Variants in regulatory elements of *PDE4D* associate with Major Mental Illness in the Finnish population

Running title: PDE4D in Finnish major mental illness

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Abstract

We have previously reported a replicable association between variants at the *PDE4D* gene and familial schizophrenia in a Finnish cohort. In order to identify the potential functional mutations alluded to by these previous findings, we sequenced 945kb of the *PDE4D* genomic locus in 96 individuals, followed by two stages of genotyping across 6,668 individuals from multiple Finnish cohorts for major mental illnesses. We identified 4,570 SNPs across the *PDE4D* gene, with 380 associated to schizophrenia ($p \leq 0.05$). Importantly, two of these variants, rs35278 and rs165940, are located at transcription factor binding sites, and displayed replicable association in the two-stage enlargement of the familial schizophrenia cohort, (combined statistics for rs35278 $p=0.0012$; OR=1.18, 95% CI 1.06-1.32; and rs165940 $p=0.0016$; OR=1.27, 95% CI 1.13-1.41). Further analysis using additional cohorts and endophenotypes revealed that rs165940 principally associates within the psychosis ($p=0.025$, OR=1.18, 95% CI 1.07-1.30) and cognitive domains of major mental illnesses. Specifically, the cognitive domain was a factor score for quantitative neuropsychological endophenotypes related to verbal learning and memory ($p=0.0078$, $\beta=-0.19$). Moreover, expression data from the GTEx database demonstrated that rs165940 significantly correlates with the mRNA expression levels of *PDE4D* in the cerebellum (p-value=0.04; post-prob=0.9; m-value=1.4), demonstrating a potential functional consequence for this variant. Thus, rs165940 represents the most likely functional variant for major mental illness at the *PDE4D* locus in the Finnish population, increasing risk broadly to psychotic disorders.

Keywords: Schizophrenia; Psychotic disorders; Mood Disorders; PDE4D, Endophenotypes

1. Introduction

The phosphodiesterase sub-family 4 are protein encoding genes belonging to the cyclic nucleotide phosphodiesterase (PDE) family. PDEs are important catalytic enzymes with a key role in many important physiological processes through regulating and mediating of a number of cellular responses to extracellular signals(1). Members of the mammalian *PDE4* subfamily are evolutionary orthologues of the Drosophila learning and memory mutant Dunce(2). Flies carrying mutant Dunce display severe learning/memory phenotypes in different learning situations, showing reduced gene activity and deficits in olfactory learning and memory(3).

Although their involvement in human neuro-pathophysiology is currently unclear, several studies(3-17) have implicated *PDE4s* in psychiatric illnesses, particularly *PDE4D* and *PDE4B*. In a GWAS study of patient-related treatment response during antipsychotic therapy, three SNPs, in high linkage disequilibrium (LD), from *PDE4D* were found to significantly associate with mediating the effects of quetiapine(4). In acrodysostosis, a rare disorder characterized by intellectual disability, skeletal and neurological abnormalities, five different point mutations within the *PDE4D* gene have been identified as the genetic cause(5, 6). Furthermore, in a genome wide association study of neuroticism, a psychological trait reported to share genetic factors with both major depression and anxiety, found that one SNP in *PDE4D* associated with higher neuroticism(7). This observation has been replicated in two additional independent cohorts, but not in two other cohorts(7). Behavioural studies on rats revealed that *PDE4D* is associated with memory impairment in a water maze task(15). These rats were trained in a spatial version of the water maze task and the data suggests that *PDE4D* may mainly act on the glutamate-NDMA pathway involved in memory processes.

There have also been findings with the *PDE4D* homolog *PDE4B*. Recently, SNPs within the *PDE4B* gene have been identified to significantly associate with schizophrenia(8) and anxiety(9) in

genome-wide analyses (8). Previously, different haplotypes within the *PDE4B* gene had been noted to associate with schizophrenia in both European and African ancestry populations(10), and the Japanese population(11). Furthermore, a heterozygous balanced t(1;16)(p31.2;q21) chromosomal translocation that directly disrupts *PDE4B* transcription, resulting in a 50% reduction of the protein, has been observed in an individual with severe chronic schizophrenia(12). In a study of a Scottish cohort with schizophrenia and bipolar cases using 26 SNPs to tag and scan the entire *PDE4B* gene, it was observed that a three-SNP haplotype associated to schizophrenia only in females(13). Moreover, knockout of *PDE4B* increases risk for schizophrenia-related phenotypes in a mouse model(14).

In Finland, *PDE4D* and *PDE4B* were first identified as associated to schizophrenia in a study that deliberately set out to study known genes of DISC1 binding partners, as the DISC1 network had already been demonstrated to be of genetic importance within this large cohort for familial schizophrenia(18, 19). In this cohort of 476 families ascertained for schizophrenia, it was observed that haplotypes in both *PDE4B* (1p31.3) and *PDE4D* (5q11.2-q12.1) associated with schizophrenia in a replicable manner(17). The variant rs7412571 located in *PDE4B* provided association (p=0.018), along with a three-SNP haplotype consisting of either the CCC alleles (p=0.029) or the CTT alleles (p=0.0022) of rs4503327, rs2503222 and rs6588186(17). The *PDE4D* haplotype, comprised of the GGACA alleles of SNPs rs13190249, rs1120303, rs921942, rs10805515 and rs10514862, was observed to be significantly over-represented in affected individuals (p=0.00084). Moreover, the SNP rs1120303 also showed replicable association (p=0.021) in this dataset(17).

These associations already noted in the Finnish families were based on SNPs designed to tag the haplotypic structure of the genes of interest, and are thus not expected to be the functional mutations but instead only represent surrogate variants. Thus, here we sought to examine in detail

PDE4D, which is the gene of the *PDE4* sub-family offering the more solid evidence for association to schizophrenia in the Finnish population, in order to identify any variants with potential functional consequences. This utilised a three-stage study design that would sequence the genomic locus of *PDE4D*, and two rounds of genotyping in ever increasing sample numbers from the familial schizophrenia cohort, so that variants of interest can be identified, verified, and replicated in an identically ascertained cohort. This design was chosen as it is expected, based on the level of the prior observations (best p=0.00084, Tomppo et al. (17)), that any association with a single variant will not surpass the genome-wide significant threshold of 5.0×10^{-8}, and thus replication of the findings is of paramount importance. Observations that passed through this three-stage design were then characterised further. We utilised seven other Finnish cohorts representing a range of major mental illness phenotypes, first individually and then jointly, to study the gene's role in psychotic and mood disorders. Further, we studied neuropsychological endophenotype data that has been collected within the familial cohorts used here.

2. Materials and Methods

2.1. Samples

The samples studied came from multiple Finnish cohorts collected to study major psychiatric disorders using a number of different study designs, including familial, twin and population-based cohorts (Supplementary Table1), and phenotypes, including multiple diagnoses alongside neuropsychological endophenotypes. In total, this joint cohort consisted of 7,024 individuals for which 6,668 have been genotyped, including 1,909 psychiatrically healthy control samples. The individual cohorts have been described in great detail previously(20-25), and are briefly described, along with their abbreviations, in the supplementary materials. These samples include two familial cohorts, for schizophrenia (FSZ n= 2,818) and bipolar disorder (BPD n= 650), a sample of twin pairs concordant and discordant for schizophrenia (Twin n= 303), three population cohorts ascertained for different aspects of psychotic disorders (FEP n= 125; MMPN n= 449; HUPC n=383), a population cohort for anxiety disorders (Anx n= 823), and a sample of population controls (Controls n= 1,117).

In order to maximise the analytical potential of the cohorts studied here we combined all, except the 207 anxiety cases, into a joint analysis of two broad major mental illness related phenotypes. These dichotomous traits were derived from the diagnoses of each affected individual, where we could classify them as either having a psychotic disorder and/or mood disorder. The psychotic disorder phenotype included those individuals with any of the following diagnoses; schizophrenia, schizoaffective disorder, schizophrenia spectrum disorders, schizophreniform, psychotic disorders, and bipolar disorders and major depression only with a specification of including psychotic features. The mood disorder phenotype included individuals with the following phenotypes; bipolar disorder type I or type II, major depression, schizoaffective disorder, and psychotic disorders where major depressive or manic episodes had been present. In total, there were 1,896 people categorised

with a psychotic disorder, and 1,227 with a mood disorder, 628 individuals could be categorised as cases under both phenotypes.

2.2. Sequencing, genotyping and SNP selection

The data to be investigated in this research have been generated through a three-stage replication study design to increase our power to detect true positives while lowering the amount of false positive findings, compared with using an overly conservative Bonferroni correction(26). Firstly, to identify variants in the familial schizophrenia cohort the 945kb genomic region of the *PDE4D* gene in a subset of families (n=20 families, 96 individuals) was sequenced using Nimblegen SeqCap EZ 6Mb target enrichment kit. All variants were aligned with bioinformatic predictions of function, with the major focus on their location in exonic and regulatory sites as predicted by UCSC genome browser build 19 (Supplementary Table 2). SNPs displaying any evidence of association in this small cohort ($p \leq 0.05$), and located in predicted functional areas of the genes were selected for follow-up by genotyping. This was performed using a Sequenom MassArray platform(27) according to manufacturer's recommendations. Stage one (S1) genotyping was performed in a sub-cohort of the schizophrenia families (n=1,122 individuals in 301 families) alongside population-based controls (n=323 individuals), in order to verify both the variant's existence, if the variation was novel to this sequencing data, and independent association ($p \leq 0.05$) to schizophrenia. Those variants that were both verified and associated in this larger cohort were further taken for Stage two (S2) genotyping, which included the rest of the schizophrenia cohort (n=1,696), alongside the other major mental illness cohorts (Total n= 2,733) and additional population-based controls (n=794). The variant rs39672 displayed significant inconsistencies in its minor allele frequency between the controls of S1 and S2 (MAF S1=0.24; MAF S2=0.39: $p=1.71\times10^{-11}$) and was thus discarded, as there was no significant geographical or gender differences between these two stages that could reasonably account for such a difference.

2.3. Association analysis

Association analysis within the individual cohorts was carried out either using Pseudomarker(28) (family-based or twin cohorts) or PLINK(29) (population-based controls). Pseudomarker analyses test for single marker (two-point) association and linkage. Furthermore, Pseudomarker can utilize data of differing epidemiological design, helping us to combine familial studies with population controls, alongside singleton cases from other cohorts. It can handle missing data, even when the genotypes of the parents are unknown. The 'association given linkage' (LD|Linkage) option of the program was used to identify association for the SNPs with the diagnosis ($p \leq 0.05$), using the three main genetic models (dominant, recessive and additive) assuming incomplete penetrance. LD|Linkage corrects for the effect of any linkage within the families that may influence the observed association. PLINK was used to analyse population-based cohorts, where the family structure was not known. The options --model and --perm produced the p-values, using permutation tests to generate significance levels empirically.

In the family cohorts for schizophrenia and bipolar disorder different liability classes (LC) of the diagnoses were used in the analyses. In the schizophrenia cohort these are LC1 constitutes schizophrenia only, LC2 added those individuals affected with schizoaffective disorder, LC3 added individuals with schizophrenia spectrum disorder, and LC4 added individuals with bipolar disorder or major depressive disorder. In the bipolar cohort, the liability classes (LC) are LC1 bipolar disorder type I, LC2 adds schizoaffective disorder, bipolar type, LC3 adds bipolar disorder type II, LC4 adds recurrent major depressive disorder. All other cohorts only used the single diagnostic criteria for which the cohort was ascertained (Supplementary Table 3-5). In the sequencing stage, association analysis was only performed using LC4, to maximise the number of cases (n=41) versus unaffected family members (n= 55).

2.4. Endophenotypes and QTDT

A total of 919 subjects from the schizophrenia (n = 811) and bipolar (n = 108) familial cohorts have been administered a comprehensive neuropsychological test battery, consisting of a series of well-validated, and internationally used, means to measure cognitive ability. From this test battery, a total of 14 quantitative neuropsychological variables with previous evidence of potential to use as an endophenotype were available(30-35). These endophenotypes that are included were: Verbal learning and memory derived from the Immediate, Short delay and Long delay recall tasks from the California Learning Test (CVLT) battery(36); Verbal skills from the Similarities and Vocabulary subtests of the Wechsler Adult Intelligence Scale–Revised (WAIS-R) battery(37); Visual working memory from the Visual span forward and backward, and Verbal working memory from the Digit span forward and backward tests from the Wechsler Memory Scale-Revised battery (WMS-R)(38); Information Processing from the Stroop colour task and colour-word task(39) and the Trail Making Test, parts A and B(40), and the Digit Symbol subtest from the Wechsler Adult Intelligence Scale–Revised (WAIS-R) battery(37). Most of the endophenotypes were normally distributed, however the Trail Making and Stroop tasks were transformed so that the scores represented speed (number of items/performance time). As the neuropsychological endophenotypes are related, they could be grouped into five first-order factors using factor analysis (Table2), and a second-order general ability factor (*g-factor*) based upon previous results(41) for cognition related traits. The factor analysis was performed with Mplus 7.3. We used confirmatory factor analysis with a maximum likelihood estimator and with robust standard errors using family as a cluster and affected versus not affected as a grouping variable. The fit of the model was acceptable (CFI= 0.932; RMSEA= 0.085).

The program QTDT(42) was used for the analysis of the endophenotypes, factors and *g-factor*. This method relies on variance components testing for any transmission distortion within the families.

Gender and affection status were treated as covariates during the analysis. The orthogonal model (-ao) together with 100,000 permutations generated the empirical p-values. The orthogonal model allows for families of any size with or without parental genotypes, whereas permutation provides robust findings in the presence of stratification providing an empirical p-value for the observed difference in the trait. The strength for this analysis comes from the consistency of the findings with regard to related endophenotypes and factors. Due to the relatedness of these traits Bonferroni correction for multiple testing would be overly conservative, thus we report the uncorrected empirical p-values and highlight those that would survive multiple test correction for 14 endophenotypes (p=0.00357) and five factors (p=0.010).

2.6. Linkage Disequilibrium (LD) analysis

In order to investigate the relatedness between our identified variants, and between our observations and those of the previous study(17), the identified variants from this study were mapped onto the previously determined LD haplotypes of *PDE4D* using the Haploview program(43). For the latter, the D′ haplotype structure of the SNPs previously studied was manually enforced onto this data with Hedrick's Multiallelic D′ (44) determined between the haplotype and the two identified SNPs. However, for the correlation between our two identified SNPs the r^2 LD was determined.

3. Results

The sequencing of *PDE4D* in 96 individuals identified 4,570 variants, of which 380 variants associated with a broad schizophrenia diagnosis ($p \leq 0.05$). Five of these variants were exonic and nine were located in regulatory regions such as a CpG island or a transcription factor binding site (TFBS) according to UCSC hg19 build (Supplementary Table 2). Thus, in the first stage (S1) of genotyping 14 variants were studied, of which three were both verified and remained significant in their association to schizophrenia in this enlarged cohort, and therefore taken forward for replication in the second genotyping stage (S2). Two potential regulatory SNPs were identified to significantly replicate in their association to schizophrenia (Table 1, Figure 1, Supplementary Table 3), and were thus studied within the combined cohort for familial schizophrenia; rs35278 ($p=0.0012$; OR=1.18, ±95% CI 1.06-1.32) and rs165940 ($p=0.0016$; OR=1.27, ±95% CI 1.13-1.41). These two SNPs are in relatively high linkage disequilibrium, $r^2=0.66$. No gender or geographical-based differences were observed in our analyses.

Association analysis in the other major mental illness cohorts revealed that rs35278 significantly associates within the familial bipolar disorder cohort with broadening liability classes, but most significantly with LC2, bipolar type I and schizoaffective, bipolar type, ($p=0.032$; OR=1.27, ±95% CI 1.04-1.56) (Supplementary Table 4). Neither SNP associated within the other cohorts (Supplementary Table 5), however, the joint cohort analysis highlights the involvement of both SNPs with a broad diagnosis of any psychotic disorder (rs35278 $p=0.023$, OR=1.13, ±95% CI 1.02-1.24; rs165940 $p=0.025$, OR=1.18, ±95% CI 1.07-1.30) (Supplementary Table 5).

Follow-up analysis of these two SNPs using quantitative neuropsychological endophenotypes demonstrated that both SNPs provide evidence for association to specific endophenotypes (Table 2). All endophenotypes showing this evidence are to some degree related, representing several

aspects of verbal learning and memory (Table 2). Although these do not survive a conservative multiple test correction for the 14 endophenotypes tested, analysis of the factors of these 14 endophenotypes does demonstrate association that would survive conservative multiple test correction for the five factors tested. Here the SNP rs165940 significantly associates with decreased performance in the factor representing verbal learning and memory (p=0.0078, β=-0.19)(Table 2). An overall variable combining information from all the endophenotypes into a joint factor representing general cognition (*g-factor*) only demonstrated a trend towards significance (rs165940 p=0.077, beta estimate=-0.13)(Table 2).

Since both the SNPs have a potential regulatory function based on their locations being predicted at transcription factor binding sites, we checked the extent of their functional consequences using tissue specific expression quantitative trait loci (eQTL) from the GTEx portal(45). Only rs165940 showed significant association with expression changes in *PDE4D* within the brain, specifically the cerebellum region (p-value = 0.04, post-prob = 0.9 and m-value = 1.4) (Supplementary Figure1). Significant modulation of expression of *PDE4D* by rs165940 is also detected in the oesophagus, heart and prostate tissues.

Discussion

Through the use of a three-stage sequencing and genotyping approach to identify, validate, and replicate potential functional mutations at the *PDE4D* gene, we have identified two SNPs of principal interest to the aetiology of major mental illness in Finland. Both SNPs displayed replicable association in the familial schizophrenia cohort, are located in predicted transcription factor binding sites, and are in relatively high LD with each other (r^2=0.66). This latter aspect makes it difficult to discern which of the two SNPs is the most likely functional mutation. The higher allele frequency of rs35278 gives it a greater power for detection in the association analyses, thus, use of additional cohorts for major mental illness highlighted only rs35278 as also associating with bipolar disorder. However, when the cohorts were combined to study psychotic and mood disorders as a whole, both SNPs again displayed significant association, to psychotic disorders. The use of neuropsychological endophenotypes indicates that both SNPs display suggestive association to measures of learning and memory, while analysis of factors of these endophenotypes again shows both SNPs displaying association, but with only rs165940 being significantly so, to reduced scores in the factor representing verbal learning and memory. Through the analysis of the functional consequences of these SNPs on the gene expression levels of *PDE4D* within the GTEx database we gained extra insight that could help to specifically separate these two variants, with only rs165940 significantly associating with gene expression levels in *PDE4D*, making it the most likely functional mutation of the two SNPs. These expression level differences could be identified in the brain, the cerebellum, but also other tissues, such as oesophagus, heart and prostate.

Since this study is an extension of one that had previously implicated *PDE4D* in this familial cohort for schizophrenia, we determined whether our current findings are in concordance with our prior observations(17). Thus we mapped our SNPs onto the SNPs and haplotypes of *PDE4D* previously used to study the common haplotypic background of the gene, as determined by D' LD. The SNPs

are located in approximately the same physical area as the haplotype, being within the same intron, intron 3 of the longest isoform of PDE4D. Furthermore, both SNPs are within some degree of D′ LD (D′=0.43,0.47) with the haplotype (Supplementary Figure 2a, b), which although modest suggests some relationship between these observations and the original finding.

It is important to note that the approach taken in this study design is both a strength, as it enables us to directly replicate our findings using an identically ascertained cohort, but also a weakness. The initial sequencing step only used 96 individuals meaning that the full scope of variation at the *PDE4D* locus within these families has not been determined, with rarer mutations (MAF<0.01) in these families likely to have been missed. However, our prior evidence at *PDE4D* implied that any mutation would be common (frequency > 5%), as the associating haplotype allele had a frequency in affected individuals from this cohort of 0.40 (control frequency=0.28), and the SNP which showed some association had a minor allele frequency (MAF) of 0.13 (control MAF=0.19). The high frequency of rs165940, not just in the cohorts studied here, Finnish familial schizophrenia cases (MAF=0.34), and psychiatrically healthy controls (MAF=0.29), but also in population genomic cohorts(46) for the Finnish population (MAF=0.32) and the non-Finnish Europeans (MAF=0.28), does in turn suggest that these variants could have been detected by large scale genome-wide approaches, and yet, to date, this is not the case. While current genetic evidence identified in familial cohorts for schizophrenia is markedly different to those identified through population-based study designs, it is worth noting that the latest consortia-based genome-wide association study has identified *PDE4B* in its study of schizophrenia(8). This is part of the same sub-family as *PDE4D* and was also identified as associating to schizophrenia in the Finnish familial schizophrenia cohort used here. The high frequency of the *PDE4D* SNPs, combined with their low odds ratio/effect size, would imply that with further increases in sample size *PDE4D* could also be identified through population-based approaches. Whereas our three-stage study design has allowed

us to identify significant variants through replication, rather than being dependent on reaching the genome wide significance threshold of 5.0×10^{-8}.

Our findings strongly support the role of *PDE4D* in psychiatric disorders, with replicable association in familial schizophrenia in Finland. Further characterisation suggested that it plays a role in both psychosis and cognitive endophenotypes of major mental illnesses. In particular we demonstrate that the SNP rs165940, through its association pattern and being identified as an eQTL for the *PDE4D* gene, make it the principal variant of interest for being the functional mutation at this locus. However, further studies into the functional consequences of this variant are essential.

Contributors

VS and WH wrote the manuscript text and prepared the manuscript tables and figures; WH designed the study; TP, JS, JL, IH, PJ, EI, ATH, ST, TDC and JK provided access to samples and data; VS, LUV, WH, MTH, ST and AOA performed the analysis. All authors have reviewed the manuscript and approved the final version to be published.

Conflict of Interest*:* The authors declare they have no conflicts of interest.

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Figure1: Odds Ratios, and their respective 95% confidence intervals, for the two SNPs across the cohorts studied and the joint analysis. All plots represent the observations for the additive genetic model, for the schizophrenia family cohort only the finding for liability class 3 is shown, while for the familial bipolar disorder cohort only liability class 2 is shown.

Tables

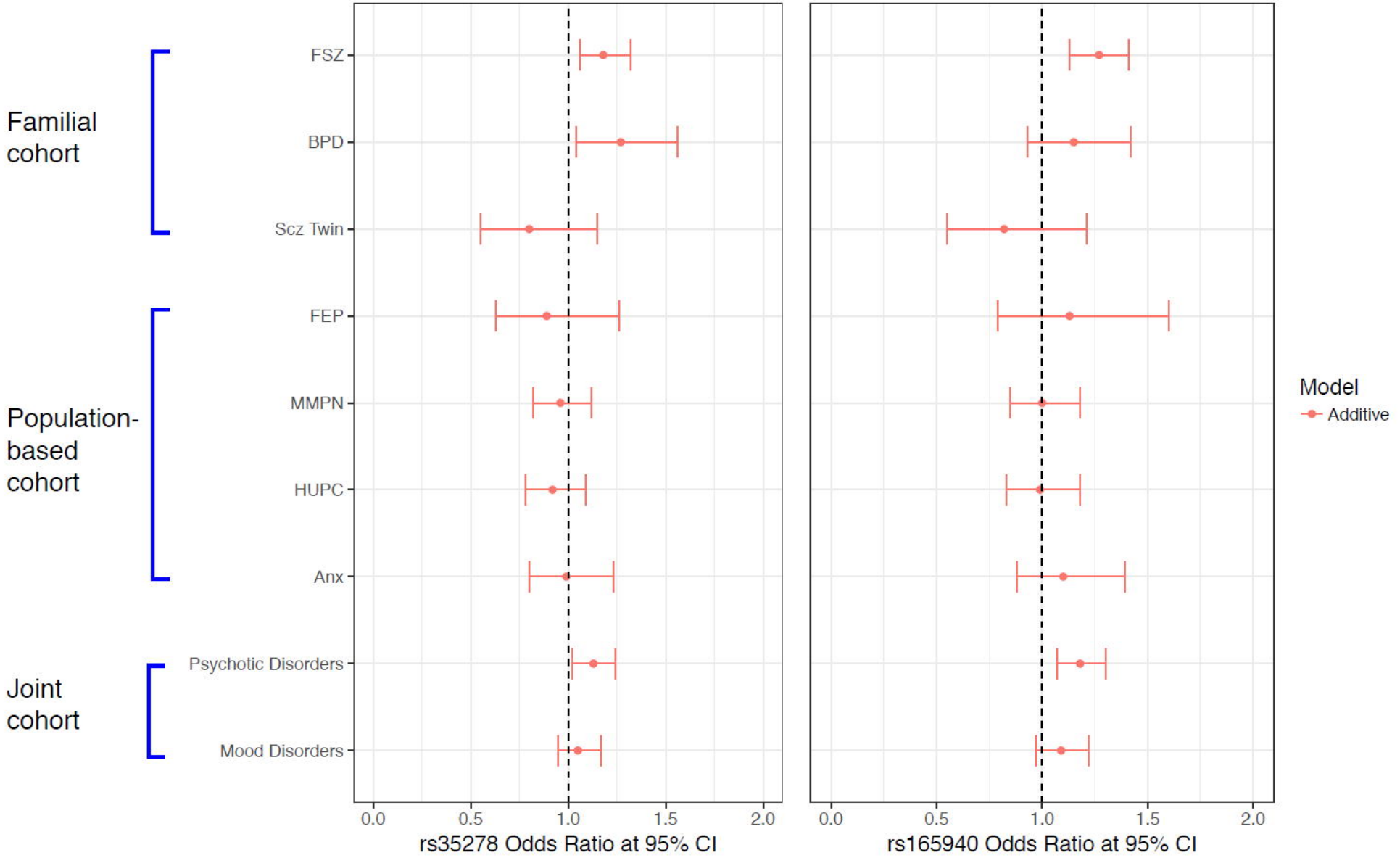
Table1: Replicated observations of association across the three-stage study design for two *PDE4D* SNPs within the Finnish familial schizophrenia cohort. P-values, odds ratios and confidence intervals are shown for liability class 3 under an additive genetic model. Values for other models and classes are provided in Supplementary Table 3.

SNP	Allele	Sequencing P-value	S1 P-value	S2 P-value	Combined (S1+S2) P-value	Odds Ratio (95% CI)	MAF SCZ Families	MAF Control
rs35278	T>G	**0.011**	**0.033**	**0.0074**	**0.0012**	**1.18 (1.06 - 1.32)**	0.437	0.392
rs165940	A>T	**0.047**	**0.0082**	**0.0084**	**0.0016**	**1.27 (1.13 - 1.41)**	0.348	0.295

Table2: Empirical p-values from the association analysis of the two *PDE4D* SNPs and the quantitative neuropsychological endophenotypes, their factors, and an overall general score for cognition.

Endophenotype	**rs35278**	**rs165940**	**Factor**	**rs35278**	**rs165940**	***g-factor***	
						rs35278	**rs165940**
WAIS-R; Vocabulary	0.061	0.14	Verbal skills	**0.021**	0.063	0.10	0.077
WAIS-R; Similarities	**0.024**	0.14					
WAIS-R; Digit symbol	0.28	0.24	Information Processing	0.38	0.31		
Stroop color naming, time	0.65	0.81					
Stroop color-word task, time	0.76	0.78					
Trail Making A, time	0.41	0.33					
Trail Making B, time	0.39	0.36					
WMS-R; Digit span forward	0.44	0.089	Verbal working memory	**0.035**	**0.020**		
WMS-R; Digit span backward	**0.027**	**0.033**					
WMS-R; Visual span forward	0.81	0.19	Visual working memory	0.41	0.23		
WMS-R; Visual span backward	0.72	0.99					
CVLT Immediate recall	**0.015**	**0.0036**	Verbal learning and memory	**0.012**	***0.0078***		
CVLT; Short delay recall	**0.010**	**0.012**					
CVLT; Long delay recall	**0.049**	**0.027**					

P-values ≤0.05 for variables and factors are marked in bold.
P-values that surpass the Bonferroni multiple test correction for either the number of endophenotypes (14) or the number of factors (5) tested are in bold and italics.


Familial cohort
Population-based cohort
Joint cohort
FSZ
BPD
Scz Twin
FEP
MMPN
HUPC
Anx
Psychotic Disorders
Mood Disorders
0.0
0.5
1.0
1.5
2.0
rs35278 Odds Ratio at 95% CI
rs165940 Odds Ratio at 95% CI
Model
Additive