Cognitive Characterization of Schizophrenia Risk Variants Involved in Synaptic Transmission: Evidence of CACNA1C's Role in Working Memory.

Running Title: SZ Synaptic Transmission Risk Variants and Cognition

Donna Cosgrove[1], Omar Mothersill[1], Kimberley Kendall[3], Bettina Konte[4], Denise Harold[2,6], Ina Giegling[4], Annette Hartmann[4], Alex Richards[5], Kiran Mantripragada[5], The Wellcome Trust Case Control Consortium, Michael J. Owen[5], Michael C. O'Donovan[5], Michael Gill[2], Dan Rujescu[4], James Walters[3], Aiden Corvin[2], Derek W. Morris[1], Gary Donohoe[1]

[1]The Cognitive Genetics & Cognitive Therapy Group, The School of Psychology and Discipline of Biochemistry, The Centre for Neuroimaging & Cognitive Genomics, National University of Ireland Galway, Ireland.
[2]Neuropsychiatric Genetics Research Group, Department of Psychiatry, Institute of Molecular Medicine, Trinity College Dublin, Ireland.
[3]Institute of Psychological Medicine and Clinical Neurosciences, Cardiff University, UK.
[4]Department of Psychiatry, Psychotherapy and Psychosomatics, Martin-Luther-University Halle-Wittenberg, Germany.
[5]MRC Centre for Neuropsychiatric Genetics and Genomics, Cardiff University, UK.
[6]School of Biotechnology, Dublin City University, Dublin, Ireland.

Corresponding Author:
Prof. Gary Donohoe
The Centre for Neuroimaging & Cognitive Genomics,
School of Psychology
National University of Ireland Galway
Email: gary.donohoe@nuigalway.ie

Tel: +353 91 495122
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Abstract

With over 100 common variants associated with schizophrenia risk, establishing the biological significance of these variants is a priority. We sought to establish the cognitive effects of risk variants at gene loci implicated in synaptic transmission by 1) identifying GWAS schizophrenia variants whose associated gene function was identified as related to synaptic transmission, and 2) testing for association between these variants and measures of neurocognitive function. We selected SNP variants, reported in the largest genome-wide association study to date, located within ~20kb of genes associated with synaptic transmission. Associations between genotype and cognitive test score were analyzed in a discovery sample of 798 Irish patients with psychosis and 190 healthy participants, and replication samples (528 UK participants; 921 German participants). Three loci showed significant associations with neuropsychological performance in the discovery samples. This included an association between rs2007044 within *CACNA1C* and poorer working memory performance (increase in errors – B [95% CI] = 0.635 – 4.535, p=0.012). In an fMRI analysis of working memory performance (n=103 healthy participants), we further found evidence that the same *CACNA1C* risk allele G was associated with decreased functional dysconnectivity between the right dorsolateral prefrontal cortex and both the right anterior cingulate and cuneus. In conclusion, these data provide some evidence to suggest that the *CACNA1C* risk variant rs2007044 is associated with poorer memory function, and that this may result from risk carriers' difficulty with top down initiated responses caused by dysconnectivity between the right DLPFC and three cortical regions.

Key Words: genetics, GWAS, fMRI, RIMS1, CACNB2

Introduction

Predictions that increasing sample sizes would also lead to an increase in genome wide significant schizophrenia (SZ) risk variants being identified[1] have been supported to date. As sample sizes available for GWAS have increased, the numbers of significantly associated common variants and genes has gone from one, *ZNF804A*[2], to a handful[3] to over 108 loci now being implicated[4]. As the purpose of gene discovery is to identify the biological pathways that increase risk and are capable of being targeted by treatments, moving from discovering to establishing functional impact is a priority for our field.

While this is often thought of at the molecular and cellular level, establishing the functional impact of genes and gene sets at other levels of analysis (i.e. neural, cognitive, and behavioral) is also likely to be important in providing insights for new treatment discovery. For example, we and others have previously sought to establish the cognitive effects of polygenic risk in pathways defined either by biological processes, e.g. variants within the cell adhesion molecule pathway[5], or by gene sets functionally related to identified SZ-risk genes (e.g. *ZNF804A)*[6]. To date, and to our knowledge, however, no study has systemically investigated the cognitive effects of SZ variants for synaptic transmission despite the fact that alterations in this process (and neurotransmitter systems, e.g. dopamine) have long been held as a key etiological hypothesis for both SZ risk and for deficits in cognition associated with the disorder[7-9]. Looking at the effect of SZ-associated variants on cognition may help identify specific neurobiological processes involved, so as to allow this aspect of disability to be more effectively targeted.

The purpose of the present study was to undertake a systematic analysis of the cognitive effects of SZ risk associated variants identified by the Schizophrenia Working Group of the Psychiatric Genomics Consortium (PGC-SCZ)[4] in genes related to synaptic transmission using a novel methodology. We did this by 1) cross-referencing PGC-SCZ identified variants whose associated gene function was bioinformatically identified as related to synaptic transmission

using OMIM and Gene Ontology, and 2) comparing the performance of carriers and non-carriers of these variants on cognitive functions typically impaired in SZ. Our hypothesis was that the identified risk variants at these gene loci would be associated with poorer performance on measures of cognition.

Methods

Neuropsychological Sample Characteristics

Cognitive performance was assessed in 939 cases and 330 healthy participants (769 males, 500 females). Cases consisted of clinically stable patients with either a 'narrow sense psychosis' diagnosis of SZ or schizoaffective disorder (SZA) (n=676) or a 'broader sense psychosis', which in addition to our SZ/SZA sample, included patients with a diagnosis of bipolar disorder with psychotic features, major depressive disorder with psychotic features, or psychosis not otherwise specified (n=263), based on Structured Clinical Interview for DSM-IV Axis I[10]. Healthy participants were recruited from the general population through local media advertisements and satisfied, on the basis of clinical interview, the criteria of having no history of major mental health problems or intellectual disability, and no first-degree relative with a history of psychosis. All participants were aged between 18 and 65 years and had Irish-born paternal and maternal grandparents, no acquired brain injury, and no substance abuse in the preceding six months. All assessments were conducted in accordance with the relevant ethics committees' approval (St. James's Hospital, Tallaght Hospital, Galway Hospital, Cork Hospital, and Beaumont Hospital Research Ethics Committees), and all participants provided written informed consent. A significant proportion of these samples were previously included in the PGC-SCZ GWAS[4].

Two replication samples were also available. Samples from Cardiff consisted of 528 European Caucasian individuals with SZ or SZA who were recruited from community mental health teams in Wales and England. A SCAN interview and case note review were carried out to confirm diagnosis according to DSM-IV criteria. The UK Multicenter Research Ethics Committee (MREC) approved the study, and all participants provided valid informed consent.

The German replication sample consisted of 362 clinically stable patients with a DSM-IV diagnosis of SZ, and 559 healthy controls, all genotyped as part of a previous study[2]. All participants provided written informed consent, were aged between 18 to 80 years, and had no history of head injury or neurological diseases. Patients were included if they had a DSM-IV diagnosis of SZ, whereas healthy controls underwent interviews to confirm the absence of any lifetime psychotic disorder or those who had first-degree relatives with psychotic disorders. Healthy participants underwent an extensive screening process described elsewhere[11] and all were European Caucasian.

Demographic variables available for all samples included age, gender and years of education. For patients, both clinical symptom severity (measured using the Scale for the Assessment of Positive Symptoms (SAPS) and the Scale for the Assessment of Negative Symptoms (SANS) and medication dosage in terms of chlorpromazine equivalents (CPZ) were also available, as previously described[11].

fMRI Spatial Working Memory Task Sample Characteristics

A subgroup of the healthy participants (n=103, all right handed) had also undergone functional imaging during performance of an fMRI spatial working memory task. This task, and the acquisition parameters used, has been extensively used by us previously[12-14] (see **Appendix I** for fuller description). This task required participants to identify whether a white dot was in the same spatial location as a red circle over a three-second delay.

Candidate SNP Selection

A novel approach was taken to gene selection in this study. Gene function was assigned to PGC2 identified genes[4] using the OMIM[15] and Uniprot[16] catalogues (**Supplementary Table 1**). Genes associated with the processes of neurotransmission, neurotransmitter secretion or transduction were identified by the lead author. Following this, 28 genes functioning in synaptic transmission were prioritized. We identified SNPs within 20kb of these genes, initially by visual inspection of the supplementary regional GWAS association plots from the PGC-SCZ[4]. This exercise shortlisted loci where the genome-wide significant association signal was at or close to a synaptic transmission gene (n=11). For these loci we identified the most significant GWAS SNP (the index SNP) as the variant for use in our cognition analyses. In the case of *CACNA1C* multiple GWAS signals relating to two LD blocks were identified; for this reason two SNPS were included in the analysis, representing the strongest signal for each LD block. To confirm *cis* effects of these SNPs on the proposed associated genes, the Braineac[17] and Ensembl[18] databases were consulted. Finally, to bioinformatically confirm functional identity, this list of genes was entered into GO and found to have >98 fold enrichment in chemical synaptic transmission (GO: 0099565). Based on this approach the following variants were identified: rs2007044 and rs2239063 (*CACNA1C*), rs7893279 (*CACNB2*), rs8042374 (*CHRNA3*), rs10520163 (*CLCN3*), rs2514218 (*DRD2*), rs9922678 (*GRIN2A*), rs12704290 (*GRM3*), rs1501357 (*HCN1*), rs1339227 (*RIMS1*), and rs4523957 (*SRR*). All of these SNPs are located within introns at these respective genes except for *RIMS1* and *DRD2* where the index SNPs are located in flanking regions[17, 18]. The gene *RIMS1* is located further away (~50kb) from the index SNP, however, we still included it in the analysis as it met all other criteria.

Neuropsychological Assessment

The second stage in the study was to undertake a cognitive analysis of the variants identified above, seeking replication of significant findings in independent samples. A full neuropsychological assessment, designed to evaluate the cognitive deficits typically reported in SZ (with five cognitive domains - general cognitive function, episodic and working memory, attention, and social cognition) was administered to each participant. Selected subtests (Vocabulary, Similarities, Block Design, and Matrix Reasoning) of the Wechsler Adult Intelligence Scale (WAIS III)[19] were used to measure general cognitive function. The Logical Memory and Faces subtests from the Wechsler Memory Scale (WMS III)[20] were used to assess verbal and visual memory recall. Working memory was assessed using the Spatial Working Memory (SWM) task taken from the Cambridge Automated Neuropsychological Test Battery (CANTAB) and the Letter-Number Sequencing subtest from WAIS III. Attentional control was assessed using the Continuous Performance Task (CPT)[21] and the Sustained Attention to Response Task (SART)[22]. Social cognitive function was assessed using three measures of social cognition: two Theory of Mind (ToM) tests and one attributional style test. ToM is the ability to attribute mental states – beliefs, intents, desires, pretending, knowledge, etc. – to oneself and others[23]. The ToM tests used were the Reading the Mind in the Eyes test (Eyes)[24] and the Hinting Task (Hint)[25]. Attributional style was measured using the Internal, Personal, Situational Attributions Questionnaire (IPSAQ)[26]. This refers to the pervasive tendency to explain personally significant positive or negative events in a particular manner, i.e. the degree to which events can be attributed to oneself or to external causes. The discovery dataset is based on a number of data collection waves. Social cognition was only included in the more recent waves of recruitment, resulting in smaller N size available for these tasks.

All individuals in the Cardiff sample were assessed using the MATRICS Consensus Cognitive Battery[27]. This includes seven cognitive domains: speed of processing, attention/vigilance, verbal and nonverbal working memory, verbal and visual memory, reasoning and problem

solving. Of note, the social cognition domain included in the MATRICS was not a measure of ToM (unlike in the Irish samples) but rather an index of emotional self-regulation.

In the German sample, IQ was measured using the German version of the WAIS Revised[28], while memory and attention was assessed using subtests from the German WMS Revised[29], the N-back[30, 31], and the Continuous Performance Test, 3-7 Version[32], respectively. These measures were selected on the basis of closely approximating the measures available in the Irish samples; unfortunately, measures of social cognition were not available for the German samples. **Supplementary Table 2** provides more detailed description of neuropsychological tests used in each sample.

Genotyping

Genotyping was performed on DNA extracted from whole blood or saliva on a subset of participants who underwent neuropsychological testing. Full GWAS data was available for 988 participants (healthy participants n=190; SZ/SZA=578; other psychoses n=220). Previous analysis of the Irish GWAS sample used in the study indicated that the sample is homogenous [33] in terms of population stratification. Age and gender of those with genotype information did not differ from that of the whole sample. A proportion of samples were genotyped with an Affymetrix 6.0 chip and the remainder on the Illumina HumanCoreExome chip. SNPs were excluded on the basis of MAF<0.1%, SNP missingness ≤2%, Hardy-Weinberg equilibrium $p \leq 10^{-6}$, the analysis for which was carried out using PLINK[34]. Imputation was carried out on these datasets separately using 1000 Genomes Phase I integrated haplotypes (Dec 2013 release) and IMPUTE2 to give ~10 million SNPs genome-wide per sample. Genotyping for the Cardiff and German samples was as previously described[35].

Statistical Analysis

Analysis of Neuropsychological data All neuropsychological analyses were carried out using SPSS 20[36]. To estimate risk variant effects on cognitive deficits associated with SZ, linear regression analyses were performed on the total sample (i.e. irrespective of diagnosis, n=988). To test associations between genotype and cognitive test score, hierarchical multiple regression with bootstrapping was performed (1000 iterations, with replacement, to identify a bias corrected and accelerated 95% confidence interval). In each case cognitive test score was entered as a dependent variable, age and sex were entered as predictors on the first step, followed by genotype on the second step. As this initial discovery sample analysis was carried out in order to identify any significant or nominally significant findings, a correction for multiple testing was not applied. Instead, nominally significant findings from cognitive analyses were taken forward for analysis to seek replication in two independent samples. Where replication of results was found in these independent samples, the associated SNPS were taken forward for neuroimaging analysis.

Analysis of fMRI data: Pre-processing and statistical analysis are detailed in **Appendix I**. First-level analysis included working memory (1 dot and 3 dots > baseline), and increased memory load (3 dots > 1 dot) contrasts. The 1 dot and 3 dots > baseline contrast considered both the 1 dot and 3 dot conditions together (i.e. all of the spatial working memory blocks) and contrasted all of these blocks against the baseline blocks (the blocks during which there was no delay between the white dot and red circle). The 3 dots > 1 dot condition considered the 3 dots blocks and contrasted these against the 1 dot blocks to examine increasing blood-oxygen-level dependent (BOLD) response associated with increasing working memory load (i.e. remembering the location of 3 white dots compared to remembering the location of 1 white dot). Contrast maps were entered into a multiple regression analysis with number of risk alleles as covariate of interest. 19 participants were excluded due to low quality or missing data

(**Appendix I**). Seed voxel correlation analysis was used to examine functional connectivity using the right dorsolateral prefrontal cortex (DLPFC) as a seed. Eigenvariates extracted from this seed were entered into a first level analysis and resulting connectivity maps entered into a multiple regression analysis. Results were examined at a $p<0.001$ (uncorrected) level and clusters were considered statistically significant at a $p<0.05$ level, family-wise error (FWE) corrected for multiple comparisons across the whole brain at the cluster level. Masks used for functional connectivity analysis were kindly provided to us by Esslinger & Paulus based on Paulus et al.[37] Functional connectivity analysis was examined using both 'global maximum' and 'next local maximum' seed selection methods to examine whether results were consistent across both methods[38] (**Appendix I**). The rationale for carrying out this connectivity analysis is that genetic effects may be more penetrant at a cortical level than a behavioral level, as we and others have previously shown for schizophrenia risk variants[37, 39].

Results

Neuropsychological Samples - Demographic and Clinical Characteristics.

For the cognitive measures employed, healthy participants scored higher than patients on all measures of IQ, attention and social cognition used in analysis except for Externalizing Bias (ANCOVA; covariates age and gender, $p<0.0001$; **Table 1**). There was a significant difference in age between the rs9922678 (*GRIN2A*) allele groups only (F=4.28, $p<0.05$; Mean values GG=41.15, GA=42.44, AA=38.28). No other between group differences were observed.

When genotype groups were compared on demographic variables, no significant between-group differences were observed for any SNP included for either gender or years of education ($p>0.05$). No between group differences were observed for diagnosis status or total positive symptom severity scores (SAPS total scores) for any SNP. For symptoms, *DRD2* rs2514218 risk genotype showed significantly higher negative symptoms (F=3.67, $p<0.05$; Mean values TT=18.61, CT=23.78, CC=25.69). No medication dosage (measured in terms of chlorpromazine equivalents), or other genotype related clinical differences were observed. Demographic and clinical characteristics for patients and healthy participants are presented in **Table 1**. A between comparison of these groups indicated differences between the healthy participants and both broad and narrow groups in terms of age, gender and education. **Supplementary Table 3** shows additional summary statistics and results comparing genotype group demographics. No age or sex associations were observed with rs2007044 in either the Cardiff or German sample.

>>Table 1<<

Effect of Genotype on Cognition

Of 11 SNPS included, significant effects were observed in the discovery sample (cases and controls) for three SNPS (**Table 2** presents the three SNPs with significant results in all participants and subgroups). For rs2007044 (located within the Calcium Channel Subunit 1AC gene; *CACNA1C*), increased risk 'G' allele dosage was associated with increased errors made on the CANTAB spatial memory task, explaining a proportion of test score variance in the entire sample (B=2.6, r^2=0.006, p<0.05). In a *post hoc* analysis to explore whether or not the signal was driven by specific patient subgroups, in the broad psychosis group, the same direction of effect was observed (B=3.634, r^2=0.012, p<0.01). This was also the case in the narrow psychosis group (SZ and SZA patients only) (B=3.578, r^2=0.011, p<0.05; see **Table 2**). No significant association was observed in healthy participants when considered alone. However, the same direction of association, i.e. reduced working memory performance in risk carriers, was observed. The same analysis was re-run in patients by including CPZ as a co-variate; this did not affect the significance of the results observed. No association between verbal working memory, as measured by Letter-Number Sequencing task and rs2007044 was observed.

For the SNP rs7893279 (located within the gene encoding the Calcium Channel Subunit B2; *CACNB2*) increased risk 'T' allele dosage was associated with lower accuracy on the Hinting Task, a measure of ToM ability (B=-0.772, r^2=0.012, p<0.01). When the broad psychosis group was considered separately, this association remained significant (B=-0.643, r^2=0.006, p<0.05). This association also remained significant in the narrow psychosis group (B=-0.826, r^2=0.009, p<0.05) and in the healthy sample (B=-0.895, r^2=0.052, p<0.01). No associations with other social cognition measures, or measures of other cognitive domains were observed.

For rs1339227 (located ~50kb from *RIMS1*, the only known gene at this locus) increased risk 'C' allele dosage was, contrary to our expectation, associated with improved Eyes Task performance, another measure of ToM ability (B=0.831, r^2=0.008, p<0.05). When the broad

psychosis group was considered separately, this association remained significant (B=1.272, r^2=0.018, p<0.01). This association remained significant also in the narrow psychosis group with the amount of variance explained increasing (B=1.807, r^2=0.034, p<0.01), suggesting that the genotype effects were more pronounced in this group. No significant association was observed in the healthy participants sample only.

>> Table 2<<

Replication Analysis

We sought replication of our spatial working memory and rs2007044 risk G genotype association (uncorrected). In the Cardiff replication sample (consisting of patients only), we sought to replicate this association between *CACNA1C* and working memory using the working memory domain score (calculated from both the Wechsler Memory Scale Spatial Span subtest and the Letter-Number Span subtest) from the MATRICS battery. Based on this analysis (three genotype groups AA, AG, GG), increased rs2007044 risk 'G' allele dosage was again associated with decreased working memory accuracy (B=-0.157, r^2=0.01; p=0.03). Because the working memory domain score in the MATRICS is composed of a verbal (letter number sequencing) and spatial (Spatial Span), we conducted a post hoc analysis to establish whether our result was being driven by either the verbal or spatial subtest. This analysis suggested that the association observed was driven by the verbal subtest performance (Letter number sequencing: B= –0.200, 95% CI -0.358 – -0.042, p=0.013) as the spatial subtest by itself was non-significant (Spatial Span: B= –0.089, 95% CI -0.222 – 0.045, p=0.192).

In the German samples, two working memory measures were available. The first consisted of performance on an n-back test (1- and 2-back), and a composite working memory score based on the Wechsler Digit Span and Spatial Span Tasks. Based on the same analysis undertaken as in the Irish and Cardiff sample, a main effect of *CACNA1C* genotype for the n-back test (2-

back condition only) such that the risk 'G' genotype carriers scored significantly below the non-risk carriers in the patients but not in controls (B=2.615, r^2=0.012; *p*=0.03). Furthermore, the *CACNA1C* variant was associated with cases only and not healthy participants, and neither was an association between *CACNA1C* and the composite Digit/Spatial Span score observed.

Finally, for the associations between social cognition and *CACNB2* (Hinting task performance) and *RIMS1* (Eyes task performance), neither risk variant was associated with social cognition as indexed by the MSCEIT 'managing emotions' subtest in the Cardiff samples, and no social cognition data was available in the German dataset.

fMRI Spatial Working Memory Analysis

Since we observed an effect of the rs2007044 risk 'G' genotype on working memory in two replication samples as well as our own, we also brought this SNP forward to investigate whether or not there was an association with altered connectivity during a spatial working memory fMRI task. There were no significant differences between genotype groups for age, years of education, spatial working memory accuracy, spatial working memory reaction time or gender in the MRI sample (*p*>0.05) (**Table 3**).

>>Table 3<<

Neural Activity Associated with Spatial Working Memory Performance in Healthy Participants

Spatial working memory (1 dot and 3 dots combined) was associated with significantly increased neural activation in the fronto-parietal attention network across our sample [40, 41]

including, bilaterally, the dorsolateral prefrontal cortex (BA 9 and 46; $t_{max(84)}$ = 19.48; p<0.05 corrected; see **Appendix I**). In addition, the 3 dots condition was associated with increased activity across several of these frontal and parietal regions compared to the 1 dot condition ($t_{max(84)}$ = 12.99; p<0.05, corrected; see **Appendix I**). There were no significant differences between genotypes on either contrast, i.e. genotypes did not influence BOLD response for the spatial working memory task.

Functional Connectivity Analysis

Decreased functional connectivity was associated with risk genotype, between the right DLPFC and three clusters incorporating the right cuneus, right anterior cingulate cortex, and left inferior frontal gyrus ($t_{max(82)}$=5.32; p<0.05, corrected; see **Appendix I**). As a QC measure, average parameter estimates were calculated for each individual, based on which five outliers were identified; after removing these outliers however findings were largely unchanged, with altered connectivity between the DLPFC and the right superior occipital gyrus/right cuneus and the right anterior cingulate cortex remaining significant ($t_{max(77)}$ =4.92; p<0.05, corrected; see **Table 4** and **Figure 1**). Finally, we examined functional connectivity of the right DLPFC across our sample using the 'next local maximum' approach (see **Appendix 1**). Results of this analysis were again supportive of the main analysis, with altered connectivity between the rDLPFC and occipital and cingulate clusters observed, albeit at a lower threshold (p<0.001, uncorrected).

>> Table 4 <<

>> Figure 1 <<

Discussion

In this study we assessed the effects of 11 SZ-associated SNPs linked with synaptic transmission genes on behavioral and cortical measures of cognition. Three SNPs were found to be significantly associated with neuropsychological test scores, two of which are linked to calcium channel genes, and one with vesicular trafficking. Carriers of the SZ-associated risk 'G' allele at rs2007044 (located in intron 3 of *CACNA1C*), made more errors on a spatial working memory task than non-risk carriers in a risk allele dose dependent manner, an effect seemingly driven by the patient sample. The same risk allele was also associated with poorer working memory performance in independent patient samples, although not for the same task as was associated with the Irish case samples. No behavioral working memory associations were found with healthy participants in any sample. Finally, fMRI connectivity analysis of this risk allele in a subset of the discovery healthy participant sample suggested a gene dosage effect of the rs2007044 risk G allele on connectivity between the right DLPFC and multiple other cortical regions.

As reviewed by Heyes et al., [42] the role of voltage-gated calcium channels in increasing risk of psychiatric disorders including SZ is currently the focus of intense investigation. Multiple intronic variants situated within the gene coding for *CACNA1C* (e.g. rs2007044, rs1006737, rs4765905) have been found to confer trans-diagnostic susceptibility to SZ, bipolar disorder and major depressive disorder[3, 43-47]. Timothy Syndrome, a disorder that includes cognitive deficits[48] has also been associated with a missense mutation in *CACNA1C*. A previously identified intronic *CACNA1C* risk variant, rs1006737[46, 49], which is in moderately high LD with the PGC identified variant rs2007044 investigated here (LD r^2=0.79), was also previously associated with poorer cognitive performance in SZ patients and controls[50-52]. In the first study that reports on the cognitive effects of rs2007044, the results we observe across three independent cohorts are highly consistent with those reported for rs1006737 by Hori et al.[51]

and Zhang et al,[53] although one finding reported by Frazier et al[54] reports the rs1006737 risk allele as being associated with an increase in cognitive function.

Neural Mechanism of *CACNA1C's* Role in Working Memory

In seeking to establish the cortical basis of the *CACNA1C* rs2007044 risk allele effects on working memory, we analyzed fMRI data obtained during working memory task performance. Based on a previously reported methodology for assessing task dependent functional connectivity[55], we found that *CACNA1C* risk genotype was associated with decreased connectivity between the dorsolateral prefrontal cortex and a distributed network of cortical regions (including the right cuneus, right anterior cingulate cortex, and left inferior frontal gyrus). This association suggested that during working memory task performance, risk carriers had difficulty with top down initiated cortical responses that resulted from dysconnectivity between the right DLPFC and other frontal and parietal regions. Although no genotype effects were evident for neural activation (as measured by BOLD activations), it has previously been suggested that functional connectivity between brain regions may represent a more sensitive intermediate phenotype in identifying neural circuits for schizophrenia risk variants compared to measures of neural activity[56]. Previous connectivity analysis for rs1006737 in working memory also reported altered functional connectivity between the right dorsolateral prefrontal cortex and the hippocampal formation, however the results obtained by Paulus et al.[37], in which the rs1006737 risk allele (A) was associated with increased connectivity between the regions implicated, are inconsistent with the present study. Our results also differed from this previous study by suggesting that the pattern of altered connectivity between the right DLPFC and other cortical regions is more widespread. The reasons for this are uncertain and may relate to differences in either the SNP or working memory task studied. Whatever the cause, by again relating *CACNA1C's* deleterious effects on working memory to pre-frontal dysconnectivity, our study highlights calcium channel deregulation as an important mechanism that contributes

to cognitive deficits. Finally, differences in in functional connectivity findings between studies are, as with other imaging analyses approaches, also likely to results from methodological differences[38]. As there is currently no gold standard method in functional connectivity research, these results should be interpreted with caution until they are replicated in an independent sample and shown to be consistent across different methodologies.

Molecular Mechanisms

CACNA1C codes for the α1c subunit of the L-type calcium channel which is particularly important to heart and brain function; in the brain, these channels are highly expressed in the granular cells of the dentate gyrus, a region synonymous with memory function[57, 58]. Although no significant findings in hippocampal regions are evident from the previous study, the α1c channel subunit encoded by *CACNA1C* is also widely expressed throughout other areas of the brain, including regions of altered connectivity identified in the present, and other, studies. These are predominantly expressed post-synaptically on the cell soma but also on the spines and shafts of neuron dendrites[59]. These channels support neuronal plasticity, through which they are involved with learning and memory.

The molecular impact of rs2007044 has not yet been explored. For the rs1006737 SNP, risk genotype has been associated with increased *CACNA1C* mRNA expression in a dosage dependent manner in induced human neurons[60] and in human dorsolateral prefrontal cortex cells[61]. In contrast, data from the Braineac database[17] shows that the rs2007044 SNP can have opposite effects – that *CACNA1C* expression is decreased in the presence of one or more risk (G) rs2007044 alleles, although this is dependent on brain region. It is currently unclear, therefore, how the behavioral and cortical SNPs effects reported here might relate to difference in expression, and thereby to calcium subunit function; although developmental stage and type of cell are likely to influence this.

The two voltage gated calcium channel genes included in this analysis – *CACNA1C* and *CACNB2* – both play a key role in synaptic transmission. Calcium channels are composed of different channel-forming subunits; each have multiple isoforms that determine specific cellular behavior[62]. Contrary to the assumption that genes coding for calcium subunits might influence the same cognitive functions, the two calcium channel SNPs included here were found to influence different domains of cognitive function – working memory and Theory of Mind respectively. To our knowledge, no other group to date has examined the effects of this *CACNB2* variant rs7893279 (or any other *CACNB2* variant) on cognition, and it was not possible to replicate these findings in the independent samples available (see discussion of limitations below). At the same time, *CACNA1C* has recently also been associated with effects on social information processing[63]. Therefore, whether this gene family has effects on more than one cognitive process warrants further investigation for potentially dissociable effects.

Study Limitations

Associations between common variants associated with risk for neuropsychiatric disorders are expected to be small and issues of both false positive and false negatives have been widely reported[64]. Here we sought to replicate our working memory finding in independent samples. While a significant effect of rs2007044 on working memory was observed in the Irish, Welsh and German samples, these associations were with different working memory tasks in each sample. In the Irish patient samples, the association was with spatial but not verbal working memory; in the Cardiff samples (consisting only of patients) the association observed was with a composite score, and in a follow up analysis with verbal but not spatial working memory; finally in the German samples, the association was with N-back (2-back) performance but not with a second composite measure, or the individual tasks from which the composite score was derived. These between sample differences between working memory tasks had not been hypothesized. It is possible that some measures may have differed in their sensitivity to gene

effects in different cohorts. What is clear, based on the post hoc analysis undertaken in the Cardiff samples, is that these differences cannot simply be attributed to verbal versus visuo-spatial effects, as for example spatial effects were strongest in the Irish samples, but verbal effects in the Cardiff samples. Finally, the lack of association with other cognitive domains may further suggest that this association is specific to, or strongest for, the working memory domain.

Given the importance of social cognition to understanding functional disability in SZ[65], our inability to replicate the association in our discovery sample between two other risk loci and ToM is noteworthy. For example, in our discovery sample, based on a measure of ToM, we found that risk allele T carriers at rs7893279 scored lower on a ToM measure than non-carriers; and also that carriers of the C risk allele at rs1339227 scored higher on a ToM task than non-carriers, both for the group as a whole and for the SZ/SZA sample considered separately. The rs1339227 SNP is ~50kb away from *RIMS1*; the closest gene in this region. The direction of this result was contrary to our expectation that risk allele dosage would be associated with poorer performance, and did not replicate in the Cardiff sample based on MSCEIT 'managing emotions' subtest performance; no social cognition measure was available in the German dataset. The lack of either the same measure or any measure of social cognition in sufficiently large datasets to enable replication of genetic findings related to social cognition is currently an important limitation for our field, especially considering our surprising findings here.

Surprisingly, none of the SNPs identified as associated with cognitive performance in our discovery sample were linked to glutamate or dopaminergic transmission, given previous candidate gene studies (e.g. *COMT*, *DTNBP1*). It is of interest to note that in a recent bioinformatics study seeking to prioritize signals identified by the PGC,[4] Pers et al.[66] generated a gene set that overlapped substantially with the set reported here. This suggests that the

findings observed through this novel approach taken here are unlikely to be accounted for purely on the basis of an idiosyncratic approach to gene prioritization.

Conclusion

In conclusion, these data, based on previously identified SZ risk variants associated with synaptic transmission, provide some additional evidence for the role of *CACNA1C* in memory function already postulated in the literature. Furthermore, this association, we suggest, may result from patient risk carriers' difficulty with top down initiated responses caused by dysconnectivity between the right DLPFC and other cortical regions. These deficits, and the potential role of calcium channel subunits in contributing to these, are not effectively addressed by current therapeutics. In this context, the approach to characterizing the functional effects of risk variants at both a behavioral and neural systems level, reported here, may assist future efforts to identify novel biological targets for treatment.

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Author Affiliations: The Cognitive Genetics & Cognitive Therapy Group, The School of Psychology and Discipline of Biochemistry, The Centre for Neuroimaging & Cognitive Genomics, National University of Ireland Galway (Cosgrove, Mothersill, Morris, Donohoe); Neuropsychiatric Genetics Research Group, Department of Psychiatry, Institute of Molecular Medicine, Trinity College Dublin (Harold, Gill, Corvin); Institute of Psychological Medicine and Clinical Neurosciences, Cardiff University (Kendall, Walters); Department of Psychiatry, Psychotherapy and Psychosomatics, Martin-Luther-University Halle-Wittenberg (Konte, Giegling, Rujescu); MRC Centre for Neuropsychiatric Genetics and Genomics, Cardiff University (Richards, Mantripragada, Owen, O'Donovan)

Group Information: The Wellcome Trust Case Control Consortium 2 investigators include: Peter Donnelly, Lesley Bates, Ines Barroso, Jenefer M. Blackwell, Elvira Bramon, Matthew A. Brown, Juan P. Casas, Aiden Corvin, Panos Deloukas, Audrey Duncanson, Janusz Jankowski, Hugh S. Markus, Christopher G. Mathew, Colin N. A. Palmer, Robert Plomin, Anna Rautanen, Stephen J. Sawcer, Richard C. Trembath, Ananth C. Viswanathan, Nicholas W.Wood, Chris C. A. Spencer, Gavin Band, Céline Bellenguez, Colin Freeman, Garrett Hellenthal, Eleni Giannoulatou, Lucinda Hopkins, Matti Pirinen, Richard Pearson, Amy Strange, Zhan Su,Damjan Vukcevic, Cordelia Langford, Sarah E. Hunt, Sarah Edkins, Rhian Gwilliam, Hannah Blackburn, Suzannah J. Bumpstead, Serge Dronov, Matthew Gillman, Emma Gray, Naomi Hammond, Alagurevathi Jayakumar, Owen T.McCann, Jennifer Liddle, Simon C. Potter, Radhi Ravindrarajah, Michelle Ricketts, Matthew Waller, Paul Weston, Sara Widaa, and Pamela Whittaker.

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Figure Legends

Figure 1. Effects of *CACNA1C* rs2007044 variant on right prefrontal connectivity during spatial working memory, global maximum approach, five outliers removed (n = 77)

Tables

Table 1. Neuropsychological Sample Participant Demographics and Subtest Scores

	Healthy Participants	Broad Diagnosis	F (Healthy V Broad)	Narrow Diagnosis	F (Healthy V Narrow)
Age, mean (SD)	35.82 (12.65)	43.00 (12.40)	81.02***	42.30 (12.47)	59.36***
Male:Female (%)	44.5:55.5	66.2:33.8	49.96***	70.4:29.6	67.01***
CPZ, mean (SD) mg/day	-	435.97 (453.17)	-	498.18 (482.18)	-
SAPS mean (SD)	-	19.86 (19.30)	-	21.86 (19.77)	-
SANS mean (SD)	-	23.58 (19.93)	-	26.38 (19.99)	-
Education Years mean (SD)	15.64 (1.82)	12.95 (2.64)	184.28***	12.73 (2.51)	191.51***
WTARR	41.90 (5.96)	33.52 (10.60)	179.36***	32.77 (10.56)	194.60***
VERBIQ	118.45 (15.80)	92.06 (20.02)	374.27***	90.36 (19.18)	433.73***
PERFIQ	117.78 (22.90)	91.20 (19.32)	356.35***	89.83 (18.43)	393.58***
FSIQ, mean (SD)	119.68 (15.43)	92.07 (19.22)	429.87***	90.31 (18.12)	515.19***
LM1	49.09 (10.10)	27.95 (12.35)	597.47***	26.52 (12.37)	627.06***
LM2	31.75 (7.44)	15.34 (8.84)	688.13***	14.40 (8.68)	732.18***
FACES1	38.93 (4.50)	33.79 (5.09)	128.61***	33.65 (5.20)	124.70***
FACES2	39.56 (4.30)	35.04 (5.28)	85.29***	35.04 (5.24)	85.14***
LNS	12.72 (3.13)	8.16 (3.40)	353.15***	7.90 (3.44)	365.55***

CANTAB SWM (errors)	19.04 (16.45)	43.05 (23.85)	189.727***	43.86 (24.21)	204.20***
SART (reaction time)	32.38 (90.80)	445.29 (112.64)	126.04***	443.83 (113.42)	122.46***
CPT d'Prime 2 Digit	3.82 (0.56)	2.69 (1.21)	19.95***	2.67 (1.22)	19.79***
CPT d'Prime 3 Digit	3.35 (0.75)	3.35 (0.75)	46.37***	1.91 (1.09)	46.72***
CPT d'Prime 4 Digit	2.25 (0.97)	1.14 (0.87)	45.76***	1.12 (0.83)	48.38***
Eyes	26.47 (3.58)	21.21 (6.21)	101.45***	20.48 (6.39)	120.43***
Hint	16.86 (1.79)	15.56 (3.31)	23.12***	15.34 (3.48)	27.94***
IPSAQ – Externalizing Bias	1.52 (4.02)	1.49 (3.85)	0.54	1.39 (3.88)	0.34
IPSAQ – Personalizing Bias	0.62 (0.24)	0.52 (0.29)	16.39***	0.51 (0.28)	18.69***

FSIQ=Full-scale IQ; CPZ=Chlorpromazine equivalents; SAPS=Scale for the Assessment of Positive Symptoms; SANS=Scale for the Assessment of Negative Symptoms. *p<0.05 **p<0.01 ***p<0.001.

Table 2. Summary of significant findings from regression analyses in the discovery sample.

rs ID	Test	Sample	n	Mean (SD)					Bootstrap 95CI		
							r^2	B	Lower	Upper	*p*
rs2007044				**AA (n=251)**	**AG (n=330)**	**GG (n=133)**					
		All	714	35.17 (23.10)	37.43 (25.55)	41.61 (23.44)	0.006	2.600	0.635	4.535	**0.012***
	SWM	**Broad**	557	38.82 (19.93)	41.91 (26.75)	47.51 (21.30)	0.012	3.634	1.133	6.184	**0.006****
		SZ/SZA	409	40.72 (22.66)	43.38 (25.76)	49.94 (21.25)	0.011	3.578	0.717	6.153	**0.013***
		Healthy	157	15.73 (12.27)	18.23 (16.42)	21.27 (17.31)	0.010	2.102	-0.632	4.891	0.138
				GG (n=6)	**GT (n=97)**	**TT (n=416)**					
rs7893279		**All**	519	17.17 (1.72)	16.43 (2.20)	15.66 (3.25)	0.012	-0.772	-1.210	-0.337	**0.003****
	Hinting	**Broad**	380	16.25 (0.96)	16.00 (2.37)	15.34 (3.55)	0.006	-0.643	-1.203	-0.122	**0.021***
	Task	**SZ/SZA**	295	16.33 (1.15)	16.02 (2.38)	15.15 (3.74)	0.009	-0.826	-1.553	-0.147	**0.017***
		Healthy	139	19.00 (1.41)	17.31 (1.51)	16.59 (1.87)	0.052	-0.895	-1.448	-0.282	**0.005****
				TT (n=63)	**TC (n=266)**	**CC (n=224)**					
rs1339227		**All**	553	22.56 (6.36)	22.32 (6.17)	23.51 (5.83)	0.008	0.831	0.300	1.568	**0.024***
	Eyes	**Broad**	392	20.20 (6.12)	20.51 (6.25)	22.48 (6.14)	0.018	1.272	0.235	2.228	**0.007****
	Test	**SZ/SZA**	273	18.84 (6.39)	19.41 (6.25)	22.28 (6.36)	0.034	1.807	0.758	2.905	**0.003****
		Healthy	161	28.00 (2.21)	26.31 (3.62)	26.37 (3.56)	0.002	-0.251	-0.977	0.496	0.501

SD=standard deviation; SWM=CANTAB Spatial Working Memory (Errors); SZ/SZA=Schizophrenia/Schizoaffective Disorder. Broad=broad sense psychosis diagnosis of bipolar disorder with psychotic features, major depressive disorder with psychotic features, or psychosis not otherwise specified; includes SZ/SZA patients. r^2 represents the percentage of variance for each neuropsychological test score explained by the genotype; B: regression coefficient; *p* values uncorrected.

Table 3. *CACNA1C* spatial working memory fMRI study: participant demographics

	Mean (SD) age	**Mean (SD) years education**	**SWM mean (SD) accuracy**	**SWM mean (SD) reaction time**	**Gender (M:F)**
AA (N=25)	30.88 (11.02)	16.96 (3.84)	64.80 (6.86)	9070.61 (2332.96)	15:10
AG (N=42)	27.86 (7.10)	17.75 (3.24)	62.17 (10.90)	8535.44 (2063.93)	15:27
GG (N=17)	29.00 (10.30)	17.66 (2.87)	64.94 (4.63)	8811.90 (2296.80)	7:10
F or χ^2	F = 0.868	F = 0.438	F = 0.972	F = 0.475	χ^2= 3.822
p	0.424	0.647	0.383	0.624	0.148

SWM = spatial working memory; F-statistic: one-way ANOVA comparing age, years of education, spatial working memory accuracy and reaction time between genotype groups; χ^2 analysis: comparing gender by genotype group.

Table 4. Clusters, including individual peaks, showing significantly decreased functional connectivity with the right DLPFC with increasing rs2007044 risk alleles – global maximum approach, five outliers removed (n = 77)

Cluster	**Extent (voxels)**	***p* value[a]**	**Cluster peaks**	**t-value**	**Z-value**	**Peak coordinates (MNI)**
1	888	<0.001	Right superior occipital gyrus	4.92	4.55	21 -73 31
			Right cuneus	4.65	4.33	15 -67 34
			Right precuneus	4.45	4.17	9 -43 46
2	121	0.02	Right anterior cingulate cortex	4.90	4.53	6 29 19

[a]*p* values are FWE-corrected for multiple comparisons at the cluster level