COMT genotype is associated with differential expression of muscarinic M1 receptors in human cortex.

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Abbreviated Title: COMT and cortical muscarinic M1 receptors

Abstract

Catechol-O-methyltransferase (*COMT*) genotype has been associated with varying levels of cognitive functioning and an altered risk of schizophrenia. COMT regulates the breakdown of catecholamines, particularly dopamine, which is thought critical in maintaining cognitive function and the aetiology of schizophrenia. This hypothesis gained support from reports that the VAL allele at rs4680 was associated with poorer performance on cognitive tests and a slightly increased risk of schizophrenia. More recently, genotype at rs4818, part of a hapblock with rs4680, has been shown to impact on cognitive ability more than genotype at rs4680 but, as yet, not the risk for schizophrenia. Here we determined if *COMT* genotype at rs4680 or rs4818, as well as rs165519 and rs737865, two synonymous single nucleotide polymorphisms (SNPs) with no known functional consequences, were associated with an altered risk of schizophrenia and if genotype at the four *COMT* SNPs was related to expression of the cortical muscarinic M1 receptor (CHRM1) because

the expression of the cortical CHRM1 has been reported to be lower in schizophrenia and is important in maintaining cognitive functioning in humans. We report that the variation in gene sequence at the four *COMT* SNPs studied was not associated with an altered the risk of schizophrenia but genotype at rs4680 and rs4818, but not rs165519 and rs737865, were associated with varying levels of cortical *CHRM1* expression in the human dorsolateral prefrontal cortex (DLPFC). These data are the first to suggest that levels of CHRM1 in the human DLPFC are, in part, determined by *COMT* gene sequence.

Keywords: schizophrenia, postmortem CNS, acetylcholine, cognition

Introduction

Cognitive deficits are a significant problem associated with a number of disorders of the human CNS and are now recognised as one of the most debilitating symptom clusters associated with schizophrenia [Vingerhoets et al., 2013]. Hence, there has been a strong interest in the potential role for catechol-O-methyltransferase (COMT) in the aetiology of schizophrenia as genetic variation in the gene for this enzyme have been associated with changes in cognitive ability [Tunbridge et al., 2006] and an altered risk for schizophrenia [Chen et al., 2004b; Egan et al., 2001; Shifman et al., 2002; Wonodi et al., 2003]. Importantly, the altered risk of schizophrenia appeared to be linked to SNP rs4680 (VAL$^{108/158}$MET); variation in gene sequence at that SNP causes a valine / methionine substitution which changes the stability of the enzyme, which in turn leads to variation in enzyme activity [Chen et al., 2004a]. COMT plays an important role in the regulating the breakdown of catecholamines [Tunbridge et al., 2006] however COMT genotype has been associated with parameters in the human CNS that go beyond a simple involvement with catecholamine metabolism. Of particular interest to the aetiology of schizophrenia was the demonstration that the *COMT* VAL allele was associated with an increased expression of tyrosine hydroxylase [Akil et al., 2003]. Tyrosine hydroxylase is a critical enzyme required for dopamine synthesis and so it was

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suggested that one mechanism by which *COMT* genotype could be associated with altered cognitive ability could be because of altered levels of dopamine in human CNS [Akil et al., 2003]. This posit is supported by increasing evidence to show cortical dopamine is important in maintaining cognitive function [Cools, 2015].

As might be expected, a number of neurotransmitter systems are involved in maintaining cognitive function in humans, with the cholinergic system being recognised as being of particular significant in the maintenance of cognitive ability [Blokland, 1995]. Moreover, there is mounting evidence that CHRM1, one of a family of five G-protein coupled receptors [Wess, 2003] activated by acetylcholine, has a major role in maintaining levels of cognition in humans [Melancon et al., 2013]. Recently, the use of CHRM1 specific allosteric modulators has shown that increasing the activity of the receptor improves cognitive function in primates [Uslaner et al., 2013] and humans [Nathan et al., 2013]. The role of the CHRM1 in the aetiology of schizophrenia has become pertinent following the finding that it, but not the CHRMs 2,3 or 4 [Dean et al., 2002; Scarr et al., 2006], is decreased in the DLPFC from people with the disorder [Dean et al., 2002; Mancama et al., 2003; Scarr et al., 2013]. Thus, given the importance of *COMT* and the *CHRM1* in cognition, we have decided to determine whether *COMT* genotype was associated with a change in risk for schizophrenia and/or levels of *CHRM1* in human DLPFC.

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Materials and Methods

To address determine if *COMT* genotype was associated with altered risk of schizophrenia we determined genotype at four SNPs in the *COMT* gene using DNA isolated from the cerebellum of 75 subjects with schizophrenia and 73 controls (Table 1). All human CNS tissue for the study was sourced through the Victorian Brain Bank Network at the Florey Institute for Neuroscience and Mental Health, Parkville, Australia and was collected with consent from the Ethics Committee of the Victorian Institute for Forensic Pathology.

Tissue was initially collected from subjects with a potential history of a psychiatric disorder and subjects with no apparent history of psychiatric disorders who were matched for age and sex. A post-mortem assessment was completed by reviewing histories, discussions with relatives and treating clinicians which, for non-psychiatric controls, was to exclude any history of significant psychiatric symptoms. For each subject with history of a psychiatric disorder the relevant clinical and neuropsychopharmacological data were recorded. This allowed for their duration of illness (DI), time from first contact with a clinical service to death, to be calculated. A diagnosis was then agreed by consensus between two senior psychiatrists and the person completing the assessment according to DSM IV criteria using the Diagnostic Instrument for Brain Studies[1,2]. For each subject, genetic racial markers were obtained and this showed that greater than 90% of cases were of European lineage with the balance being Han Chinese.

CNS tissue was only collected from people who had been seen alive up to 5 hr prior to being found dead and where cadavers had been refrigerated within 5 hours to ensure slowing of any autolysis of the CNS [3]. Hence, the post-mortem interval (PMI) was calculated as the time between witnessed death and autopsy or the midpoint between the subject being found and being last seen alive and autopsy. To further ensure optimum CNS preservation each hemisphere was processed in a standardised manner to ensure the tissue was frozen to -70°C within 30 min of autopsy[4]. For each case CNS pH was measured as an indicator of the quality of tissue preservation[5] as this measure is recognised as giving a good indication as to the biochemical integrity of tissue, which is often not related simply to PMI[6].

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To quantify levels of CHRM1 mRNA, 6 x 10 mm sections were cut from the BA 9 of each subject, fixed in 4% paraformaldehyde, placed in fresh 0.25% acetic anhydride in 0.1 M triethanolamine HCl (pH 8.0), dehydrated, delipidated and equilibrated in 95% ethanol before being dried. To increase specificity, the oligonucleotide probe contained a mix of three antisense sequences, the

probes were complementary to bases 4–51, 721–768 and 811–853 of human CHRM1 mRNA (accession no.: X52068). The oligonucleotides were labelled with [35S]ATP using the NEN Oligonucleotide 30 end labelling system and purified using a NENSORB 20 purification cartridge (Perkin Elmer, Glen Waverly, VIC, Australia). For hybridisation, the probe was prepared at $1x10^6$ dpm of [35S]ATP per 50ml of hybridisation buffer (formamide (50%), NaCl (600mM), Tris-HCl (80mM), ethylenediamine tetraacetate (4mM), sodium pyrophosphate (0.1%), SDS (0.2%), sodium heparin (2mgml_1), dextran sulphate (10%) and dithiothreitol (100mM)) and incubated with tissue sections for 20 h at 37 1C, in a humidified chamber. Two sections were incubated with the radioactive oligonucleotide probe (total binding) while two sections were incubated with radioactive probes in the presence of excess non-radioactive probes (non-specific binding). Hybridisation to mRNA was confirmed by incubating two sections, incubated in 0.02% RNaseA, with the radioactive probe (negative control). After hybridisation, all sections were washed and dried before being apposed to BAS-SR2025 imaging plates (Fujifilm, Tokyo, Japan), with [14C]microscales (GE Healthcare, Rydalmere, NSW, Australia) for 4 weeks before being scanned in the BAS 5000 high resolution phosphorimager (Fujifilm). The signal intensity of the phosphoimages were measured against those on the [14C]microscales using AIS image analysis software. Results were expressed as TB minus NSB in dpm $x10^3$ per mg estimated wet weight tissue equivalents.

To determine *COMT* genotype, 25mg of cerebellar tissue was homogenised in 80µl phosphate buffered saline (PBS) and DNA extracted using the QIAamp DNA Mini kit (QIAGEN) according to the manufacturer's instructions. The quality and concentration of the DNA was assessed using a Nanodrop (Thermo Fisher Scientific, Massachusetts). Samples were genotyped for four *COMT* SNPs and 60 unlinked ancestry informative markers (AIMs) representing three HapMap phase III populations (Northern/Western European, Han Chinese, and Yoruba in Nigeria) with the Sequenom MassARRAY MALDI-TOF genotyping system using Sequenom iPLEX Gold chemistries

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according to manufacturer's instructions. Primer sequences are given in Supplementary Table 1. Genotype analysis was performed in a semi-automated manner using the Typer 4.0 Analyser Software (Sequenom Inc., San Diego, CA). Any ambiguous genotype calls resulted in the sample being excluded and thus due to bad reads genotypes at the four SNPs were not available for all cases.

Results

We did not find any evidence to suggest that genotype at each SNP conveyed a changed risk for schizophrenia (Table 2).

As we have reported previously [Scarr et al., 2013], three distinct layers of *CHRM1* probe hybridisation intensity could be detected in human DLPFC and therefore the intensity of all layers were measured. In comparing *COMT* genotype to cortical *CHRM1* mRNA levels we showed there was significant variance in levels of oligonucleotide probe hybridisation with genotype for rs4818 (layer 1: $F_{2,127} = 3.27$, $p = 0.04$; layer 2: $F_{2,127} = 2.96$, $p = 0.05$; layer 3: $F_{2,127} = 3.38$, $p = 0.03$) and rs4680 in hybridisation layer 1 (layer 1: $F_{2,139} = 3.28$, $p = 0.04$)(Figure 1). In addition, variation in levels of probe hybridisation to *CHRM1* mRNA was close to significance in layer 2 ($F_{2,\ 139} = 2.83$, $p = 0.06$) and layer 3 ($F_{2,\ 139} = 2.24$, $p = 0.06$) but there was no significant evidence of variation in oligonucleotide probe hybridisation and *COMT* genotype for rs737865 (layer 1: $F_{2,141} = 0.18$, $p = 0.80$; layer 2: $F_{2,\ 141} = 0.36$, $p = 0.70$; layer 3: $F_{2,\ 141} = 0.11$, $p = 0.90$) or rs165599 (layer 1: $F_{2,138} = 0.64$, $p = 0.52$; layer 2: $F_{2,\ 138} = 0.75$, $p = 0.47$; layer 3: $F_{2,\ 138} = 0.92$, $p = 0.40$).

The variance in hybridisation with rs4818 genotype in layer 1, 2 and 3 was due to lower levels of hybridisation in subjects homozygous for C vs. those who were homozygous G (layer 1: $p = 0.02$, layer 2: $p = 0.02$ and layer 3: $p = 0.02$; Figure 1A).

The variance in hybridisation with rs4860 genotype in layer 1 was due to lower levels of hybridisation in subjects homozygous for C vs. those who were homozygous G ($p = 0.04$; Figure 1B) and a tendency to lower levels of hybridisation in subjects homozygous for C vs. those who were homozygous G in layers 2 and 3 (layer 2: $p = 0.05$; layer 3: $p = 0.06$).

Discussion

In this study we did not find any altered risk for schizophrenia associated with *COMT* genotype. We acknowledge that for a genetic studies, the number of cases we have studied is low, however our data agrees with a significant number of other studies (for review see [Okochi et al., 2009]) and GWAS studies [Moskvina et al., 2009; Schizophrenia Working Group of the Psychiatric Genomics et al., 2014] that do not show an association between *COMT* genotype and an altered risk for schizophrenia.

Our major finding is that *COMT* genotype at rs4818 and rs4860, but not rs165599 and rs737863, is associated with levels of *CHRM1* mRNA in the human DFPLC. This finding is of particular interest because the CHRM1 and DFPLC are important in regulating cognition and *COMT* genotype at rs4818 and rs4860, but not rs165599 and rs737863, is associated cognitive functioning in humans [Roussos et al., 2008].

Our data shows that subjects homozygous for C vs. those who were homozygous G at rs4818 genotype in layer 1, 2 and 3 have lower levels CHRM1 in their DLPFC. Notably, the C allele at rs4818 has been shown to be associated with improved performance in the Stockings of Cambridge task but poor outcomes in the Iowa Gambling Task [Roussos et al., 2008], despite both tasks targeting the prefrontal cortex. It has been argued that the DLPFC plays an important role in category fluency and planning tasks which would impact on performance on the Stockings of Cambridge task [Meier et al., 2010] whilst deficits in working memory and forward planning

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impact decision making in the Iowa Gambling Task [Bechara et al., 2005]. Our demonstration of an association between *COMT* genotype and *CHRM1* expression opens the potential for a role for the CHRM1 in the mechanisms linking *COMT* genotype to functional outcomes mediated by the DLPFC. The mechanism by which sequence variation at rs4818, a synonymous SNP, may be linked to *CHRM1* expression is unclear but it has been suggested that variance at rs4818, and other *COMT* SNPs, may act to alter *COMT* RNA structure, translation to protein [Nackley et al., 2006] and activity. Thus, a change in COMT activity cannot be excluded as being involved in the link between genotype and *CHRM1* expression.

Subjects homozygous for C vs. those who were homozygous G at rs4860 had lower levels of *CHRM1* mRNA in the outer laminae of the DFPLC and a tendency to lower levels mRNA in the middle and inner laminae of that CNS region. Significantly, the lowest levels of cortical *CHRM1* mRNA is associated with the homozygous MET variant which is linked to lower levels of *COMT* expression and activity [Chen et al., 2004a]. Hence, as with sequence variation at rs4818, it may be that COMT activity is involved in the mechanism linking genotype to *CHRM1* expression. However, it is interesting that the same *COMT* genotype has been associated with lower levels of tyrosine hydroxylase [Akil et al., 2003] and therefore possibly lower dopamine levels. By contrast, *Chrm1* knockout mice are hyperdominergic [Gerber et al., 2001] and blockade of the CHRM1 is associated with an increase in cortical dopamine release [Li et al., 2005]. Hence, lower CHRM1 expression in individuals who are homozygous C at rs4860 could be an effort to compensate for a relatively hypodopaminergic state due to decreased levels of tyrosine hydroxylase.

In conclusion, we report an association between *COMT* genotype and levels of cortical CHRM1 in human CNS. As the two genotypes we have shown to have this association are linked with cognitive deficits in humans [Roussos et al., 2008] our data suggests that one mechanism linking *COMT* genotype to cognition could be varying levels of cortical CHRM1. As cortical CHRM1

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allosteric modulators are suggested as a treatment for improving cognitive deficits [Melancon et al., 2013], our data argues determining whether there is an association between *COMT* genotype and responsiveness to such drugs would be worthwhile because the association between *COMT* genotype and *CHRM1* expression in human cortex may make it a useful pharmacogenomic marker to correlate with drug responsiveness.

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Figure Legend: Levels (mean ± SEM) of [^{32}S]CHRM1 oligonucleotide probes to human DLPFC with *COMT* genotype at four single nucleotide polymorphisms.

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Table 1: Demographic, CNS collection and antipsychotic drug treatment data on subjects with schizophrenia and age sex matched controls from whom tissue was obtained for this study. Numeric data is shown as mean ± SEM.

SNP	Diagnoses	Reads	M / F	Age	pH	PMI	DI	SUI	FRADD	LEAD
		(n)		(yr.)		(hr.)	(yr.)	(yes / no)		
Rs4680	Schizophrenia	74	52 / 22	45 ± 2.0	6.24 ± 0.03	42 ± 1.4	18 ± 1.6	34 / 40	711 ± 91	10.6 ± 1.8
	Controls	71	56 / 15	46 ± 1.9	6.31 ± 0.02	40 ± 1.6		0 / 71		
	p		0.25	0.85	0.06	0.59				
rs4818	Schizophrenia	69	56 / 13	45 ± 2.1	6.25 ± 0.03	42 ± 1.6	18 ± 1.8	31 / 38	758 ± 105	11.0 ± 1.9
	Controls	64	45 / 19	45 ± 1.9	6.32 ± 0.02	40 ± 1.7		0 / 64		
	p		0.16	0.85	0.10	0.58				
rs165599	Schizophrenia	75	58 / 15	44 ± 2.0	6.23 ± 0.03	41 ± 1.5	18 ± 1.6	35 / 40	713 ± 97	9.2 ± 1.6
	Controls	71	50 / 21	45 ± 1.9	6.31 ± 0.02	41 ± 1.6		0 / 71		
	p		0.25	0.75	0.04	0.74				
rs737865	Schizophrenia	71	56 / 15	45 ± 1.9	6.24 ± 0.03	41 ± 1.4	18 ± 1.6	31 / 40	718 ± 92	10.0 ± 1.6
	Controls	73	53 / 22	46 ± 1.9	6.31 ± 0.02	40 ± 1.6		0 / 73		
	p		0.30	0.75	0.04	0.74				

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Abbreviations: DI = duration of illness, F = female, FRADD = Final Recorded Antipsychotic Drug Dose expressed as chlorpromazine equivalents per day, LEAD = Lifetime Exposure to Antipsychotic Drugs expressed as chlorpromazine equivalents per year x 10^{-3}, M = male, PMI = postmortem interval, Reads = successful nucleotide read, SUI = suicide.

Note: Analyses (One way ANOVA, post hoc Tukey's multiple comparisons) of data specific only to subjects with schizophrenia showed that DI (F = 1.00; d.f. = 3,277; p = 0.39), FRADD (F = 0.05; d.f. = 3,192; p = 0.98) and LEAP (F = 0.15; d.f. = 3,277; p = 0.89) did not vary significantly between the group used for each of the four *COMT* SNPs studied.

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Table 2: The frequency (%) of *COMT* genotypes at 4 SNPs as measured using DNA from cerebellum from subjects with schizophrenia and healthy controls.

Diagnoses	rs4818					rs4860					rs165599					rs737865				
	CC	CG	GG	χ^2, df	p	AA	AG	GG	χ^2, df	p	GG	GT	TT	χ^2, df	p	CC	CT	TT	χ^2, df	p
Schizophrenia	34	44	22	1.57,2	0.45	27	47	26	0.40,2	0.40	25	42	33	0.70,2	0.70	5	31	47	3.20,2	0.20
Controls	45	38	17			23	48	29			20	48	32			6	33	61		

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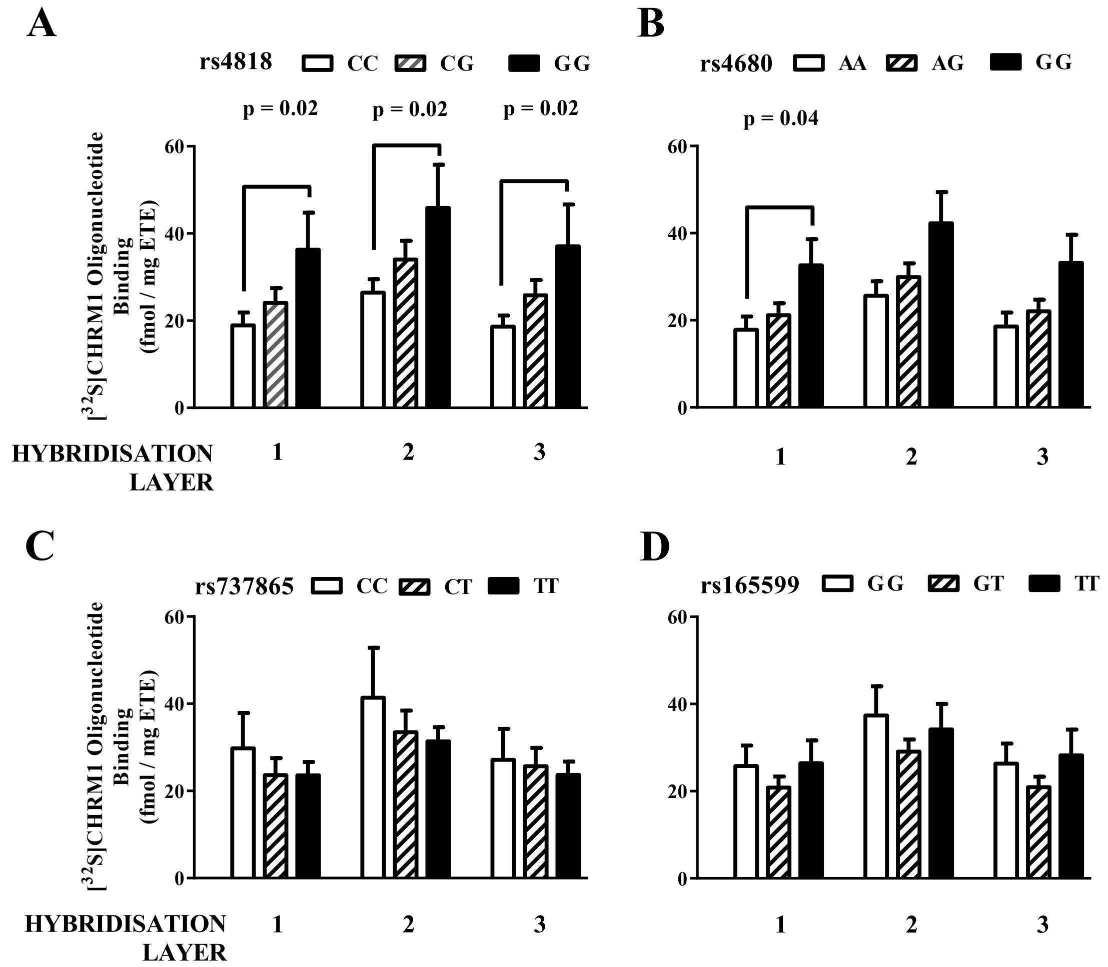

Figure 1 .

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