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Cholinergic muscarinic M_4 receptor gene polymorphisms: A potential risk factor and pharmacogenomic marker for schizophrenia

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ABSTRACT

Although schizophrenia is a widespread disorder of unknown aetiology, we have previously shown that muscarinic M_4 receptor (CHRM4) expression is decreased in the hippocampus and caudate-putamen from subjects with the disorder, implicating the receptor in its pathophysiology.
These findings led us to determine whether variation in the *CHRM4* gene sequence was associated with an altered risk of schizophrenia by sequencing the *CHRM4* gene from the brains of 76 people with the disorder and 74 people with no history of psychiatric disorders. In addition, because the CHRM4 is a potential target for antipsychotic drug development, we investigated whether variations in *CHRM4* sequence were associated with final recorded doses of, and life-time exposure to, antipsychotic drugs.
Gene sequencing identified two single nucleotide polymorphisms (SNPs; rs2067482 and rs72910092) in the *CHRM4* gene. For rs2067482, our data suggested that both genotype (1341C/C; $p = 0.05$) and allele (C; $p = 0.03$) were associated with an increased risk of schizophrenia. In addition, there was a strong trend ($p = 0.08$) towards an association between *CHRM4* sequence and increased lifetime exposure to antipsychotic drugs. Furthermore, there was a trend for people with the C allele to be prescribed benzodiazepines more frequently ($p = 0.06$) than those with the T allele.
These data, albeit on small cohorts, are consistent with genetic variance at rs2067482 contributing to an altered risk of developing schizophrenia which requires more forceful pharmacotherapy to achieve a clinical response.

1. Introduction

Schizophrenia is a complex psychiatric disorder (American Psychiatric Association, 2000). Although numerous studies have attempted to elucidate the cause of this disorder, the aetiology and pathophysiology remain ambiguous, making it difficult to design optimal treatments. Twin (Cardno et al., 2012) and adoption studies (see Ingraham and Kety, 2000) show that schizophrenia is a heritable disorder; this underpins the hypothesis that the genesis of schizophrenia should be able to be predicted by genetic factors (Agerbo et al., 2012). The genetic liability is further demonstrated by data showing that the risk of developing schizophrenia in the general population is less than 1% and rises incrementally if a single (7%) or both parents (27.3%) have the disorder (Gottesman et al., 2010). However, when considering the genetic load and schizophrenia it is important to note that approximately 90% of adults with schizophrenia do not have a parent with the disorder (Carter et al., 2002) and that only 3.8% of family groups affected by schizophrenia have more than one affected member (Lichtenstein et al., 2006). This data provides strong support for the concept that genetic risk is not the only factor that dictates whether an individual develops the disorder and that schizophrenia occurs in people with a genetic liability for the disorder who have encountered some environmental factor that has triggered the onset of illness (Tsuang, 2000). Thus, in order to better understand the epidemiology of the disorder, significant effort has been invested in identifying the genes that contribute to the genetic liability for the syndrome.

Current antipsychotic agents are effective in a proportion of patients but are not beneficial to many people with the disorder and cause unwanted side-effects (Tandon et al., 2008). Therefore, considerable energy is being expended in developing new drugs to treat schizophrenia. It is, therefore, significant that the muscarinic M_4 receptor (CHRM4) is viewed as a potential target to produce antipsychotic effects by a different mechanism of action to currently available

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drugs (Bymaster et al., 2002). A proof of principle drug trial supported this theory; xanomeline, a CHRM1/M4 preferring agonist (Shannon et al., 2000), reduced levels of psychotic symptoms in a group of people with schizophrenia who were treatment resistant (Shekhar et al., 2008). Importantly, pre-clinical studies (Gomeza et al., 1999; Salamone et al., 2001; Tzavara et al., 2003; Tzavara et al., 2004; Jeon et al., 2010) strongly support the hypothesis that the antipsychotic actions of xanomeline arise from its activation of CHRM4 (Woolley et al., 2009). Given these data, the discovery that muscarinic receptors have more than one potential drug binding site (Clark and Mitchelson, 1976) reinvigorated the development of drugs for these receptors. Importantly, these alternative (allosteric) binding sites, unlike the orthosteric sites, have not been preserved across evolution (Langmead et al., 2008). Therefore, they offer the potential to generate compounds that specifically activate CHRM4. CHRM4 allosteric activators are reported to be efficacious in models used to predict antipsychotic activity, such as conditioned avoidance response (Leach et al., 2010), prepulse inhibition (Chan et al., 2008) and amphetamine-induced hyperlocomotion (Brady et al., 2008). These preclinical data add further credibility to the concept that CHRM4 may be a viable drug target for the treatment of schizophrenia.

The involvement of CHRM4 in the pathophysiology of schizophrenia is supported by data showing that CHRM4 are decreased in the caudate-putamen (Dean et al., 2000) and hippocampus (Scarr et al., 2007) from people with the disorder. These data are the basis for our current study designed to determine if changes in the *CHRM4* gene sequence are associated with an altered risk of schizophrenia. In addition, given the potential of CHRM4s as a novel target for antipsychotic drug development, and their role in modulating dopaminergic activity (Tzavara et al., 2004; Jeon et al., 2010), we determined whether changes in *CHRM4* gene sequence was associated with the pharmacotherapy prescribed for the person.

2. Materials and methods

2.1. Tissue collection and donor information

Approval for this study was obtained from both the Ethics Committee of the Victorian Institute of Forensic Medicine and the Mental Health Research and Ethics Committee of Melbourne Health.

Cadavers were refrigerated within 5 h of being found. CNS tissue was rapidly frozen to −70 °C within 30 min of removal at autopsy. Where death was witnessed, the post-mortem interval (PMI) was the time between death and autopsy. If the death was not witnessed, PMI was taken as the interval midway between the person last being seen alive and being found dead. The pH of the CNS tissue was measured as described previously (Kingsbury et al., 1995); cerebellar tissue was homogenised in 10 volumes of distilled water and the pH measured, in duplicate, at room temperature. Case history reviews were completed using the Diagnostic Instrument for Brain Studies, achieving a diagnostic consensus using DSM-IV (American Psychiatric Association, 2000) criteria. The duration of illness (DOI) was calculated as the time from first psychiatric service interaction to death. Age at onset was calculated by subtracting DOI from age at death. Comprehensive medication histories were collated and the most recently prescribed dosages of antipsychotic drugs converted to chlorpromazine equivalents (Atkins et al., 1997; Woods, 2003). An estimate of life time exposure to antipsychotic mediation was obtained by multiplying the chlorpromazine equivalent by the number of years of treatment and dividing these by a constant, in this case, 1000 (See Table 1).

2.2. DNA extraction and sequencing

The DNA used was originally extracted for studies determining polymorphisms in the *CHRM1* (Scarr et al., 2009). In brief, approximately 200 mg of cerebellar tissue was excised from 78 subjects who had schizophrenia. Tissue was also collected from the same region from 74 subjects with no history of psychiatric illness (controls) and DNA extracted using an established technique (Strauss, 1999).

Primers were synthesized by GeneWorks (Thebarton, SA, Australia) to cover the whole *CHRM4* sequence in two amplicons, designed against GenBank accession number NM_000741.2: primer set A (primers: M4-A-F 5′-ccagagaatgtccctctgtca and M4-A-R 5′-ctgagctggactcattgg aag) and primer set B (M4-B-F 5′-tcctcaagagcccactaatga and M4-B-R 5′-tccagatgtccattcttccag). 100 ng of DNA was amplified in a 50 μL volume with 25 pmol of each primer, 10 nmol of dNTP, 1.5 U of Platinum Taq DNA Polymerase (Life Technologies, Mulgrave, VIC, Australia), 1.5 mM $MgCl_2$ and 5 μL of 10× buffer. The PCR programme consisted of: 5 minutes (min) at 94 °C, followed by 30 cycles of 30 seconds (s) at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a 7 min final extension at 72 °C. Products were validated by agarose electrophoresis, and cleaned up with 2 μL of Exo SAP-IT (Millennium Science Pty Ltd., Surrey Hills, VIC, Australia). The Exo-Sap treated product was sequenced with 1.0 μL of BigDye® Terminator v3.1 Ready Reaction Mix (Life Technologies, Mulgrave, VIC, Australia), with forward and reverse primers. Unincorporated dye and primers were removed using Qiagen DyeEx2.0 Spin Kit/DyeEx96 Kit, the samples were dehydrated, resuspended in 20 μL formamide, denatured for 5 min at 95 °C and sequenced on a 3130xl genetic analyser with POP7 polymer (Life Technologies, Mulgrave, VIC, Australia).

2.3. Statistical analyses

Categorical data were analysed and variance in frequencies identified using χ^2 test or Fisher's exact test as appropriate. Differences of continuous variables between cohorts were evaluated using Students' *t*-test or the Mann–Whitney *U* test. Differences of continuous variables between genotypes were evaluated using an ANOVA, the Kruskal–Wallis test, or the General Linear Model (GLM). The criterion for significance was set at $p<0.05$ for all of the tests. With the exception of the GLMs which were performed using Minitab 15 (Minitab, Minitab Inc., State College, PA, USA), all analyses were carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean ± S.E.M.

3. Results

3.1. CHRM4 sequencing

CHRM4 sequencing failed to give conclusive results in two subjects. Thus, this study used DNA from 76 people with schizophrenia and 74 controls. Whilst eight SNPs have been reported in *CHRM4* (rs35646260, rs7107481, rs16938505, rs16938502, rs2229163, rs2067482, rs11823766 and rs72910092), we only found two polymorphisms; rs2067482 (NC_000011.9:g.46406767G>A c.1341C>T) and rs72910092 in the 3′ UTR (NC_000011.9:g.46406493C>T c.*175C>T). For rs2067482, the frequencies of the C/C homozygote, C/T heterozygote and T/T homozygote were 0.713, 0.253 and 0.033, respectively. For rs72910092, the frequencies of the C/C homozygote and C/T heterozygote were 0.973 and 0.027.

3.2. Disease frequency

The genotype and allele distributions of the *CHRM4* polymorphisms in subjects with schizophrenia and control subjects are shown in Table 2.

3.2.1. rs2067482 (NC_000011.9:g.46406767G>A c.1341C>T)

The distribution of the rs2067482 genotypes were in Hardy–Weinberg equilibrium ($\chi^2=0.496$, d.f. = 1, $p=0.48$; see Table 2) (Rodriguez et al., 2009). There was variation in both genotype ($\chi^2=6.07$, d.f. = 2, $p=0.048$; see Table 2) and allelotype (Fisher's

Table 1
A summary of the demographic data (mean ± S.E.M.) of the cohort used for *CHRM4* sequencing. The data is presented both by diagnosis and genotype at rs2067482. Data is not presented for rs72910092 because of its low frequency in this cohort.

	Control	Schiz	p	Control genotypes (rs2067482)			Schizophrenia genotypes (rs2067482)			
				C/C	C/T	T/T	C/C	C/T	T/T	p
Age (years)	45.6 ± 2.0 (n = 74)	44.3 ± 1.9 (n = 76)	0.728	44.6 ± 2.4 (n = 46)	47.8 ± 3.4 (n = 25)	42.3 ± 12.8 (n = 3)	45.6 ± 2.2 (n = 61)	40.2 ± 4.2 (n = 13)	34.0 ± 11.0 (n = 2)	0.67
PMI (h)	40.88 ± 1.6 (n = 74)	40.6 ± 1.5 (n = 76)	0.977	40.5 ± 2.2 (n = 46)	42.1 ± 2.5 (n = 25)	36.2 ± 6.1 (n = 3)	35.6 ± 1.6 (n = 61)	42.8 ± 3.6 (n = 13)	55.3 ± 12.8 (n = 2)	0.56
Brain weight (g)	1415 ± 17.4 (n = 65)	1424 ± 18.7 (n = 64)	0.953	1380 ± 20 (n = 40)	1464 ± 31 (n = 22)	1532 ± 29 (n = 3)	1419 ± 21 (n = 51)	1436 ± 41 (n = 11)	1473 ± 132 (n = 2)	0.07
Brain pH	6.3 ± 0.02 (n = 74)	6.3 ± 0.03 (n = 76)	0.091	6.3 ± 0.03 (n = 46)	6.3 ± 0.04 (n = 25)	6.3 ± 0.14 (n = 3)	6.3 ± 0.04 (n = 61)	6.2 ± 0.06 (n = 13)	6.4 ± 0.04 (n = 2)	0.33
Gender (M/F)	58/16 (n = 74)	55/21 (n = 76)	0.45	32/14 (n = 46)	23/2 (n = 25)	3/0 (n = 3)	46/15 (n = 61)	7/6 (n = 13)	2/0 (n = 2)	0.45
Suicide/non-suicide	0/74 (n = 74)	38/38 (n = 76)					29/32 (n = 61)	7/6 (n = 13)	2/0 (n = 2)	0.92
DOI (years)[a]		18.04 ± 1.6 (n = 74)					19.2 ± 1.9 (n = 59)	14.2 ± 3.2 (n = 13)	9.0 ± 3.0 (n = 2)	0.22
Age at onset		26.1 ± 1.1 (n = 74)					26.2 ± 1.3 (n = 59)	26 ± 2.3 (n = 13)	25.0 ± 8 (n = 2)	0.80
FRAPD[c]		649 ± 74 (n = 69)					679 ± 84 (n = 56)	429 ± 145 (n = 11)	1025 ± 725 (n = 2)	0.11
LETA[b]		10.6 ± 1.8 (n = 67)					11.2 ± 2.0 (n = 54)	8.0 ± 4.5 (n = 11)	7.1 ± 3.5 (n = 2)	0.08
Atypical/typical antipsychotics		6/63 (n = 69)					5/51 (n = 56)	1/10 (n = 11)	0/2 (n = 2)	1.0
Anticholinergic/non-anticholinergic		29/47 (n = 76)					22/39 (n = 61)	6/7 (n = 13)	1/1 (n = 2)	0.44
Benzodiazepines/non-benzodiazepines		26/50 (n = 76)					24/37 (n = 61)	2/11 (n = 13)	0/2 (n = 2)	0.04

Schiz: schizophrenia
[a] DOI: duration of illness; not recorded for two subjects.
[b] LETA: lifetime exposure to antipsychotic drugs; not available for nine subjects.
[c] FRAPD: final recorded dose of antipsychotic drugs, converted to chlorpromazine equivalents; not recorded for seven subjects.

Exact test, p = 0.027; see Table 2) with diagnosis. This variation was due to an increased frequency of C/C, with concomitant decreased C/T frequency, in schizophrenia.

3.2.2. rs72910092 (NC_000011.9:g.46406493C>T c.*175 C>T)

The distribution of the rs72910092 genotypes was also in Hardy–Weinberg equilibrium, (χ^2 = 0.028, d.f = 1, p = 0.87; see Table 2) (Rodriguez et al., 2009). Neither genotype (Fisher's exact test, p = 0.99) or allelotype (Fisher's exact test, p = 0.99) frequencies were altered in schizophrenia. The low incidence of genetic variation at this SNP in our cohorts precluded any analyses at the level of haplotype and meant that it was not possible to investigate the association of this polymorphism with the potential confounding factors related to the tissue collection or donor demographics.

Table 2
Genotype and allele incidence for rs2067482 and rs72910092 in controls and people with schizophrenia.

	Genotype							Allele		
rs2067482	C/C	C/T	T/T	HWP	Total	p1	p2	C	T	p3
Control	46	25	3	0.03	74			117	31	
Schizophrenia	61	13	2	1.47	76	0.48	0.048	135	17	0.027
rs72910092	C/C	C/T	T/T	HWP	Total	p1		C	T	p3
Control	71	2	0	0.01	73			144	2	
Schizophrenia	72	2	0	0.01	74	0.87	0.99	146	2	0.99

HWP = Hardy–Weinberg proportion.
p1 values are the outcome of χ^2 tests for Hardy–Weinberg equilibrium, calculated using the Online Encyclopedia for Genetic Epidemiology studies (Rodriguez et al., 2009).
p2 values are the outcome of χ^2 tests for genotype frequency between control subjects and people with schizophrenia.
p3 values are the outcome of Fisher's Exact test for allelotype frequency between control subjects and people with schizophrenia.

3.3. Demographic data

Despite no *a priori* matching, analyses of age, PMI, brain pH, brain weight and ratio of males to females revealed no significant differences between groups (p = 0.64, 0.88, 0.12, 0.75 and 0.45 respectively; see Table 1). Having found no differences between diagnostic groups, data from both cohorts were combined to determine any interactions with genotype. There were no significant differences in age (K = 0.798, p = 0.67), PMI (F = 0.588, d.f. = 2, p = 0.56), brain pH (F = 1.41,d.f. = 2,147, p = 0.25), brain weight (F = 2.69, d.f. = 2, p = 0.072) or gender ratio (χ^2 = 2.25, d.f. = 2, p = 0.33) with genotype.

Within the schizophrenia cohort, only two people had the 1341T/T genotype, thus, the small sample size precluded them from genotype comparisons. There were no differences in genotype (χ^2 = 0.17, d.f. = 2 p = 0.92) or allelic (Fisher's exact test, p = 0.30) frequencies with suicide, duration of illness (Mann–Whitney U = 299.5, p = 0.22, medians; C/C = 15.0, C/T = 10.0) or age at onset (Mann–Whitney U = 365.5, p = 0.80, medians; C/C = 23.0, C/T = 24.0).

3.4. Neuropsychopharmacology

The low frequency of T/T at rs2067482 precluded genotype analyses at the level of antipsychotic drug exposure (chlorpromazine equivalents; 300 and 1750 mg per day respectively). The D'Agostino & Pearson omnibus normality test showed that final recorded drug doses were highly skewed; neither genotype passed the test for normality (C/C; K2 = 28.2, p<0.0001, C/T; K2 = 14.9, p<0.001). Analysis of chlorpromazine equivalents between genotype revealed no significant differences (Mann–Whitney U = 214, p = 0.115, medians; C/C = 500.0, C/T = 200.0; see Fig. 1A). However, the analysis of the lifetime exposure to antipsychotic drugs revealed a trend towards differences (Mann–Whitney U = 198, p = 0.08, medians; C/C = 6.4, C/T = 2.4; see Fig. 1B). A power analysis (http://www.biomath.info/power/ttest.htm)

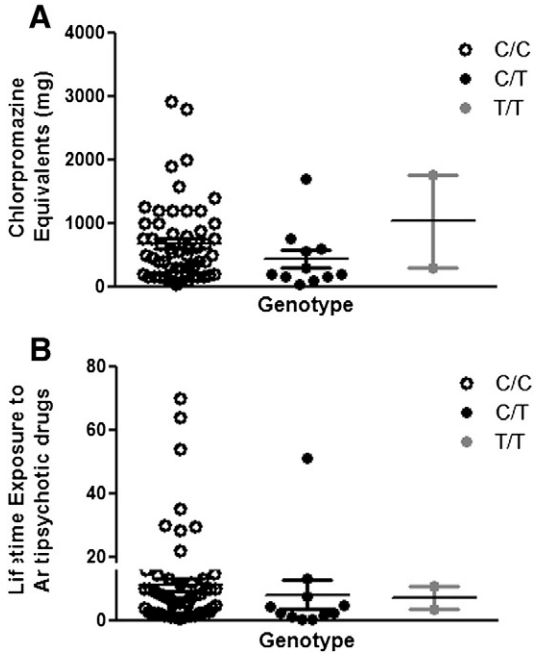

Fig. 1. A) Final recorded doses of antipsychotic drugs, normalised to chlorpromazine equivalents, prescribed to people with schizophrenia separated by genotype. B) Life time exposure to antipsychotic medication for people with schizophrenia, separated by allele at rs2067482.

indicated an additional three subjects with the 1341C/T genotype were required to achieve a power level of 0.8 with an alpha of 0.05.

Six subjects with schizophrenia had been prescribed atypical antipsychotic drugs. Importantly, Chi-square calculations are only valid when all expected values are greater than 1.0 and at least 20% of the expected values are greater than 5. These conditions were not met because only two subjects with Sz had the 1341T/T genotype, thus chi-square calculations could not be carried out at the level of genotype, so allelotype analyses were completed. There was no association between frequency of treatment with atypical antipsychotics and allelotype (Fishers exact test, $p = 1.0$).

In addition to antipsychotic drugs, a significant number of subjects had been prescribed anti-cholinergic agents and/or benzodiazepines, therefore we assessed whether there were differences in prescription patterns with variation in CHRM4 sequence. The conditions for chi-square calculations at the level of genotype were not met, therefore allelotype analyses were completed.

There was a trend towards a difference in frequency of prescription of benzodiazepines (Fishers exact test, $p = 0.055$); people with the C allele were prescribed benzodiazepines more often than those with the T allele (see Table 3). It is possible that this may reflect an allelic dosage effect since neither T/T individual was prescribed benzodiazepines.

There was no significant association of anticholinergic prescription with allele frequency (Fisher's exact test, $p = 0.44$).

Table 3
Allele incidence for rs2067482 in people with schizophrenia who were or were not prescribed benzodiazepines.

	Allele		
	C	T	p
Schizophrenia — benzodiazepines	50	2	
Schizophrenia — no benzodiazepines	85	15	0.055

p values are the outcome of Fishers exact test.

4. Discussion

In this study we examined the association of *CHRM4* polymorphisms with schizophrenia susceptibility and psychotropic treatment. For rs72910092, genotype did not indicate an altered risk for schizophrenia. By contrast, the C/C frequency at rs2067482 was significantly increased in people with schizophrenia. Clearly, this study has a small sample size and the findings must be replicated in a larger cohort. However, a recent genome wide association study reported that a high proportion of their most altered SNPs, albeit none reaching statistical significance, were located within the region of chromosome 11 that includes the *CHRM4* gene (Rietschel et al., 2011), suggesting this region of the human genome might be particularly associated with an altered risk of developing disorders such as schizophrenia. Although variations in *CHRM4* did not show any associations with the CATIE response phenotypes (Need et al., 2009), after "correcting for poor quality SNPs" there were no tags for *CHRM4* according to the HapMap CEU (Utah residents with Northern and Western European ancestry from the CEPH (Centre d'Etude du Polymorphisme Humain) collection) cohort, thus only participants with *CHRM4* SNPs similar to those of the Yoruba in Ibadan, Nigeria were included (supplemental methods(Need et al., 2009)).

We also report a strong trend towards an association between the C/C genotype and a higher lifetime exposure to antipsychotic drugs (medians; C/C = 6.4, T/T = 2.4). This failed to achieve significance because of the data for one C/T individual (full data set; Mann–Whitney $U = 198$, $p = 0.083$, medians; C/C = 6.4, C/T = 2.4; without this individual; Mann–Whitney $U = 146.5$, $p = 0.023$, medians; C/C = 6.4, C/T = 2.25). The difference in lifetime exposure to antipsychotic drugs does not appear to simply be due to the typicality of antipsychotic drugs prescribed. However, this finding must be treated with caution as only six subjects received second generation drugs, five of whom had the C/C genotype.

We also found that benzodiazepines tended to be prescribed more frequently to people with the C allele than to those with the T allele. This is of interest because clinically, benzodiazepines are prescribed to people with schizophrenia to control acute behavioural disturbances, compensate for a poor response to antipsychotics or because they are thought to allow a lower dose of antipsychotic medication to be prescribed (Paton et al., 2000). The latter reason does not hold true in our cohort, where the patients more likely to be prescribed benzodiazepines (C allele), tended to have a greater lifetime exposure to antipsychotic drugs. Thus, these data suggest that patients who carry the C allele may have a worse clinical presentation, resulting in forceful pharmacotherapy to control their disorder. However, a search of the F-SNP database (Ingraham and Kety, 2000) did not yield any information about the functional significance of this SNP leaving unanswered the question of the biological basis of this association.

This study reports two significant findings; i) C/C at rs2067482 appears to increase the risk of schizophrenia and ii) if such people develop the disorder it is more likely to be severe (i.e. associated with higher life-time doses of antipsychotic drugs and prescriptions of benzodiazepines). These findings need to be considered as preliminary, in part because of the sample size which is small for a genetic study and because the data related to the treatment received by these people was accessed from medical records as part of a post-mortem assessment rather than directly from the individuals. However, this data is a strong justification for a similar study in a larger clinical population. Despite these limitations, this pilot data is timely as CHRM4 allosteric modulators are being developed as potential therapeutics for schizophrenia (Brady et al., 2008; Chan et al., 2008; Leach et al., 2010). Although our small cohort requires more extensive studies on large clinical populations, our data suggests that such drugs may be useful in people with a relatively severe form of the disorder, particularly if *CHRM4* genotypes are associated with apparent changes in CHRM4 function. Our data also raises the possibility that variation at rs2067482 may prove useful in predicting response to such therapeutics.

In conclusion, our data suggests that the rs2067482 polymorphism may help to predict susceptibility to schizophrenia and/or therapeutic responsiveness. Moreover, this data and that from studies on *CHRM1* (Liao et al., 2003; Scarr et al., 2012) suggest that changes in CHRM sequences may be linked to symptom severity. Importantly, new drugs are being developed that specifically activate CHRM1 and CHRM4, and are suggested as potential treatments for schizophrenia (Conn et al., 2009). Therefore, studies on *CHRM4/M1* variance in larger cohorts of people with schizophrenia are required to assess whether these variants could be employed as diagnostic differentiators to identify sub-groups of individuals within the schizophrenia syndrome (Ibrahim and Tamminga, 2011) or as pharmacogenetic markers that would facilitate our progress towards personalised medicine for people with the disorder.

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Contributors

Authors Scarr & Dean designed the study. Authors Um and Cowie were responsible for genotyping and genetic analysis. Author Scarr drafted the manuscript and performed all of the analyses other than those related to the gene frequency and Hardy–Weinberg equilibrium (JYU). All authors contributed to and have approved the final manuscript.

Conflict of interest

There are no competing financial interests in relation to the work described in this paper.

TFC & JYU report no competing interests. The following authors have previously received remuneration: ES received honorarium from Astra-Zeneca and travel support from GSK. BD received travel support from GSK, honorarium from Pfizer, Eli Lilly and MSD.

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