence that sequence variations in this region of the genome are associated with schizophrenia.

The degree of sequence variation in MAP2K7 exons in our samples was remarkably low. The 5′UTR, exons and 3′UTR were sequenced from overlapping amplicons of gDNA. This lack of heterogeneity is suggestive of a selective sweep and implies that sequence variations in this gene can impair evolutionary fitness. This is consistent with a selective sweep at the Map2k7 gene locus in the mouse (57). Interestingly, rs4804833 and rs3679 fit precisely the theoretical predictions for sequence variations underlying complex disease, in terms of allele frequency and lack of the surrounding genetic variation (58). We note that the reported population allele frequency information currently available in dbSNP and linked databases for rs4804833 and rs3679 varies dramatically for related populations, deviating dramatically from the Hardy–Weinberg equilibrium in some cases. This probably reflects the low numbers of subjects currently genotyped at this locus and the low call rate (HapMap release #28 data), and the information would be expected to improve as more subjects are added. In the future, data emerging from deep sequencing efforts focused on patients with schizophrenia and control subjects will provide further information on MAP2K7 gene structure, and its relationship to disease.

We found that the proportion of patients homozygous for the rare allele was particularly high in the subgroup with early onset of symptoms (16 years old or lower). The number of subjects in this group was fairly low, and this observation would benefit from replication in a cohort of samples specifically studying this genetic variation in early-onset patients. However, the findings are intriguing, since early-onset of symptoms may be linked to a more severe symptomatology.

The association data, and the lack of other sequence variations detected by our re-sequencing, suggested that one of the genotyped SNPs could be a functional mutation. It remains possible that another SNP, e.g. in the areas of the large first two introns which were not sequenced here, could be the functionally relevant sequence variation. However, the lack of overt inter-species conservation in these intronic regions implies that this maybe unlikely. In the PFC from subjects heterozygous for alleles of the 3′UTR SNP rs3679, transcripts bearing the risk allele were present at higher abundance than those bearing the common allele. Since the 3′UTR regulates mRNA stability, the risk allele may enhance MAP2K7 mRNA stability and hence increase levels. However, the genomic region immediately surrounding rs4804833 shows a remarkable degree of inter-species sequence conservation, and hence may be important for regulating alternative splicing of the exon adjacent to rs4804833 (the majority of SNPs affecting exon splicing are not within consensus splice sites) (59). Transcripts containing exon b (MAP2K7 γ isoforms) were indeed more abundant in the PFC from subjects with the risk allele compared with the common allele. Taken together, these results provide a strong indication that this SNP is a cis-acting factor regulating splicing/expression of the γ isoforms, and hence may act as a functional mutation affecting MAP2K7/MKK7 activity.

Mechanisms and functional consequences of schizophrenia-related MAP2K7 sequence variations

The primer extension assay targeting the 3′UTR SNP rs3679 revealed clearly elevated expression of transcripts containing
the risk allele. The 3'UTR has a well-established role in the regulation of mRNA stability. Very little genetic variation was detected in the 3'UTR in our samples. In particular, the samples homozygous for the rare rs3679 allele, which were over-represented in the patient group, did not show any other polymorphic alleles in the 3'UTR. This may indicate that rs3679 is the functional mutation, affecting mRNA stability and leading to increased levels of transcripts containing the rare allele. However, in view of the almost complete linkage disequilibrium between rs3679 and rs4804833, immediately upstream of exon b, the findings are also consistent with the view that rs4804833 is the functional mutation. Indeed, the extraordinary conservation of sequence at this region across diverse species supports the suggestion that it plays some important functional role. It may be significant that the rare rs4804833 (A) allele introduces a consensus site (TCAGTCTN) for the cap signal for transcription initiation just in advance of exon b. If this were to increase transcription from MAP2K7 genes with the rare allele, this would explain both the increased levels of exon b-containing transcripts, and the elevated expression of all transcripts from the risk haplotype as assessed by the primer extension assay. We therefore favour the interpretation that it is the rs4804833 sequence variation that is acting as a functional mutation.

The functional significance of an increased presence of exon b in mature MAP2K7 transcripts is not completely clear, although these γ isoforms appear to be more potent at activating JNK (60–62). Interestingly, these γ isoforms have very recently been specifically linked to actin regulation and altered neurodevelopment (62,63).

Although the MAP2K7 risk genotype appears to act to increase MAP2K7 expression levels, the qRT–PCR analysis showed a profound down-regulation of this kinase in tissue from the patients with schizophrenia. There are two possible reasons for this paradox. First, all the tissue samples derived from patients who had been on long-term antipsychotic treatment. It is conceivable that chronic antipsychotic treatment in adulthood down-regulates MAP2K7/MKK7 expression. However, this is unlikely, as rats treated with chronically antipsychotic drugs do not show effects on this pathway (64,65) (unpublished data). Alternatively, a consequence of elevated MAP2K7/MKK7 signalling due to genetic factors early in development may be to initiate a compensatory down-regulation of pathway genes, for example, to protect the CNS against adverse (pro-apoptotic) effects of elevated JNK activity. This would be consistent with our knowledge of the effects of elevated signalling in the Raf-MEK-ERK pathway (66). Indeed, we confirmed that in embryonic cortical neurones, increased MAP2K7 activation leads to a dramatic reduction in MAP2K7 expression (Supplementary Material, Fig. S6), supporting the concept that tight regulation is important for this signalling pathway during CNS development. Thus, we speculate that sequence variations in MAP2K7, combined with environmental triggers, lead to altered pathway activity, and consequent down-regulation of MAP2K7, in patients with schizophrenia. This is of interest considering existing knowledge of the activation of MAP2K7-JNK signalling by ‘stress stimuli’ (67). While it will be extremely important for this report to be followed up by further genetic association studies for MAP2K7, the findings we report here linking MAP2K7 signalling to schizophrenia raise the prospect that dysfunction of MAP2K7-JNK signalling, caused by altered activity of one or more genes in the pathway, contributes to causing schizophrenia, and hence that novel drug treatments, aimed at restoring the function of this pathway, may be a highly effective strategy for treating the disease.

**MATERIALS AND METHODS**

**Post-mortem tissue sample preparation**

Post-mortem PFC tissue (BA 9 or 10, matched between groups) was available from 18 patients and 18 matched control subjects.
Sequencing of MAP2K7 regions of exons and the 5′ in the VILOTM cDNA synthesis kit (Invitrogen) following the GAPDH, actin, cycler. Four reference (housekeeping) genes were employed: Taqman gene-specific Assays-by-Design (ABI), or custom assays where the target assay was not available, and an ABI cycler. Four reference (housekeeping) genes were employed: GAPDH, actin, β2-microglobulin and 18S rRNA.

Genotyping of post-mortem tissue samples
The post-mortem brain-derived cDNA samples were genotyped for the rs3679 3′ UTR SNP as above, and these genotypes were also used to infer the genotype of the rs4804833 intronic SNP, due to almost 100% linkage disequilibrium between the two loci (Haplovieart 0.95 and our data D′ 0.99). The samples were assigned a number at the first-strand cDNA synthesis stage so that genotyping was performed blind to sample ID and disease status.

Allele-specific expression analysis of rs3679 heterozygote subjects
Allele-specific expression of rs3679 heterozygotes was analysed using the SNAPSHOTM Multiplex Kit (Applied Biosystems) SBE of a single fluorescent nucleotide. Further details are provided in Supplementary Material.

Phenotypic analysis of Map2k7Hz mice
Mice heterozygous for a functional deletion of the Map2k7 gene have been described previously (43,44). Mice were backcrossed onto the C57Bl6J strain. All studies were conducted using WT and HZ littermates, of mixed sexes, and aged 8–12 weeks. Initially, mice were tested on elements from the SHIRPA protocol (33) to screen for any overt behavioural abnormalities. For the open field test, the apparatus consisted of perspex arenas (dimensions 40 cm × 40 cm × 40 cm) situated on an infra-red light box. Movement within the arenas was tracked by an overhead infrared detecting camera using Ethovision video tracking software. Mice were placed in each arena and assessed for locomotor activity for 90 min to enable investigation of habituation to the environment and locomotor activity per se. Mice were also tested using the discrete paired-trial variable delay T-maze working memory paradigm developed by Aultman and Moghaddam (34). This task incorporates important elements of clinical working
memory, being sensitive to retention interval and proactive interference, and is dependent on the integrity of the PFC. All animals were initially handled for 5 min each over a 2-day period. Following the initial 2 days of handling, each animal was given 5 min exposure to T-maze environment with both arms baited with liquid reward (70 μl 50% condensed milk) and given a further 5 min of handling. On the final 2 days of habituation, all animals were given five trials with both arms baited. Training on the task involved a randomly chosen forced run in which animals were given access to only one arm of the maze and rewarded after entering that arm. After a 10 s intertrial delay (retention interval), they were presented with the choice run, during which they had access to both arms and were rewarded for entering the arm that they had not entered on the previous forced run (a correct response). After this choice run and an intertrial interval of 40 s, animals were exposed to the next forced run and so on. Ten paired trials were performed each day until the mice achieved 70% correct responding. Thereafter, mice were subjected to three variable intertrial intervals of 5, 15 and 30 s over 3 consecutive days (three trials per day). The order of forced choice presentation was randomized across days. The number of correct arm choices was monitored as was the number of perseverative errors.

AUTHOR CONTRIBUTIONS

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

NOTE ADDED IN PROOF
Consistent with the down-regulation of MAP2K7 we observe, in conjunction with genetic influences increasing expression, it has recently become clear that loss-of-function mutations in TGF-beta vasculopathies are associated with increased expression of TGF-beta signalling pathway genes (88).

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Conflict of Interest statement. None declared.

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