

Association between the *CHRM2* gene and intelligence in a sample of 304 Dutch families

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The *CHRM2* gene is thought to be involved in neuronal excitability, synaptic plasticity and feedback regulation of acetylcholine release and has previously been implicated in higher cognitive processing. In a sample of 667 individuals from 304 families, we genotyped three single-nucleotide polymorphisms (SNPs) in the *CHRM2* gene on 7q31–35. From all individuals, standardized intelligence measures were available. Using a test of within-family association, which controls for the possible effects of population stratification, a highly significant association was found between the *CHRM2* gene and intelligence. The strongest association was between rs324650 and performance IQ (PIQ), where the T allele was associated with an increase of 4.6 PIQ points. In parallel with a large family-based association, we observed an attenuated – although still significant – population-based association, illustrating that population stratification may decrease our chances of detecting allele–trait associations. Such a mechanism has been predicted earlier, and this article is one of the first to empirically show that family-based association methods are not only needed to guard against false positives, but are also invaluable in guarding against false negatives.

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Individual performance across a single aspect of cognitive ability is highly predictive of performance on other aspects of cognitive ability. Indeed, about 40% of the population variance of measures of these individual cognitive processes can be accounted for by a single general intelligence factor (Plomin *et al.* 2004). Multivariate genetic analyses indicate

that this general intelligence factor is highly heritable (Boomsma & van Baal 1998; Cherny & Cardon 1994; Plomin *et al.* 1994; Posthuma *et al.* 2001) and that there is a substantial overlap in the genes influencing different aspects of cognitive ability. This implies that genes associated with one aspect of cognitive ability are likely to be associated with other aspects as well. As noted by Plomin *et al.* (2004), these quantitative genetic findings make general intelligence an excellent target for molecular genetic research.

In spite of its high heritability, reports on the actual genes influencing intelligence are scarce. Recently, Comings *et al.* (2003) reported an association between a variant of the cholinergic muscarinic receptor 2 (*CHRM2*) gene explaining 1% of the variance in scores on full-scale IQ (FSIQ) and years of education. We recently conducted an autosomal genome scan for intelligence using two independent, unselected samples consisting of 329 Australian families and 100 Dutch families, totalling 625 sib pairs (Posthuma *et al.* 2005). Although the most promising regions were 2q and 6p, we also found modest evidence for linkage with performance IQ (PIQ) at 7q31–36 right above the *CHRM2* gene.

The cholinergic muscarinic receptor family (M1–M5) belongs to the superfamily of G-protein-coupled receptors. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of acetylcholine (ACh) release and cognitive processes, including learning and memory (Hulme 1990; Volpicelli & Levey 2004; Wess 1996). On the basis of its putative role in cognitive ability, we genotyped three tagging single-nucleotide polymorphisms (SNPs) in the *CHRM2* gene in a sample of 667 Dutch individuals from 304 twin families. The current sample overlaps only marginally (17%) with the sample used in the linkage analysis. A family-based genetic association test was used, which allows evaluating evidence for association that is free from spurious effects of population stratification (Abecasis *et al.* 2000; Fulker *et al.* 1999; Posthuma *et al.* 2004).

Materials and methods

Subjects

All twins and their siblings were part of two larger cognitive studies and were recruited from the Netherlands Twin Registry, which ensures population-based sampling (Boomsma 1998). We have previously shown such a population to be representative of the total population with respect

to intelligence (Posthuma *et al.* 2000). Informed consent was obtained from the participants (adult cohort) or from their parents if they were under 18 years of age (young cohort). The current study was approved by the Institutional Review Board of the VU University Medical Center. None of the individuals tested suffered from severe physical or mental handicaps, as assessed through surveys sent out to participants or their parents every 2 years.

Young cohort

The young cohort consisted of 177 twin pairs, born between 1990 and 1992, and 55 siblings. The twins were 12 (mean = 12.42, SD = 0.16) years of age, and the siblings were between 8 and 15 years old at the time of testing. There were 41 monozygotic male (MZM) twin pairs, 28 dizygotic male (DZM) twin pairs, 56 monozygotic female (MZF) twin pairs, 25 dizygotic female (DZF) twin pairs, 27 dizygotic opposite-sex (DOS) twin pairs, 28 male siblings and 27 female siblings. Participation in this study included a voluntary agreement to provide buccal swabs for DNA extraction. Buccal swabs were obtained from 391 children.

Adult cohort

A total of 793 family members from 317 extended twin families participated in the adult cognition study (Posthuma *et al.* 2001). Participation in this study did not automatically include DNA collection; however, part of the sample (284 subjects) returned to the laboratory to provide blood for DNA extraction. Mean age was 37.3 years (SD = 12.5). There were 20 MZM twin pairs, 11 DZM twin pairs, 1 DZM triplet, 14 MZF twin pairs, 22 DZF twin pairs, 17 DOS twin pairs, 23 female siblings, 23 male siblings and 59 subjects from incomplete twin pairs (18 males and 41 females).

Cognitive testing

In the young cohort, cognitive ability was assessed with the Dutch adaptation of the Wechsler Intelligence Scale for Children-Revised (WISC-R) (Wechsler 1986) and consisted of four verbal subtests (similarities, vocabulary, arithmetic and digit span) and two performance subtests (block design and object assembly).

In the adult cohort, the Dutch adaptation of the Wechsler Adult Intelligence Scale III-Revised (WAISIII-R) (Wechsler 1997) assessed IQ and consisted of four verbal subtests (VIQ: information, similarities, vocabulary and arithmetic) and four performance subtests (PIQ: picture completion, block design, matrix reasoning and digit-symbol substitution). The correlation between VIQ and PIQ is usually around 0.5 (0.53 in our data), implying that only 25% of the variance in PIQ and VIQ is shared. Thus, a substantial part of the variance in these two measures is non-overlapping, and theoretically, they are expected to capture different aspects of cognitive ability. We therefore included VIQ and PIQ as measures of the two different aspects of intelligence as well as FSIQ as a general measure of intelligence.

In both cohorts, VIQ, PIQ and FSIQ were normally distributed (Table 1).

DNA collection and genotyping

The DNA isolation from buccal swabs was performed using a chloroform/isopropanol extraction (Meulenbelt *et al.* 1995). DNA was extracted from blood samples using the salting out protocol (Miller *et al.* 1988).

Zygoty was assessed using 11 polymorphic microsatellite markers (Het > 0.80). Eight subjects were not included in further association analyses due to genotypic incompatibilities on the marker alleles (i.e. more than four different marker alleles observed within one family).

SNPs were selected based on their minor allele frequency (MAF) and genotypic correlation (ρ) as obtained from a randomly selected Caucasian sample (http://www.celeradiagnostics.com/cdx/applera_genomics). MAF had to be >0.10 to avoid the rare heterozygous genotypes, and SNPs with a $\rho > 0.85$ compared with any of the other SNPs were not selected to avoid redundancy.

SNP genotyping was performed blind to familial status and phenotypic data.

Three tagging SNPs were selected, rs2061174, rs324640 and rs324650, using SNP Browser version 2.0.4 (http://www.applied_biosystems.com/support/software/snplex/) (NCBI build 34) (Fig. 1).

SNP genotyping was performed as part of an SNPLex assay. We here focus on the SNPs in the *CHRM2* only, as

Table 1: Means and standard deviations of IQ in the young and adult cohorts

| | Young cohort | | Adult cohort | |
|----------------|----------------|----------------|----------------|----------------|
| | Total sample | Genotyped | Total sample | Genotyped |
| <i>N</i> | 409 | 391 | 793 | 276 |
| Age (SD) | 12.37 (0.95) | 12.36 (0.90) | 37.60 (13.00) | 37.40 (12.42) |
| Mean PIQ (SD) | 101.40 (12.85) | 101.66 (12.96) | 100.96 (12.50) | 100.04 (12.40) |
| Mean VIQ (SD) | 98.42 (19.04) | 98.90 (19.02) | 92.78 (13.83) | 93.03 (14.36) |
| Mean FSIQ (SD) | 99.81 (15.20) | 100.21 (15.21) | 95.74 (11.62) | 95.59 (12.04) |

FSIQ, full-scale IQ; PIQ, performance IQ; VIQ, verbal IQ.

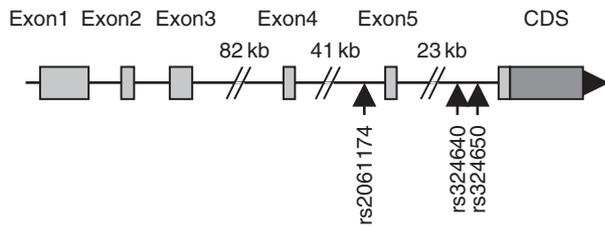


Figure 1: Location of single-nucleotide polymorphisms (SNPs) within the *CHRM2* gene on chromosome 7.

this gene was selected based on its putative role in cognition and its position under one of our linkage peaks. The SNPLex assay was conducted following the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Stock genomic DNA (gDNA) solutions (50 ng/ μ l) were diluted after fragmentation to a final concentration of 18.5 ng/ μ l. Diluted gDNA aliquots (2 μ l) were spotted and dried down in 384-well plates (Bioplastics, Landgraaf, the Netherlands). Previous to the oligo ligation assay (OLA) reaction, reagents were phosphorilated and diluted (1 : 1). The OLA reaction was performed in a total volume of 5 μ l, which contained 37 ng of gDNA, 0.5 μ l of ligation buffer, 0.025 μ l of 48-SNPLex ligase and 1 μ l of activated ligation probe pool. The PCR conditions were 3 min at 90 $^{\circ}$ C, 30 cycles of 15 seconds at 90 $^{\circ}$ C, 30 seconds at 60 $^{\circ}$ C and 30 seconds at 51 $^{\circ}$ C (2% ramp), followed by denaturation at 99 $^{\circ}$ C for 10 min. After this step, a purification step was conducted after which the OLA products were ready to be amplified in a final volume of 10 μ l. Exonuclease I 0.1 μ l and lambda exonuclease 0.2 μ l (Applied Biosystems) were added and incubated at 37 $^{\circ}$ C for 90 min followed by a deactivation step at 80 $^{\circ}$ C for 10 min. The purified OLA products were diluted (2 : 3) for further amplification. Amplification of OLA products was performed in 10 μ l, which contained 2 μ l of diluted OLA reaction, (\times 1) SNPLex amplification master mix and (\times 20) SNPLex amplification primers. The OLA amplification conditions were 95 $^{\circ}$ C for 10 min, followed by 95 $^{\circ}$ C for 15 seconds, 63 $^{\circ}$ C for 60 seconds for 30 cycles. After the hybridization step, analysis of the fluorescence intensity was performed in an aliquot (7.5 μ l) using ABI Sequencer 3730 (Applied Biosystems). All pre-PCR steps were performed on a cooled block. Reactions were carried out in Gene Amp 9700 Thermocycler (Applied Biosystems). Data were analyzed using GENEMAPPER v3.7 (Applied Biosystems).

Statistical analyses

Allele frequencies of the three selected SNPs were estimated in both young and adult cohorts using PEDSTATS (<http://www.sph.umich.edu/csg/abecasis/PedStats>) in which a Hardy-Weinberg test is implemented, based on an exact calculation of the probability of observing a certain number of heterozygotes conditional on the number of copies of the minor SNP allele.

We first determined the heritability of IQ scores in the young and adult cohorts using the standard strategy of comparing MZ and DZ twin resemblance. Specifically, we used the variance decomposition framework implemented in Mx (Posthuma *et al.* 2003).

Genetic association tests were conducted using the program QTD T which implements the orthogonal model proposed by Abecasis *et al.* (2000) (see also Fulker *et al.* 1999; Posthuma *et al.* 2004). This model allows to decompose the genotypic effect into orthogonal between-family (β_b) and within-family (β_w) components and also models the residual sib-correlation as a function of polygenic or environmental factors. MZ twins can be included and are modeled as such, by adding zygosity status to the datafile. They are not informative for the within-family component (unless they are paired with non-twin siblings) but are informative for the between-family component. The between-family association component is sensitive to population admixture, whereas the within-family component is significant only in the presence of linkage disequilibrium (LD) due to close linkage. Spurious associations may arise in a population that is a mix of two or more genetically distinct subpopulations. Any trait that is more frequent in one of the subpopulation(s) (e.g. because of assortative mating or cultural differences) will show a statistical association with any allele that has a different frequency across those subpopulation(s) (e.g. as a result of different ancestors or genetic drift). In practice, more than two subpopulations may have combined, and it will not be obvious from the combined populations whether the sample is stratified and in what way.

If population stratification acts to create a false association, the test for association using the within-family component is still valid. More importantly, if population stratification acts to hide a genuine association, the test for association using the within-family component has more power to detect this association than a population-based association test. To correct for multiple testing, the Monte-Carlo permutation framework as implemented in QTD T is used to calculate empirical *P*-values (Abecasis *et al.* 2000). Empirical *P*-values provide an indication of the empirical levels of type I errors, conditional on the observed, multivariate data.

Results

Comparison of MZ and DZ twin similarities for IQ measures showed that the observed variation in IQ could be attributed to additive genetic variance and unique environmental variance and not to shared environmental variance. Heritabilities of PIQ, VIQ and FSIQ were 0.73 (95% CI 0.63–0.80), 0.70 (95% CI 0.59–0.78) and 0.80 (95% CI 0.72–0.85), respectively, in the young cohort. Using the complete adult cohort, the respective heritabilities for PIQ, VIQ and FSIQ were 0.71 (95% CI 0.62–0.77), 0.78 (95% CI 0.72–0.83) and 0.78 (95% CI 0.72–0.83). These heritability estimates are comparable with those reported previously for these age cohorts in the Dutch population (Bartels *et al.* 2002; Posthuma *et al.* 2001).

In total, 667 subjects were available for SNP genotyping. On the basis of blind controls and MZ checks, no genotyping errors were found. For SNP rs2061174, 2.8% of the genotypes could not be called (648 genotypes succeeded), for SNP rs324640, 4.0% of the genotypes could not be called (640 genotypes succeeded) and for SNP rs324650, 5.7% of the genotypes could not be called (629 genotypes succeeded). SNP rs2061174 (A/G) in intron 4 had an MAF of 0.34. Two SNPs in intron 5, rs324640 (A/G) and rs324650 (A/T), had similar MAFs between 0.48 and 0.49. Observed haplotype frequencies were estimated using HAPLOVIEW 3.11 that implements the EM-algorithm (<http://www.broad.mit.edu/mpg/haploview/>). Only one twin from each MZ pair was included. LD was calculated from the estimated haplotype frequencies. The two SNPs in intron 5, lying 4 kb apart, are in very strong

LD ($r^2 > 0.90$). The SNP in intron 4 lies about 28 kb apart from the SNPs in the downstream region and shows lower LD with the two SNPs in intron 5 ($r^2 < 0.35$).

Genotypic means per cohort are summarized in Tables 2a and b. The three SNPs were in Hardy–Weinberg equilibrium in both cohorts as well as in the combined cohort. As the heritabilities were comparable across cohorts, as well as the allele frequencies and the directions of the genotypic effects, we pooled the two cohorts for the association tests.

The models used in QTDT included the effects of age and sex on the means and modeled additive allelic between- and within-family effects. When testing for the presence of population stratification (i.e. equivalence of the between- and within-family effects), we found significant evidence for the presence of population stratification in the association

Table 2a: Means (SD) per genotype for performance IQ (PIQ), verbal IQ (VIQ) and full-scale IQ (FSIQ) in the young cohort

| | Genotype | | | Total N |
|----------------|----------------|----------------|-----------------|---------|
| rs2061174 | GG | AA | AG | |
| Frequency | 0.43 | 0.45 | 0.12 | |
| Mean PIQ (SD) | 100.16 (13.75) | 102.20 (12.55) | 105.26 (11.57) | 381 |
| Mean VIQ (SD) | 99.81 (18.71) | 97.56 (19.75) | 101.00 (18.32) | 382 |
| Mean FSIQ (SD) | 99.64 (15.52) | 99.93 (15.40) | 103.62 (14.16) | 381 |
| rs324640 | AA | AG | GG | |
| Frequency | 0.28 | 0.50 | 0.22 | |
| Mean PIQ (SD) | 100.60 (14.02) | 101.81 (12.82) | 102.67 (12.27) | 381 |
| Mean VIQ (SD) | 98.78 (18.82) | 98.41 (18.82) | 100.38 (18.96) | 382 |
| Mean FSIQ (SD) | 99.18 (16.35) | 100.10 (14.67) | 101.88 (15.42) | 381 |
| rs324650 | AA | AT | TT | |
| Frequency | 0.29 | 0.50 | 0.21 | |
| Mean PIQ (SD) | 100.50 (14.15) | 101.76 (12.74) | 102.96 (12.051) | 379 |
| Mean VIQ (SD) | 97.67 (19.47) | 98.94 (18.93) | 100.05 (18.81) | 380 |
| Mean FSIQ (SD) | 98.56 (16.11) | 100.35 (14.83) | 101.89 (15.05) | 379 |

Table 2b: Means (SD) per genotype for performance IQ (PIQ), verbal IQ (VIQ) and full-scale IQ (FSIQ) in the adult cohort

| | Genotype | | | Total N |
|----------------|----------------|----------------|----------------|---------|
| rs2061174 | AA | AG | GG | |
| Frequency | 0.45 | 0.44 | 0.11 | |
| Mean PIQ (SD) | 98.59 (12.55) | 100.95 (12.79) | 102.76 (9.21) | 259 |
| Mean VIQ (SD) | 91.94 (14.08) | 94.15 (15.71) | 93.62 (9.69) | 260 |
| Mean FSIQ (SD) | 94.06 (12.08) | 97.27 (12.67) | 96.03 (8.12) | 256 |
| rs324640 | AA | AG | GG | |
| Frequency | 0.26 | 0.44 | 0.30 | |
| Mean PIQ (SD) | 98.17 (13.39) | 100.68 (12.64) | 101.09 (11.5) | 252 |
| Mean VIQ (SD) | 91.26 (16.770) | 93.28 (13.76) | 93.559 (13.33) | 251 |
| Mean FSIQ (SD) | 94.60 (12.23) | 95.71 (12.92) | 96.17 (10.78) | 248 |
| rs324650 | AA | AT | TT | |
| Frequency | 0.27 | 0.45 | 0.29 | |
| Mean PIQ (SD) | 98.84 13.07 | 100.41 (12.78) | 100.07 (12.51) | 238 |
| Mean VIQ (SD) | 92.33 16.13 | 93.22 (13.23) | 93.39 (13.70) | 239 |
| Mean FSIQ (SD) | 95.33 11.72 | 95.48 (12.48) | 95.94 (11.09) | 235 |

between rs324650 and both PIQ and FSIQ ($P < 0.05$), indicating the association effects across the total population are not equal to the association effects as found within families. As it seems obvious that population stratification is caused by pooling the two age cohorts, we also tested for population stratification in each cohort separately and found evidence for population stratification for the same SNPs, in the same direction (i.e. between-family effects smaller than within-family effects), in the young cohort (rs324650 with PIQ: rs324650 with FSIQ: $P < 0.05$), but not in the adult cohort. The within-family effects were comparable across both cohorts.

Using the within-family association, a significant association of PIQ with all three SNPs was found. The strongest effect was seen with the T allele of rs324650 ($P < 0.001$), which was associated with an increase of 4.6 IQ points among family members (Table 3). Put otherwise, the difference between AT and TT genotypes or AT and AA is 4.6, whereas the difference between AA and TT genotypes is estimated at 9.2.

Notably, the effect sizes of the increaser alleles were all reduced, although still significant, in the total association test as compared with the effect sizes based solely on the within-family association. As within-family associations are not sensitive to spurious associations due to population stratification, whereas between-family associations are, this means that stratification acted to hide a true association. Figure 2 shows the observed mean difference in IQ points between different genotypes for individuals within families, for associations significant at the 0.01 level.

Discussion

To investigate the possible role of the *CHRM2* gene in intelligence, we employed a family-based genetic association test. Significant evidence was found for an association between the *CHRM2* gene and IQ, showing an effect size of 4.6 IQ points for the increaser allele of SNP rs324650 ($P < 0.001$).

We also found that the effect sizes based on the within-family effects were 1.5-2.5 times as large as the population-based effect sizes, suggesting that population stratification resulted in an underestimation of the genuine allelic effect.

The attenuation of allelic effects due to population stratification occurs when across subpopulations higher trait values tend to go together with a lower frequency of the increaser allele, or vice versa. This is consistent with findings from mouse model systems in which it has been shown that the same allele at the same locus may cause a major disease in one mouse strain, but no disease phenotype in a strain with a different genetic background (e.g.Linder 2001; Liu *et al.* 2001; Montagutelli 2000). The same has been reported for effects on gene expression in different environmental backgrounds (Cabib *et al.* 2000; Crabbe *et al.* 1999). In humans, the presence of different genetic (or environmental) backgrounds that derive from mixed strata may differentially affect the expression of gene variants (G × E interaction). As non-Mendelian traits are likely to be influenced by multiple (risk-) factors which in turn are likely to interact with each other, neglecting the presence of population stratification may realistically hide genuine allele-trait associations,

Table 3: Tests for genetic association between the *CHRM2* gene and intelligence

| | Within-family association | | | Population-based association | | |
|-----------|---------------------------|---------------------------------------|--|------------------------------|---------------------------------------|--|
| | <i>N</i> | χ^2 (nominal <i>P</i> -value) | Genotypic effect (increaser allele) | <i>N</i> | χ^2 (nominal <i>P</i> -value) | Genotypic effect (increaser allele) |
| rs2061174 | | | | | | |
| PIQ | 175 | 7.3 (<0.01) | 3.7 (G) | 648 | 9.0 (<0.01) | 2.4 (G) |
| VIQ | 175 | 0.4 | 1.2 (G) | 648 | 0.0 | 0.2 (G) |
| FSIQ | 174 | 2.4 | 2.3 (G) | 644 | 2.3 | 1.4 (G) |
| rs324640 | | | | | | |
| PIQ | 209 | 7.7 (<0.01)* | 3.7 (G) | 640 | 5.2 (<0.05)* | 1.8 (G) |
| VIQ | 209 | 1.9 | 2.5 (G) | 640 | 1.2 | 1.1 (G) |
| FSIQ | 207 | 4.6 (<0.01) | 3.1 (G) | 636 | 2.9 | 1.4 (G) |
| rs324650 | | | | | | |
| PIQ | 193 | 12.1 (<0.001)* | 4.6 (T) | 629 | 6.0 (<0.05)* | 1.9 (T) |
| VIQ | 193 | 3.9 (<0.05) | 3.6 (T) | 629 | 1.8 | 1.4 (T) |
| FSIQ | 191 | 8.0 (<0.01)* | 4.1 (T) | 625 | 4.0 (<0.05)* | 1.7 (T) |

FSIQ, full-scale IQ; PIQ, performance IQ; VIQ, verbal IQ.

Note: *N* denotes the number of individuals. For the within-family association test, it denotes the number of individuals informative for the within-family association, i.e. those individuals who occur in families with more than one genotype. The *N* for the between-family association differs slightly from the added totals of Tables 2a and 2b as QTDT assumes equal genotypes for monozygotic twins and includes non-typed MZ cotwins with IQ scores.

*Statistically significant based on 1000 Monte-Carlo permutations.

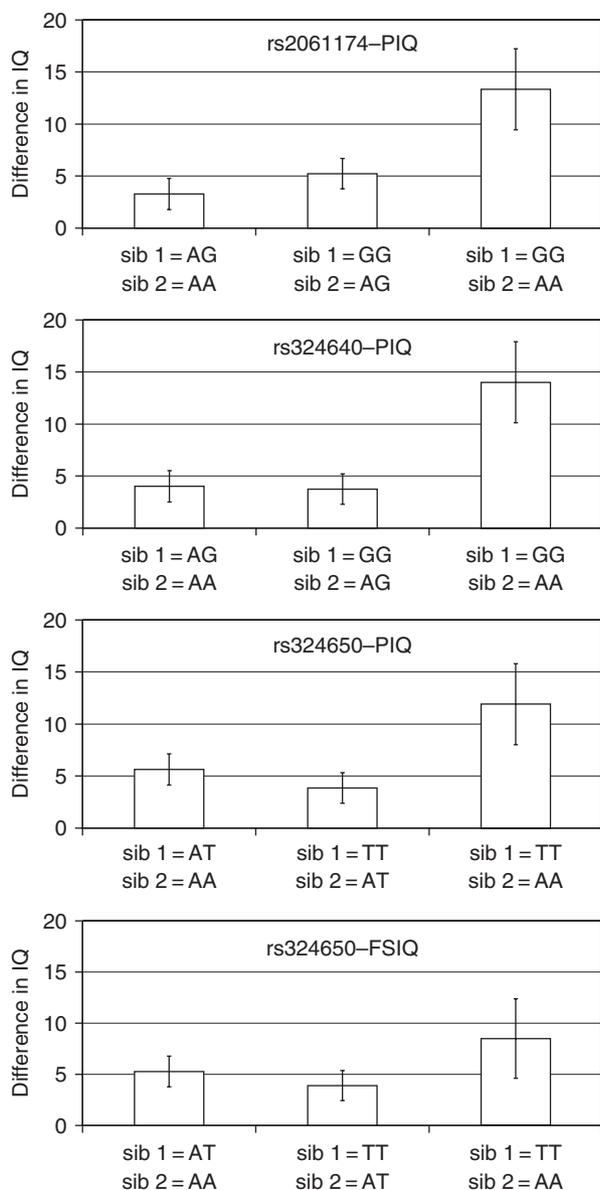


Figure 2: Observed mean difference in IQ scores between siblings (i.e. within family pairs) with different genotypes for those single-nucleotide polymorphisms (SNPs) in the CHRM2 gene that show a significant association. Sibling pairs include dizygotic pairs and non-twin sibling pairs. The number of pairs on which the difference scores are based is: rs2061174: 97 (AG-AA), 64 (GG-AG) and 15 (GG-AA); rs324640: 87 (AG-AA), 97 (GG-AG) and 9 (GG-AA); rs324650: 75 (AG-AA), 98 (GG-AG) and 11 (GG-AA). FSIQ, full-scale IQ; PIQ, performance IQ.

and may be responsible in part for the difficulties in replicating reported associations. We previously predicted this phenomenon based on theory and simulations (Posthuma *et al.* 2004) and now show it to occur in practice as well.

The most significant association was seen with rs324650 where the presence of the T allele was associated with an

increase of 4.6 points for PIQ. This extends earlier findings of Comings *et al.* (2003) who found a weaker association between the *CHRM2* gene and both total IQ score and years of education, which was only significant after stratifying on the parental origin of transmission. The current study therefore looked at different SNPs than the SNP that was used by Comings *et al.* (2003). Their SNP was in the 3' UTR region and is not classified as a tagging SNP. The SNPs used in the current sample are tagging SNPs, two of which (rs324640 and rs324650) are in LD with the SNP used in the study by Comings *et al.* (2003). These two SNPs are also the ones that show a significant association with IQ in the current study, albeit stronger than in the study by Comings *et al.* (2003). This study thus provides further evidence of a role of the cholinergic muscarinic receptor in cognition.

The M2 subtype cholinergic muscarinic receptor is, like the M1 and M4 subtypes, predominantly expressed in the CNS (Volpicelli & Levey 2004). The M2 receptors are predominantly located at the presynaptic level (Levey *et al.* 1991; Mrzljak *et al.* 1993), spread throughout the brain but with the highest levels in the cerebral cortical, forebrain cholinergic nuclei, cervical spinal cord region, cerebellum and thalamus (Flynn & Mash 1993; Piggott *et al.* 2002; Spencer *et al.* 1986; Wei *et al.* 1994). M2 receptors are selectively coupled to G-proteins of the Gi/Go family, which mediate the inhibition of voltage-sensitive Ca²⁺ channels. Furthermore, the M2 receptor subtype is likely to have an additional role in cholinergic modulation of excitatory and inhibitory hippocampal circuits acting as autoreceptor (Akam *et al.* 2001; Kitaichi *et al.* 1999a, 1999b; Rouse *et al.* 2000; Shapiro *et al.* 1999; Zhang *et al.* 2002), inhibiting ACh release from cholinergic terminals.

It is well known that exposure to a novel environment causes pronounced ACh release at the level of the neocortex and hippocampus and that these high levels of ACh are necessary for memory formation (Miranda *et al.* 2000; Pepeu & Giovannini 2004; Ramirez-Lugo *et al.* 2003). Many studies in animals confirm the importance of cholinergic activity for the acquisition and retrieval of several learning tasks (Orsetti *et al.* 1996; Vannucchi *et al.* 1997). Importantly, M2-knockout animal models and studies using selective receptor antagonist agents have shown enhancement in performance in several tasks with cognitive components (Carey *et al.* 2001; Quirion *et al.* 1995; Seeger *et al.* 2004). Finally, higher M2 distribution volumes have been found in post-mortem and *in vivo* studies in Alzheimer's disease (AD) patients compared with healthy controls (Cohen *et al.* 2003).

The association found in the present study as well as by Comings and coworkers was all with SNPs in non-coding regions of the gene. We found association with SNPs located in intron 4 (rs2061174) and intron 5 (rs324640 and rs324650) of the *CHRM2* gene. Comings *et al.* (2003) found an association between the *CHRM2* gene and IQ with an SNP in the 3' UTR of the gene. The transcription of the *CHRM2* gene is complex. Krejci *et al.* (2004) determined that the 5' UTR of *CHRM2* consists of four non-coding regions

whose different combinations give rise to eight splice variants. In addition, expression is regulated by two promoters. One promoter regulates expression at the cardiac cell level, whereas the second promoter could be considered neuron specific. Experiments using reporter genes demonstrated that additional regulatory sequences are present further upstream of the proximal promoter(s) and, even more interesting, within the intronic regions (Krejci *et al.* 2004).

On the basis of the findings from animal and functional studies (Carey *et al.* 2001; Krejci *et al.* 2004; Miranda *et al.* 2000; Pepeu & Giovannini 2004; Quirion *et al.* 1995; Ramirez-Lugo *et al.* 2003; Seeger *et al.* 2004), we hypothesize that a non-coding polymorphism might be involved in the regulation of expression or alternative splicing of the *CHRM2* gene. This polymorphism may subsequently affect mAChR2 transcription, as well as the fine-tuning negative feedback of this particular receptor, making it less reactive to ACh increases during cognitive processing. Another possibility is that SNP rs324650 is in strong LD with the causative regulatory variant in the *CHRM2* gene.

Identifying genes for variation in the range of normal intelligence could provide important clues to the genetic etiology of disturbed cognition in, for example, autism, reading disorder and attention deficit and hyperactivity disorder (ADHD). It is worth to mention the first genome-wide linkage screen in autism, performed by the International Molecular Genetic Study of Autism Consortium, involving sib pairs from the United Kingdom. Interestingly, the strongest linkage signal for autism occurred at 7q near the *CHRM2* gene (for a review on linkage scans for autism, see Wassink *et al.* 2004). With regard to attention problems, it is of note that a number of recent findings clearly implicate deviant ACh neurotransmission in attentional processing (Beane & Marrocco 2004; Yu & Dayan 2005). Thus far, candidate gene approaches for attention disorder have focused only on genetic variation in nicotinic receptors (Sacco *et al.* 2004; Todd *et al.* 2003). The results in the current study tentatively suggest that muscarinic signaling may be involved as well.

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