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ORIGINAL RESEARCH

No Association Between Cholinergic Muscarinic Receptor 2 (*CHRM2*) Genetic Variation and Cognitive Abilities in Three Independent Samples

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Abstract Cognitive ability has a substantial genetic component and more than 15 candidate genes have been identified over the past 8 years. One gene that has been associated with general cognitive ability is the cholinergic muscarinic 2 receptor (*CHRM2*). In an attempt to replicate this finding we typed marker rs8191992 (the originally reported *CHRM2* SNP) in two population based cohorts—one Scottish aged over 50 years ($N = 2{,}091$) and the other English comprising non-demented elderly participants ($N = 758$)—and a family-based Australian adolescent sample ($N = 1{,}537$). An additional 29 SNPs in *CHRM2* were typed in the Australian sample and a further seven in the English cohort. No significant association was found between *CHRM2* and diverse measures of cognitive ability in any of the samples. In conclusion, this study does not support a role for *CHRM2* in cognitive ability.

Keywords Association analyses · *CHRM2* · Cognitive ability · Genetics · Intelligence

Introduction

Data collected from tens of thousands of twins and siblings both reared together and reared apart, and after adjusting for shared environments (including maternal womb environments), indicate that the heritability of general cognitive ability (the IQ or *g-factor*) is approximately 50% in adolescents (Bouchard and McGue 2003; Devlin et al. 1997) with other studies indicating a higher heritability in old age (Deary et al. 2006) and lower earlier in childhood

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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(McClearn et al. 1997; Polderman et al. 2006). Over the last 8 years some specific genes have been implicated to explain this heritable variance (Deary et al. 2006; Payton 2006). Several neurotrophic factors have been indicated, including the extensively studied brain-derived neurotrophic factor (*BDNF*) (Miyajima et al. 2008; Pertusa et al. 2008; Savitz et al. 2006). However, the majority of these genes have been neurotransmitter-related, catechol-*O*-methyltransferase (*COMT*) being an example reported to influence cognition by multiple independent groups (Barnett et al. 2007, 2008; Bruder et al. 2005; de Frias et al. 2005). For most other genes there have been few attempts at replication and/or a failure to replicate, including *DRD2* and *IGF2R* (for review, see Payton 2006). One such gene that falls into the latter category is the cholinergic muscarinic receptor 2 (*CHRM2*).

Of the five muscarinic receptor sub-types (differing in structure, function and distribution), M1 and M2 (the most abundant) have been associated with cognitive ability. M1 receptor agonists (AF102B, AF150(S) and AF267B-1) which increase synaptic ACh levels are related to improved cognition in various animal Alzheimer's disease models (Fisher et al. 2002) and antagonists of presynaptic M2 receptors (which decrease ACh) enhance cognitive ability in rodents and non-human primates (Carey et al. 2001). *CHRM2* has been shown to be involved in long-term potentiation (Calabresi et al. 1998), which is thought to be a fundamental mechanism in learning and memory (Silva 2003), and single nucleotide polymorphisms (SNPs) have been associated with visual evoked brain oscillations suggesting at least a role for *CHRM2* in higher mental functioning in humans (Jones et al. 2006), although reports that *CHRM2* polymorphisms are associated with alcoholism and depression (Comings et al. 2002; Edenberg and Foroud 2006; Luo et al. 2005; Wang et al. 2004) suggest a non-specific phenotype.

Together with post-mortem evidence that showed an increase in muscarinic M1 binding sites in striatal areas of Alzheimer's disease patients (Aubert et al. 1992) and increased M2 receptor density in frontal and temporal cortex of patients with psychotic symptoms (Lai et al. 2001), evidence for the role of ACh in memory and learning gave credence to a report by Comings et al. (2003) which linked variation in *CHRM2* with measures of intelligence. In their study of 828 Caucasian adults, marker rs8191992 (1890A > T) was associated with IQ and with years of education [a correlate of IQ; (Deary et al. 2007)], accounting for one percent of the variance in full-scale IQ (Comings et al. 2003). Positive findings at this locus for performance IQ have subsequently been reported in the Collaborative Study on the Genetics of Alcoholism (COGA) cohort (Dick et al. 2007) and in a Dutch family cohort (Gosso et al. 2006). By contrast, Harris et al. (2007) found no support for association of marker rs8191992 to general ability measured at age 11, nor at ages 64 or 79, nor to executive function, memory or learning (nominal *P*-values 0.16–0.78) in a Scottish sample of healthy elderly subjects ($N = 437$).

In the Dutch cohort, marker rs324650 showed the strongest association with IQ; the T increaser allele associated with a 0.3 SD performance IQ increment (4.6 IQ points). This SNP was not associated with any measure of cognitive ability in the COGA sample. Marker rs2061174 was significant in both studies but the increaser allele was different between the two studies. Dick et al. (2007) argued that multiple variants in *CHRM2* contribute to variation in IQ since SNPs in different regions of the gene have been reported. For instance, SNPs located in introns 4–5 and introns 5–6 have been supported in the COGA and Dutch cohorts, with SNPs in the 3′ UTR of the gene also supported in the COGA and Comings et al. (2003) samples. The COGA study, which undertook more extensive genotyping of *CHRM2* also found evidence of association with SNPs located in introns 3–4 and downstream of intron 6.

Clearly, further replication studies are warranted to clarify the relationship between *CHMR2* and cognition. Here, we attempted to replicate the original association reported by Comings and colleagues with IQ in three independent samples from Australia ($N = 1{,}537$), England ($N = 758$) and Scotland ($N = 2{,}091$) and to examine association at 29 additional tagging SNPs in the Australian sample.

Materials and methods

Samples

Australian cohort

Twins and their non-twin siblings were initially recruited as part of ongoing studies of melanoma risk factors (McGregor et al. 1999; Zhu et al. 1999) and cognition (Wright and Martin 2004). Twins and their families were representative of the Queensland population for mole count (Zhu et al. 1999) and intellectual ability (Luciano et al. 2004). Participants were excluded if parental report indicated a history of significant head injury, neurological or psychiatric illness, substance abuse or dependence, or chronic use of medication with known effects on the central nervous system. Cognitive measures were available for 1,537 individuals (48.7% male) from 730 families comprising 204 monozygotic (MZ) pairs, 404 dizygotic (DZ) pairs, 122 unpaired twins and 199 siblings. Participants were predominantly Caucasian of Anglo-Celtic descent

and ranged in age from 15 to 22 years (mean age: 16.2 ± 0.4 for twins; 17.3 ± 1.2 for siblings) at the time of testing. Written informed consent was obtained from each participant and their parent/guardian (if younger than 18 years) prior to testing and participants agreed to donate a blood sample for DNA isolation and genotyping. Zygosity was assessed using nine polymorphic DNA microsatellite markers (AmpF1STR Profiler Plus Amplification Kit, Applied Biosystems, Foster City, CA) and three blood groups (ABO, MNS, and Rh), giving a probability of correct assignment greater than 99.99% (Nyholt 2006). Parental genotypes but no parental IQ scores were available.

English cohort

The 758 elderly Caucasian volunteers involved in this study form part of the Dyne Steele DNA bank for cognitive genetic studies and comprise 234 males and 524 females. On entry to the study the age range was 50–85 years and the mean age was 63.2 ± 6.4 years. At the beginning of the study all volunteers achieved the maximum score on the mini mental state examination. Details on the recruitment, sample composition and cognitive tests are described in detail elsewhere (Rabbitt et al. 2004). Volunteers gave written consent for the use of their DNA in the investigations performed.

Scottish cohort

This included participants from the aspirin for asymptomatic atherosclerosis (AAA) Trial—a randomised controlled trial of aspirin for the reduction of cardiovascular events and death in people with asymptomatic atherosclerosis. For further details about participant selection and recruitment see (Price et al. 2008; Stewart et al. 2006). In short, the sample was aged over 50 years with no history of cardiovascular disease but with a ratio of systolic blood pressure in the ankle to that in the arm (i.e., ankle-brachial index) of 0.95 or less, indicative of atherosclerotic burden and increased risk of developing symptomatic cardiovascular disease (Fowkes et al. 2008; Heald et al. 2006). In addition to symptomatic vascular disease or major illness, subjects were excluded if they had a contra-indication to aspirin therapy, including a haematocrit measurement <38% for men and <35% for women. Of the 3,350 subjects recruited into the trial, 2,312 were assessed for cognitive ability at baseline using one cognitive test and at 5 years follow-up using a battery of six cognitive tests. In this study, 2,091 participants—who completed at least three tests—had DNA available for analysis.

Measures

Australian cohort

Measures of IQ were assessed with the shortened version of the Multi-dimensional Aptitude Battery (MAB) comprising three verbal (information, vocabulary, arithmetic) and two performance (spatial, object assembly) subtests (Jackson 1984; Jackson 1998). Scaled scores for overall intelligence quotient [full-scale IQ (FIQ)] as well as measures of verbal (VIQ) and nonverbal IQ (PIQ) were compiled following the manual instructions and were normally distributed.

English cohort

Tests of fluid intelligence (novel problem solving) comprised the Alice Heim intelligence tests parts one and two (AH1 and AH2) (Heim 1970). Participants completed the Mill Hill Vocabulary Test (Raven 1965) of verbal intelligence based on two parallel lists of words (parts A and B). A general factor was extracted from these tests using principal components analysis and was taken as a measure of general cognitive ability, *g*. Measures of full scale IQ and *g* have been shown to be strongly correlated (~0.90) in previous studies (Jensen 1998). Verbal IQ was represented by the composite of the Mill Hill Vocabulary Test parts A and B (Raven 1965); while the composite of the Alice Heim intelligence test parts 1 and 2 (Heim 1970) was primarily an index of nonverbal IQ. Extensive data on demographics and health have also been archived. Details on the cognitive tests are described in detail elsewhere, although here we do not report on follow-up measures in this sample to avoid confounding of differential cognitive decline due to dementia (Rabbitt et al. 2004).

Scottish cohort

Non-verbal reasoning was measured by Raven's Standard Progressive Matrices (RAVENS) (Raven et al. 1998) and executive function by the Verbal Fluency Test (VFT) (Lezak 1982). The Auditory Verbal Learning Task (AVLT) (Lezak 1982) tested immediate and delayed memory (total score on the first five trials). Processing speed was measured using the Digit Symbol Test (DST) from the WAIS (Wechsler 1981), and Part B of the Trail Making Test (TMT), which also assesses mental flexibility. Verbal ability was measured by combining synonyms of the Junior and Senior versions of the Mill Hill Vocabulary Test (Raven 1965) at base-line and the National Adult Reading Test (NART), often used as an estimate of pre-morbid IQ (Crawford et al. 2001), at follow-up. A *g* factor was derived from the Ravens, VFT, AVLT, Mill Hill, DST and TMT tests and was based on the first unrotated principal

component which explained 50.5% of common variance. Similarly, a verbal factor was derived from the VFT, AVLT, Mill Hill, and NART; and a nonverbal factor from the Ravens, DST and TMT tests. Identifying the same phenotype is of critical importance in replicating association studies. The *g*-factors from diverse batteries appear close to indiscriminable (Jensen 1998), suggesting that studies testing association with the first principal component of an ability battery will be testing the same target. Similar arguments can be made for the other high-level latent constructs tested in research on *CHRM2* such as verbal and performance IQ. At the level of single tests of ability, such as the Ravens or WAIS-R, correlations are more modest, but still around 0.7 (cf: Jackson 1998 for the relationship of the MAB (used in the Australian sample) to WAIS full-scale scores).

Genotyping

Australian cohort

Thirty-five SNPs across the *CHRM2* locus were selected on the basis of data available at the time: (1) eight SNPs were chosen from *CHRM2* association studies (Jones et al. 2004; Wang et al. 2004), and (2) twenty-seven haplotype-tagging SNPs were chosen from the International HapMap Project public database (http://www.hapmap.org/; Phase II dbSNP Build 124) for coverage of *CHRM2* by Haploview (version 3.2) (Barrett et al. 2005) and TAMAL (Hemminger et al. 2006) software. Assays were designed using the MassARRAY Assay Design (version 3.0) software (Sequenom Inc., San Diego, CA) and typed using iPLEX chemistry on a Compact MALDI-TOF Mass Spectrometer (Sequenom Inc., San Diego, CA). Forward and reverse PCR primers and primer extension probes were purchased from Bioneer Corporation (Daejeon, Korea). Genotyping was carried out in standard 384-well plates with 12.5 ng genomic DNA used per sample. Allele calls for each 384-well plate were reviewed using the cluster tool in the SpectroTyper software (Sequenom Inc.) to evaluate assay quality. Parental genotypes but no parental phenotypes were available. Genotype error checking, sample identity and zygosity assessment were completed in PEDSTATS (Wigginton and Abecasis 2005). Five SNPs failed during the assay design or provided unreliable genotype data and were excluded from further analyses.

English cohort

An automated denaturing high performance liquid chromatography based technique (Transgenomic WAVE^{TM}, Crewe, UK) was employed to screen the *CHRM2* locus for polymorphisms (Underhill et al. 1997). Thirty randomly selected DNA samples were used for WAVE screening. This number allows for 95% power to detect polymorphisms with frequencies of 5% or higher. Amplification of *CHRM2* was performed using 600 base pair (bp) fragments that overlapped by approximately 50 bp for accurate sequence alignment. Gene sequence was obtained from the National Centre for Biotechnology Information (NCBI, Bethesda, USA), accession number NT_007933. A total of nine approximately 600 bp fragments were used to screen a 5 kb region of the *CHRM2* gene that spanned 2.5 kb upstream, 1.5 kb of the single coding exon and 1 kb downstream. Sequencing was performed using the ABI 377 Prism. Sequence results were viewed using Chromas software version 1.45 (www.technelysium.com.au) and aligned using the multiple sequence alignment editing software Genedoc (version 2.6.001) (www.psc.edu/biomed/genedoc). Genotyping of four SNPs identified through the screening plus four additional SNPs selected from the literature was performed using the SNaPshot^{TM} method (Perkin Elmer Applied Biosystems). Laboratory work was performed under the ISO 9001:2000 quality management requirement.

Scottish cohort

For the AAA Trial sample, genotyping was carried out by KBioscience, using their in-house chemistry of Competitive Allele Specific PCR (KASPar).

Statistical analysis

For the family-based Australian sample, tests of total association with VIQ, PIQ and FIQ were conducted in the program QTDT (Abecasis et al. 2000a, b) which involves maximum-likelihood modeling of the data using a variance-components framework. Total association considers transmission within and between families, specifying an additive model against the null hypothesis of no linkage and no association. All traits were corrected for sex and age effects by fitting covariates in the regression model. MZ twins can be included and are modeled as such by adding zygosity status to the data file and while they are not informative to the within-family component (unless they are paired with non-twin siblings) they are informative for the between-family component. The between-family association component is not robust to population admixture whereas the within-family component is unaffected by spurious associations due to population structure. Thus, if population structure creates a false association, the test for association using the within-family component is still valid, though usually less powerful. Additional analyses of the Australian sample were performed in QTDT to check for population stratification using a variant of the

orthogonal model which evaluates population stratification by comparing the between- and within-family components of association. As the English and Scottish cohorts are population based, the within-family test can not be conducted and hence only a simple regression test for association was performed using PLINK and included sex and age as covariates (Purcell et al. 2007).

In the Australian sample, we have approximately 97% power ($\alpha = 0.05$) to detect overall association with a SNP with minor allele frequency (MAF) of 0.35 that explains 1% of variance in our traits under an additive model and against a background sibling correlation of 0.30 (Purcell et al. 2003). In the English and Scottish samples, we have 95 and 100% power, respectively, to detect a gene effect size of 1% with a significance level of 0.05. Pair-wise marker-marker linkage disequilibrium (LD) was assessed using the r^2 statistic in Haploview 3.31 (Barrett et al. 2005).

Results

Descriptive

In total, 30 *CHRM2* SNPs spanning a region of 160.7 Kb were genotyped in the Australian cohort. Mendelian inconsistencies and discordant MZ genotypes identified using PEDSTATS made up 0.15% of the data and were removed from analysis. Call rates of >97% were achieved for all SNPs except rs1424572 (93.4%) and rs1378650 (96.2%) in this sample. The physical locations of and inter-marker linkage disequilibrium (LD) between the 30 SNPs are schematically presented in Fig. 1. Seven haplotype blocks spanning small clusters of SNPs (2–4 SNPs) were observed according to the criteria of Gabriel et al. (2002). This pattern of LD is similar to the data (on a smaller number of people) from the HapMap Project public database for CEPH families of European origin.

Mutation screening of the *CHRM2* gene in 30 randomly selected English subjects identified one SNP (rs324651) located −354 bp upstream of the transcription initiation site of the single coding exon, and 3 SNPs located in the 3′untranslated region (UTR) at nucleotides 1696 (rs8191992), 1951 (rs8191993) and 2323 (rs6962027). Polymorphism details were submitted to NCBI dbSNP for assignment of an rs number. These SNPs and a further four SNPs (rs2350780, rs2061174, rs324640, rs324650) were genotyped through the 758 elderly English subjects. Call rates of approximately 82% were achieved for SNPs identified during the *CHRM2* screening stage with ~99% call rates attained for latter four SNPs. Moderate to strong LD (D′ 0.7–0.9) was observed between the 5 SNPs spanning intron 5 to the 3′UTR (Fig. 1). A 96% call rate was achieved for rs8191992 in the Scottish cohort.

In all cohorts, no SNP showed significant deviation from Hardy–Weinberg equilibrium (HWE) at a $P < 0.001$ level (Haploview version 3.31; Barrett et al. 2005). SNP marker information, including genetic map position, location within *CHRM2* and minor allele frequencies within the three cohorts are tabulated in Table 1. Allele frequencies for the five SNPs typed in both the English and Australian samples and for rs8191992 typed in all samples did not differ.

SNP association analyses

While different IQ tests were used to study cognitive ability across the three samples, mental ability tests are positively correlated (upwards of 0.30) and in a large study of varying cognitive tests were shown to load on a general cognitive factor (Carroll 1993). Significant mean effects were shown for sex and age in the Australian cohort with males scoring higher than females and older participants performing better, whereas in the older English sample younger participants performed better and females scored higher than males. In the Scottish cohort, age was negatively associated with all measures except the NART (which was not significant) and positively associated with the Mill Hill. Women performed better than men on AVLT, DST, NART and *g*, whereas men showed superior performance on TMT and Ravens. Sex and age were included as covariates in the tests of association.

As there was no evidence for significant population stratification in the Australian sample, excluding results for rs1378650 for PIQ ($P = 0.02$) and rs1424572 for VIQ ($P = 0.04$), we proceeded with the total-test of association (Table 2). Overall, no significant association was detected between 30 *CHRM2* SNPs and IQ measures. No significant association between general cognitive ability and measures of verbal and non-verbal intelligence was observed in the population-based tests of additive effects in the English and Scottish cohorts (Table 3).

Discussion

Our results did not support an association between cognitive ability and variation in *CHRM2* in neither young nor old cohorts of Anglo–Celtic origin. The original SNP reported by Comings et al. and typed in all three of our cohorts (rs8191992) was not significantly associated with any of the IQ measures in any of the cohorts. Like the study of Dick et al. (2007) we have also performed a comprehensive analysis of the *CHRM2* gene and its relation to a diverse battery of cognitive ability and general ability measures, and we found no support at any of the previously reported regions. It is important to understand the

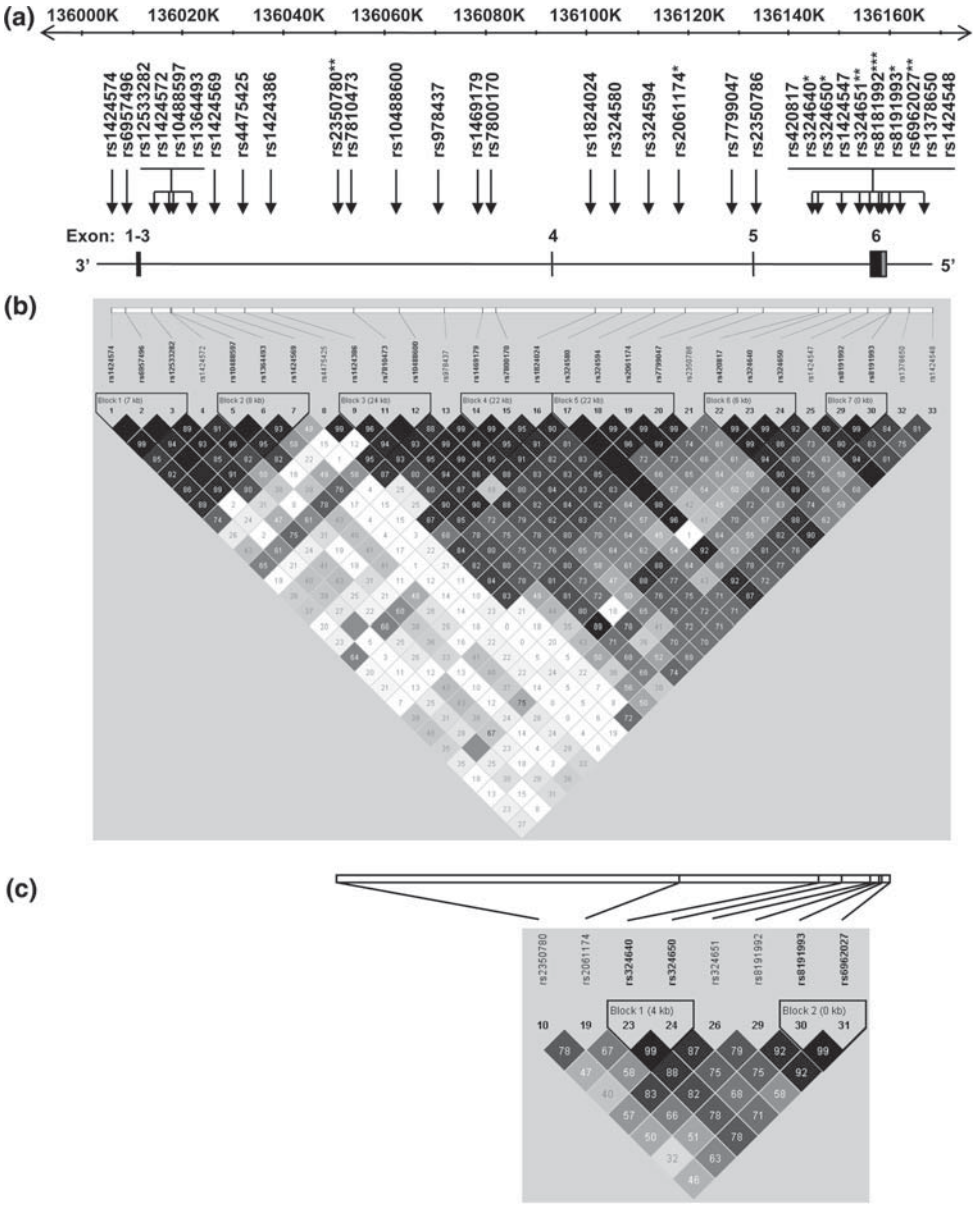

Fig. 1 Variation in the human *CHRM2* gene. **a** The gene structure of *CHRM2* showing the location of 31 SNPs, not including rs11773032 and rs12721506 whose MAFs were <0.2%, typed in the Australian, English and Scottish cohorts. SNPs marked with one asterisk were typed only in the Australian and English cohorts while SNPs marked with two asterisks were typed only in the English cohort. SNPs marked with three asterisks were typed in the three cohorts. Exons are numbered from 1 to 6 and relative exon size is denoted by the width of the vertical bars. **b** Pairwise marker-marker linkage disequilibrium (*LD*) between 28 SNPs genotyped in the Australian cohort was generated using Haploview (Barrett et al. 2005). LD causes tightly linked genetic variants to be highly correlated (Abecasis et al. 2005). Shading represents regions of low (*white*) to high LD (*black*). **c** LD between 8 SNPs genotyped in the English cohort

implications of data available now from seven comparably sized, independent samples: the original positive result, two positive replications, and, now, four failures to replicate.

Three features seem note worthy: The status of the original marker rs8191992; the effect of multiple testing on false-discovery rates; and differences in the nature of the samples tested, especially the possible importance of a background of substance abuse.

The SNP rs8191992 reported by Comings et al. (2003) reached nominal significance ($P = 0.05$) with performance IQ when tested in the COGA study (Dick et al. 2007). This study tested 27 SNPs and 3 phenotypes. Correction even in part for range of SNPs and phenotypes tested would not allow this association to remain significant. The Comings marker was not tested by Gosso et al. (2007) but it was tested in an elderly sample of 437 Scottish participants [79.1 years (SD = 0.6 years)] against measures of verbal and nonverbal reasoning, executive function and verbal memory and learning (Harris et al. 2007), and no significant support for association was found. Given marginal support in one study, the weight of evidence is against an association of rs8191992 and cognitive ability.

As is inevitable in studies of this nature, a large number of comparisons have been made across diverse SNPs. Taken as a whole, some 100+ comparisons have been made in the seven studies to date (including the present study). Seen in this light, one would expect approximately

Table 1 *CHRM2* gene marker information

SNP	Gene location[a]	LD block[b]	Chromosomal location (bp)[c]	SNP alleles	Australian		English		Scottish	
					MA	MAF	MA	MAF	MA	MAF
rs1424574	Upstream	Block 1	136,006,288	A/G	G	0.15	nd	nd	nd	nd
rs6957496	Promoter	Block 1	136,009,092	A/G	G	0.09	nd	nd	nd	nd
rs12533282	Intron 3	Block 1	136,014,233	A/G	G	0.16	nd	nd	nd	nd
rs1424572	Intron 3		136,017,934	C/T	C	0.30	nd	nd	nd	nd
rs10488597	Intron 3	Block 2	136,018,187	C/T	C	0.21	nd	nd	nd	nd
rs1364493	Intron 3	Block 2	136,022,368	G/T	T	0.10	nd	nd	nd	nd
rs1424569	Intron 3	Block 2	136,026,671	C/T	T	0.47	nd	nd	nd	nd
rs4475425	Intron 3		136,032,454	A/G	A	0.25	nd	nd	nd	nd
rs1424386	Intron 3	Block 3	136,037,740	A/G	G	0.38	nd	nd	nd	nd
rs2350780	Intron 3		136,050,224	A/G	nd	nd	G	0.37	nd	nd
rs7810473	Intron 3	Block 3	136,053,712	A/G	G	0.41	nd	nd	nd	nd
rs10488600	Intron 3	Block 3	136,062,713	C/T	T	0.15	nd	nd	nd	nd
rs978437	Intron 3		136,071,433	C/T	C	0.34	nd	nd	nd	nd
rs1469179	Intron 3	Block 4	136,078,932	A/T	T	0.50	nd	nd	nd	nd
rs7800170	Intron 3	Block 4	136,081,575	A/C	A	0.50	nd	nd	nd	nd
rs1824024	Intron 4	Block 4	136,100,949	A/C	C	0.34	nd	nd	nd	nd
rs324580	Intron 4	Block 5	136,106,099	C/T	T	0.07	nd	nd	nd	nd
rs324594	Intron 4	Block 5	136,112,578	C/T	C	0.25	nd	nd	nd	nd
rs2061174	Intron 4	Block 5	136,118,655	A/G	G	0.32	G	0.32	nd	nd
rs7799047	Intron 4	Block 5	136,128,813	C/G	G	0.31	nd	nd	nd	nd
rs2350786	Intron 5		136,133,825	G/A	A	0.27	nd	nd	nd	nd
rs420817	Intron 5	Block 6	136,144,658	C/T	T	0.48	nd	nd	nd	nd
rs324640	Intron 5	Block 6	136,146,251	A/G	G	0.49	G	0.48	nd	nd
rs324650	Intron 5	Block 6	136,150,916	A/T	T	0.47	T	0.46	nd	nd
rs1424547	Intron 5		136,154,392	C/T	C	0.03	nd	nd	nd	nd
rs324651	Intron 5		136,156,516	G/T	nd	nd	T	0.10	nd	nd
rs11773032[d]	Exon 6		136,157,084	A/G	A	0	nd	nd	nd	nd
rs12721506[e]	Exon 6		136,158,223	A/G	G	0.00	nd	nd	nd	nd
rs8191992	3'UTR	Block 7	136,158,563	A/T	T	0.47	T	0.45	T	0.44
rs8191993	3'UTR	Block 7	136,158,818	C/G	G	0.34	G	0.33	nd	nd
rs6962027	3'UTR		136,159,190	A/T	nd	nd	T	0.46	nd	nd
rs1378650	Downstream		136,162,406	A/G	A	0.47	nd	nd	nd	nd
rs1424548	Downstream		136,167,015	C/T	T	0.38	nd	nd	nd	nd

Note: SNPs genotyped in multiple samples are indicated in bold. Monomorphic or rare (MAF < 1%) SNPs excluded from association analyses are indicated in italics. The minor allele (MA) and MAF are listed for each SNP

[a] Position within or near gene

[b] LD block in Australian sample. SNPs without a block number listed are located between blocks

[c] Position in nucleotides on chromosome 2 as estimated in dbSNP (Build 127)

[d] Non-synonymous coding variant (Gly73Ser)

[e] Synonymous coding variant (Lys452Lys)

the number of positive findings reported to date by chance. This factor plays especially heavily, as none of the reported support for association corrects for multiple testing and many are close to the nominal threshold for significance without such correction. Gosso et al. (2007) did not test the Comings SNP, but the SNPs which showed evidence of association in their sample also showed evidence of population stratification, as well as an inconsistent direction of the increaser allele across their samples (for rs2061174) and no correlation with *CHRM2* gene expression in the brain. Also regarding inconsistency in sub-sample effects, it is unclear whether Comings et al. (2003) adjusted for the

Table 2 *P*-values resulting from tests of population stratification and total association with *CHRM2* markers and verbal IQ (VIQ), performance IQ (PIQ) and full-scale IQ (FIQ) in an Australian family-based sample

SNP	Gene location	Population stratification			Total association		
		VIQ	PIQ	FIQ	VIQ	PIQ	FIQ
rs1424574	Upstream	0.43	0.34	0.35	0.74	0.62	0.84
rs6957496	Promoter	1.00	0.57	0.70	0.15	0.15	0.11
rs12533282	Intron 3	0.31	0.42	0.35	0.50	0.82	0.92
rs1424572	Intron 3	**0.037**	0.58	0.17	0.62	0.64	0.92
rs10488597	Intron 3	0.33	0.27	0.23	0.32	0.27	0.86
rs1364493	Intron 3	0.29	0.14	0.14	0.30	0.45	1.00
rs1424569	Intron 3	0.53	0.84	0.86	0.37	0.74	0.79
rs4475425	Intron 3	0.53	0.92	0.74	0.84	0.52	0.53
rs1424386	Intron 3	0.29	0.18	0.17	0.17	0.49	0.30
rs7810473	Intron 3	0.61	0.29	0.32	0.13	0.70	0.37
rs10488600	Intron 3	0.39	1.00	0.69	0.92	0.54	0.67
rs978437	Intron 3	0.55	0.86	0.66	0.72	0.69	0.89
rs1469179	Intron 3	0.72	0.43	0.75	0.81	0.65	0.81
rs7800170	Intron 3	0.63	0.29	0.65	0.55	0.81	1.00
rs1824024	Intron 4	0.66	0.81	0.69	0.32	0.71	0.84
rs324580	Intron 4	0.13	0.81	0.38	0.72	0.52	0.82
rs324594	Intron 4	0.42	0.19	0.24	0.52	0.47	0.86
rs2061174	Intron 4	0.86	0.24	0.40	0.68	0.69	0.89
rs7799047	Intron 4	0.45	0.19	0.23	0.54	0.60	0.92
rs2350786	Intron 5	0.79	0.19	0.51	0.92	0.30	0.45
rs420817	Intron 5	0.36	1.00	0.67	0.12	0.82	0.39
rs324640	Intron 5	0.34	1.00	0.68	0.17	0.74	0.42
rs324650	Intron 5	0.50	1.00	0.79	0.25	0.89	0.56
rs1424547	Intron 5	0.57	0.63	0.60	0.22	0.82	0.41
rs12721506	Exon 6	–	–	–	0.92	0.06	0.23
rs8191992	3′UTR	0.27	0.28	0.89	0.14	0.65	0.34
rs8191993	3′UTR	0.16	0.65	0.70	0.09	0.65	0.28
rs1378650	Downstream	0.40	**0.022**	0.32	0.16	0.65	0.35
rs1424548	Downstream	0.62	0.28	0.66	0.48	0.54	0.48

Note: All tests of association were performed in QTDT and include correction for age and sex, as described in "Materials and methods". *P*-values < 0.05 are indicated in bold

Table 3 *P*-values from the tests of total association modelling additive effects with *CHRM2* markers and general, verbal, and nonverbal cognitive ability in English and Scottish population samples

SNP	Gene location	English			Scottish		
		g	Verbal	Nonverbal	*g*	Verbal	Nonverbal
rs2350780	Intron 3	0.41	0.21	0.85	nd	nd	nd
rs2061174	Intron 4	0.23	0.13	0.55	nd	nd	nd
rs324640	Intron 5	0.90	0.53	0.76	nd	nd	nd
rs324650	Intron 5	0.63	0.29	0.98	nd	nd	nd
rs324651	Intron 5	0.82	0.94	0.52	nd	nd	nd
rs8191992	3′UTR	0.74	0.98	0.59	0.59	0.56	0.77
rs8191993	3′UTR	0.66	0.53	0.97	nd	nd	nd
rs6962027	3′UTR	0.44	0.46	0.57	nd	nd	nd

mean effect of sex in their combined analysis of mothers and fathers, since in either sex alone no association was detected for IQ although this may have been due to lower power in these sex specific analyses. In terms of a target phenotype, the traits reaching nominal significance vary both within and across study, for instance from performance IQ (Gosso et al. 2006) to verbal IQ (Gosso et al. 2007) for rs324650. If *CHRM2* was related to general ability, we would expect the strongest association with this general trait, and consistent, but smaller associations with specific abilities such as verbal and performance. As such, these findings warrant further investigation. Finally, though differences in the SNPs chosen often make comparisons difficult, across studies the particular SNPs reaching nominal significance show little if any consistency. Where a number of SNPs have been tested in a large sample, for instance the five SNPs typed in both our Australian and English cohorts, none were nominally significant nor were any trends observed between any of the other SNPs and cognitive ability in either of the cohorts.

Turning to the possible role of sample background phenotype, it is important to note that the original sample studied by Comings et al. (2003) consisted of parents of twins with substance abuse. Given the heritability of this trait, the parental sample will be enriched also for genetic risk for substance abuse. Similarly, the study by Dick et al. (2007) sampled families with alcoholic probands and in a later study using the same sample, Dick et al. (2008) reported stronger associations between *CHRM2* and a general externalizing factor (derived from lifetime symptom counts of alcohol dependence, illicit drug dependence, childhood conduct disorder and adult antisocial personality disorder plus novelty and sensation seeking traits) than those observed with the cognitive phenotypes. The report by Dick et al. (2008) suggesting that *CHRM2* predisposes for externalizing behavior, taken together with the present null finding in three un-selected samples, may suggest that previously association between *CHRM2* and cognition may be specific to populations with externalizing phenotype behaviors.

Our samples were unselected, and we found no significant support for the original SNP or for tag-SNPs in *CHRM2*. The only other reported study of *CHRM2* to use an unselected sample was that of Gosso et al. (2007), who did not test the putative SNP identified by Comings et al. (2003) which was later tested in the COGA sample. Drawing short of a conclusion that *CHRM2* is unrelated to normal cognition, then, it seem invaluable to examine the Comings' SNP in other populations selected for substance abuse and or alcoholism, or to measure and use alcohol intake as a covariate. It is possible that *CHRM2* plays a role in cognition under circumstances of substance abuse, perhaps interacting with alcohol consumption, with a protective effect shown in heavy users of alcohol, but no effect in non-users.

In summary, although CHRM2 does appear to be related to dementia in animal models, and initial genetic work suggested it was associated with human intelligence our findings in three independent cohorts differing in age from early adulthood thru middle age to early-old age must cast doubt on the involvement of *CHRM2* in intelligence, at least in the normal population.

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