HHS Public Access

Author manuscript

Mol Psychiatry. Author manuscript; available in PMC 2011 October 01.

Published in final edited form as:

Mol Psychiatry. 2011 April ; 16(4): 407–418. doi:10.1038/mp.2010.24.

Genetic Variation in Cholinergic-Muscarinic-2 Receptor Gene Modulates Muscarinic$_2$-Receptor Binding *In Vivo* and Accounts for Reduced Binding in Bipolar Disorder

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Abstract

Genetic variation in the cholinergic-muscarinic2 (M_2)receptor gene (*CHRM2*) has been associated with the risk for developing depression. We previously reported that M_2-receptor distribution volume (V_T) was reduced in depressed subjects with bipolar disorder (BD) relative to depressed subjects with major depressive disorder (MDD) and healthy controls (1). In the current study we investigated the effects of six single nucleotide polymorphisms (SNP) for *CHRM2* on M_2-receptor binding to test the hypotheses that genetic variation in *CHRM2* influences M_2-receptor binding and that a *CHRM2* polymorphism underlies the deficits in M_2-receptor V_T observed in BD. The M_2-receptor V_T was measured using PET and [^{18}F]FP-TZTP in unmedicated, depressed subjects with BD (n=16) or MDD (n=24) and healthy controls (n=25), and the effect of genotype on V_T was assessed. In the controls one SNP (with identifier rs324650, in which the ancestral allele adenine (A) is replaced with one or two copies of thymine (T), showed a significant allelic effect on V_T in the pregenual and subgenual anterior cingulate cortices in the direction AA<AT<TT. In contrast, in BD subjects with the TT-genotype V_T was significantly lower than in BD subjects with the AT-genotype in these regions. The BD subjects homozygous for the T-allele also showed markedly lower V_T (by 27 to 37% across regions) than healthy controls of the same genotype. *Post hoc* analyses suggested that T homozygosity was associated with a more severe illness course, as manifested by lower socioeconomic function, poorer spatial recognition memory and a greater likelihood of having attempted suicide. These data represent novel preliminary evidence that reduced M_2-receptor V_T in BD is associated with genetic variation within *CHRM2*. The differential impact of the M_2-receptor polymorphism at rs324650 in the BD and HC samples suggests interactive effects with an unidentified vulnerability-factor for BD.

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Disclosure Statement: No author has potential conflicts of interest related to this work.

Keywords

Depression; Anxiety; Muscarinic M2 binding; *CHRM2*; G-protein coupled receptor; [^{18}F]FP-TZTP; Positron Emission Tomography

Introduction

A variety of indirect evidence has implicated the central muscarinic-cholinergic system, and more specifically the type-2 muscarinic (M_2) receptor, in the pathophysiology of depressive symptoms arising in major depressive disorder (MDD) and bipolar disorder (BD) [reviewed in (1)]. We previously used positron emission tomography (PET) and [^{18}F]FP-TZTP (3-(3-((3-fluoropropyl)thio)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine) to investigate muscarinic cholinergic receptor binding in MDD and BD, and found that this radioligand's distribution volume (V_T) was reduced in the cingulate cortex in BD subjects relative to both healthy controls and MDD subjects (1). In the cingulate cortex (and in most brain regions) [^{18}F]FP-TZTP binding is relatively selective for M_2-receptors(2). Moreover, [^{18}F]FP-TZTP binds to M_2-receptors as an agonist, putatively accounting for this radioligand's sensitivity to intrasynaptic ACh concentrations (3). The abnormal reduction in [^{18}F]FP-TZTP binding in the cingulate cortex of BD subjects thus suggested that either the intrasynaptic acetylcholine concentration was increased or the density or affinity of M_2-receptors was decreased in bipolar depression.

Conceivably, these observations in BD may reflect genetic variation in the gene coding for the M_2-receptor (*CHRM2*). *CHRM2* contains several single nucleotide polymorphisms (SNPs) that have been associated with the risk for developing major depressive episodes (4–6). Genetic variation in the 3′ region of the *CHRM2* gene (A/T 1890, rs8191992) has been associated with MDD in females (5). In families with both an alcohol-dependent proband and relatives with MDD, Wang et al. (4) showed an association between two SNPs in intron 4 of the *CHRM2* gene and depression. Wang et al. (4) also identified a T-T-T haplotype (rs1824024-rs2061174-rs324650) that was under-transmitted to individuals who manifested both alcohol dependence and co-morbid MDD, and this group subsequently identified the risk-influencing locus for affective disorders as rs324650 in European-Americans (6). In a study of MDD cases not selected on the basis of having co-morbid alcoholism, however, no association was identified between this haplotype and MDD (7). Although no direct association between *CHRM2* and BD has been reported, linkage and sib-pair studies found associations between the risk for BD and genetic variation in the vicinity of *CHRM2*. The *CHRM2* gene is located in the q31–35 region of chromosome 7 (8) and evidence for linkage was reported for 7q31 (LOD=2.08)(9, 10) and 7q34 (LOD=2.78) in affected sib-pair analyses of families of BD probands (11). Moreover, a study of 27 SNPs across the *CHRM2* gene demonstrated an association between *CHRM2* and "externalizing psychopathology" as a broader conceptualization of psychiatric disorders that encompassed symptoms or syndromes that occur comorbidly with mood disorders, such as substance dependence (12).

In addition, *CHRM2* function has been associated with cognitive domains that are impaired in individuals with BD. Neuropsychological studies have shown that BD subjects manifest

impairments in attention, memory and social cognition, which in some cases appear trait-like, insofar as they are evident in unaffected relatives of bipolar probands (13). For example, deficits in verbal memory have been identified in currently depressed subjects with MDD or BD, as well as in unaffected twin and non-twin siblings of BD subjects (14–16). The *CHRM2* gene conceivably may influence function across a range of cognitive domains through its role in generating or modulating evoked electrophysiological oscillations (17–19), as the development of theta and delta event-related oscillations which play critical roles in decision making (20, 21), selective attention (22), recognition memory and episodic retrieval (23–27) are dependent upon muscarinic cholinergic receptor stimulation. Consistent with such a far-reaching influence, genetic variation in *CHRM2* has been shown to influence performance intelligence quotients (PIQ)(28–32).

Acetylcholine neurotransmission has been linked to the regulation of mood (33–35), sleep (36, 37) and neuroendocrine function (38–41) by preclinical and clinical evidence. In studies of MDD and BD, increasing cholinergic transmission via administration of muscarinic receptor agonists or acetylcholinesterase inhibitors exacerbates depressive symptoms in both illnesses and reduces manic symptoms in BD (42–44). Moreover, neurophysiological responses to muscarinic receptor–agonist challenge are exaggerated both in subjects with current depression and in subjects with remitted MDD or BD relative to controls (45, 46). Since the muscarinic cholinergic system has been shown to play roles in evaluating and learning the salience of sensory stimuli (47), the increased muscarinic sensitivity evidenced in individuals with mood disorders conceivably may contribute to the altered perceptions of emotionally-valenced events reported in these conditions (48).

In healthy humans, administration of the M_2 antagonist procaine, which putatively increases intrasynaptic ACh concentrations, elicits a spectrum of robust emotional responses, ranging from sadness, anxiety and fear to euphoria (49), resembling the spectrum of emotional symptoms manifested in BD. These responses were associated with physiological activation of limbic structures, primarily the anterior cingulate cortex (ACC)(50), a region densely innervated by cholinergic projections from the basal forebrain that also has been implicated in the pathophysiology of MDD and BD by neuroimaging and neuropathological evidence. In this and other structures the M_2-receptor is expressed both presynaptically and postsynaptically, and the presynaptic M_2-receptor constitutes one of the predominant muscarinic inhibitory autoreceptor subtypes (i.e., receptor stimulation decreases ACh release)(51), conferring it with a major influence over cholinergic transmission.

The current study characterized relationships between the V_T values obtained in our previous study and six SNPs within *CHRM2* to test the hypothesis that genetic variation in this gene influences M_2-receptor receptor binding to [^{18}F]FP-TZTP in healthy humans. For SNPs where such an effect was demonstrated we additionally examined interactions between genetic variation in *CHRM2*, diagnosis and regional [^{18}F]FP-TZTP V_T to test the hypothesis that a genotype-by-diagnosis interaction accounts for the abnormal reduction in regional V_T observed in BD (52). Finally, *post hoc* analyses examined the effects of variation in *CHRM2* on performance on tests of intelligence (28–31), memory, attention or executive function (53–55) and explored interactions between V_T, genetic variation, cognitive function and mood disorders.

Materials and Methods

Participants

Subjects ages 18 to 50 who either were psychiatrically healthy (n=25) or met Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for a current major depressive episode (n=40) were recruited through advertisements in local media, the NIMH Outpatient Clinic or the Howard University School of Medicine. The depressed subjects additionally met criteria for either BD (n=16) or recurrent-MDD (n=24) using the DSM-IV criteria. Exclusion criteria included exposure to psychotropic drugs including nicotine or medications with anticholinergic activity within the three weeks prior to scanning, major medical or neurological illnesses, lifetime history of substance dependence including nicotine, substance abuse within 1 year, and current pregnancy or breast feeding. Additional exclusion criteria applied to the healthy control sample included having a personal or family history of a major psychiatric disorder. Subjects provided written informed consent as approved by the NIMH IRB.

Clinical Assessments

Mood and anxiety symptoms were assessed using the Montgomery-Asberg Depression (MADRS)(56), the Hamilton-Anxiety (HAM-A)(57) and the Young Mania Rating scales (YMRS)(58). Socioeconomic status (SES) scores were determined based on the level of education and employment attained (59). The family history of psychiatric disorders was assessed using the Family Interview for Genetics Studies (60). All assessments were performed at the time of scanning.

Genotyping

Blood was sampled in all subjects for genotyping. Genotype data spanning the coding region and ~2 kb of flanking sequence were downloaded from the International HapMap Project (version 1.0, accessed 11/2004). Based on these data, a relatively uncorrelated set ($r^2<0.8$) of common markers (minor allele frequency >7.5% in persons of European descent) was selected for genotyping. Six SNPs were selected to assess variation in the *CHRM2* gene: *rs7810473; rs1824024; rs2061174; rs2350786; rs324650 and rs8191992*. These SNPs were included on a chip that included a larger set of 768 SNPs in 68 candidate genes, as detailed in McMahon et al. (61). Samples were shipped to Illumina, Inc., San Diego, California, where they were genotyped on an assay(62) with >99% success and >99 % of possible genotypes returned, including blind duplicate genotypes, all of which matched exactly.

A set of 344 unlinked SNPs was used to control for ethnic differences. Using STRUCTURE (63), we estimated probability of membership in each of three ancestral populations (20,000 burn-in steps followed by 20,000 replications). These values then were used as covariates in subsequent analysis.

PET image acquisition and processing

A detailed account of the image acquisition and processing and of the modeling of distribution volume (V_T) was reported in Cannon et al[1]. Briefly a 120 minute dynamic PET scan was acquired using a GE Advance scanner in 3D mode (3D spatial resolution=6

mm full-width at half-maximum) following injection of 352–389 MBq of high specific activity [^{18}F]FP-TZTP(3, 64). Arterial blood was sampled during scanning. MRI scans were obtained using a GE Signa Scanner (3.0 Tesla) and co-registered to the PET images to provide an anatomical framework for image analysis. PET data were corrected for partial-volume effects frame-by-frame before kinetic modeling(3). The primary outcome parameter was the [^{18}F]FP-TZTP $V_{T,}$, which is proportional to the product of receptor density and affinity. The arterial input function for [^{18}F]FP-TZTP was generated by quantifying the plasma concentration of parent [^{18}F]FP-TZTP using a hexane-extraction procedure(65) in 28 serial blood samples drawn at increasing intervals from a radial artery cannula (number_frame duration [in minutes]: 6_0.25, 5_0.5, 2_1, 3_2, 1_3, 5_5, 2_10, and 4_15). Using quantitative tracer kinetic modeling and a one-tissue compartment model the regional V_T (modeled as K_1/k_2 where K_1 is the rate of delivery of [^{18}F]FP-TZTP and k_2 is the rate of clearance) and K_1 values were obtained from the arterial input function and the regional tissue time radioactivity concentration curves (3). V_T was corrected for protein binding of the parent radioligand by dividing by the plasma free fraction (f_p). The V_T was modeled from the averaged radioactivity concentrations within 10 regions-of-interest (ROI): whole brain, subgenual anterior cingulate (sgACC), pregenual anterior cingulate (pgACC), posterior and dorsal cingulate cortices, amygdala, hippocampus, ventral striatum, lateral orbital cortex and primary visual cortex.

Cognitive Assessments

The intelligence quotient (IQ) was assessed by the Weschler Abbreviated Intelligence Scale(WAIS; performance and verbal T-scores). Attention and executive function were evaluated using the computerized Rapid Visual Information Processing (RVIP) and Intradimensional/Extradimensional Shift (ID/ED) tasks, respectively. We assessed memory using the Delayed Match to Sample (DMS) and spatial recognition memory (SRM) tasks. These computerized tasks were from the Cambridge Neuropsychological Test Automated Battery (CANTAB; Cambridge Cognition Ltd, Cambridge, UK) and were presented on an Advantech computer (Model PP-120T-RT) with a 10.5 inch touch-screen monitor.

Statistics

The hypothesis that genetic variation in *CHRM2* influenced M_2-receptor binding was tested in *healthy controls* by examining effects on V_T at each SNP allele-wise using two-sample t-tests in the 10 ROI listed above. The hypothesis that an interaction between diagnosis and genetic variation in *CHRM2* accounted for the lower V_T in depressed BD subjects (1) was tested using a linear mixed-model with an unstructured model for covariance in the regions where the greatest M2-receptor binding deficits were observed in the BD subjects in our original study: the pgACC and sgACC. Corrections for multiple testing in these two regions were performed by calculating the false discovery rate (FDR) adjusted p-value (66). Results with $p_{FDR}<0.05$ were considered significant. All uncorrected p-values given are denoted p_{UNC}. The specificity of these findings to the ACC was assessed *post hoc* by examining the relationships between genotype and V_T in the other eight ROI examined in the HC sample. The normality of the V_T data was assessed using the Shapiro-Wilk test, which showed the data were normally distributed ($0.98<W<0.99$, $0.23<p<0.92$) without influential outliers (based on an individual V_T value >mean±3*SD). Gender distribution across genotype was

examined using Pearson's Chi-squared test and mean age across diagnostic groups was examined using ANOVA.

For any SNP where an interaction between diagnosis and V_T was significant, *post-hoc* exploratory analyses were conducted using Pearson's Chi-squared tests to assess the relationships between genotype and: 1) the likelihood of having a past suicide attempt, 2) the presence of a first-degree relative with BD, and 3) current psychosocial function, as reflected by the socioeconomic status scores. In addition, secondary analyses explored relationships between V_T, genotype and performance on tasks of intelligence (28–31), memory, attention or executive function (53–55) using Pearson's or Spearman's correlations depending on the normality of distribution of the performance variables. Eight variables were assessed: performance IQ (PIQ T-score), verbal IQ (VIQ T-score), attention performance (RVIP: correct detections of the target sequences and omission errors), memory performance (DMS: % total correct; SRM: % correct), and executive function performance (ID/ED Shift-completed stage trials {i.e. number of trials taken to complete a stage, adjusted for stages completed} and errors {adjusted for trials completed}). IQ (PIQ p_{UNC}=0.26) and performance on the task of attention (RVIP correct detections p_{UNC}=0.76, RVIP omission errors p_{UNC}=0.63) were normally distributed. Therefore, their relationship to V_T was examined using Pearson's bivariate correlations. Performance on tasks of memory (DMS percent correct p_{UNC}=0.051, SRM percent correct p_{UNC}=0.003), executive function (ID/ED shift stage trials p_{UNC}=0.0001, and errors p_{UNC}=0.0001) and verbal IQ (p_{UNC}=0.061) were considered non-normally distributed and Spearman's correlations performed thereafter. Due to the large number of comparisons, results were not reported unless p-values would remain significant after applying FDR corrections for eight tests.

Finally, based on the literature reporting relationships between the *CHRM2* gene and intelligence, an exploratory analysis was conducted to assess associations between average IQ (PIQ+VIQ T-scores) and genotype for all six *CHRM2* SNPs.

Results

Mean age (HC 33±6.5, MDD 34±8.6, BD 32±7.7, F=0.28, p_{UNC}=0.77) and gender distribution (group{n female/total n}, HC{14/25}, MDD{18/24}, BD{12/16}) did not differ significantly across diagnostic groups (χ^2=2.54, p_{UNC}=0.28). Genotype frequencies did not differ significantly across subject samples for any of the six SNPs (table 1). Three of the BD subject had BD Type I. Only one BD subject had a psychotic episode in the past. On ratings of depression severity and anxiety symptoms the MDD and BD groups did not differ significantly from each other (p>0.2) but rated higher than the HC group (p<0.001) (MADRS MDD: 22±6.9, BD: 25±8.1, HC: 0.3±0.7; HAMA MDD:13±5, BD: 16±6, HC: 0.3±0.7). The BD group had a higher YMRS score (4.8±3.5) than the MDD (3.2±2.0) and HC groups (0.2±0.6, p_{UNC}=1.15×10^{-}8). The HC group had a higher SES score (53±8.6) than the BD (42±10) or MDD (44±12, p_{UNC}=0.002) groups and the MDD and BD group did not differ significantly from each other.

Genotype in five of the six *CHRM2* SNPs assessed did not relate significantly to [^{18}F]FP-TZTP binding among the HC subjects. In healthy controls the allele-wise testing revealed

higher V_T associated with the T-allele for SNP rs324650 in the pgACC and sgACC (Fig. 1). Similar associations were observed in the whole brain, amygdala, ventral striatum and lateral orbital cortex ($p_{uncorrected}<0.05$) but these did not remain significant after applying corrections for multiple comparisons. In contrast, in the MDD or BD samples no significant allele-based difference existed in any region.

The interaction between diagnosis, rs324650 genotype and V_T was significant in the pgACC ($F=3.62$, $p_{FDR}=0.04$) and reached a trend level in the sgACC ($F=3.89$, $p_{FDR}=0.06$). Post-hoc exploratory analyses showed similar relationships in the amygdala ($F=4.30$, $p_{UNC}=0.02$), hippocampus ($F=5.42$, $p_{UNC}=0.01$; Fig. 2) and lateral orbital cortex ($F=3.68$, $p_{UNC}=0.04$). The interaction was accounted for by reduced [^{18}F]FP-TZTP V_T in BD subjects who were T-homozygotes relative to HC-subjects of the same genotype ($0.013<p_{UNC}<0.022$ in the regions listed above). The T-homozygous BD subjects also showed lower V_T relative to both BD-heterozygotes ($0.031<p_{UNC}<0.046$) and to HC-heterozygotes ($0.006<p_{UNC}<0.019$, Fig. 2). *Post-hoc* assessments revealed no other genotype-by-diagnosis interactions involving the five other *CHRM2* SNPs examined ($p>0.1$).

Of the clinical variables considered the proportion of cases with past suicide attempts differed by genotype at rs324650 (Table 2). Five of the six BD subjects homozygous for the T-allele previously had attempted suicide, compared to only three of the ten BD subjects who were A-carriers ($p=0.039$). The proportion of BD subjects who had a first-degree relative with BD showed a non-significant trend toward being higher in T-homozygotes than in A-carriers ($p=0.053$). Finally, the BD subjects homozygous for the T-allele showed lower socioeconomic status scores (mean±SD: 33±9.7, $t=3.8$, $p=0.002$) than A-carrier BD subjects (mean±SD: 48±6.0). The MADRS scores did not differ significantly between the T-homozygotes and A-carriers from the BD sample ($t=-0.65$, $p_{UNC}=0.52$).

Performance on tests of attention and memory was impaired in the BD and MDD groups versus the control group (Fig. 3A). The number of omission errors on the RVIP task and the percentage of correct responses on the DMS task differed across groups ($F=5.12$, $p=0.009$ and $F=3.58$, $p=0.035$, respectively). These differences were attributable to poorer performance in the MDD and BD groups relative to the HC group (Fig. 3A) and not to differences between the MDD and BD groups. Performance on the ID/ED Shift task, intelligence, memory or attention tests did not correlate significantly with V_T in any region in the HC, BD or MDD groups.

Assessments of the relationship between *CHRM2* rs324650 genotype and cognitive performance revealed that BD subjects homozygous for the T-allele showed poorer spatial recognition memory on the SRM ($t=3.36$, $p=0.005$; Fig. 3B) relative to A-carrier BD subjects. These subgroups did not differ on the other neuropsychological test measures considered. In MDD, spatial working memory performance (DMS percent correct) was poorer in those possessing the rs324650 AA-genotype relative to T-carriers ($F=7.81$, $p=0.003$). In healthy controls none of the cognitive performance measures differed significantly across *CHRM2* rs324650 genotypic variants.

Exploratory analyses of the association between genetic variation at other *CHRM2* markers and cognitive performance showed that in the entire study sample, individuals homozygous for the T-allele of SNP rs2061174 had a higher average IQ (122±12, F=3.9, p=0.026) than C-carriers (CT: 113±13; CC: 112±11).

Discussion

Genetic variation within the *CHRM2* gene influenced the binding of [^{18}F]TZTP to M_2-receptors in healthy controls and accounted for the abnormal reduction in M_2-receptor binding previously reported in subjects with bipolar depression. This genetic variance was associated with a SNP involving an adenine-to-thymine substitution at marker rs324650 within the *CHRM2* gene. Our data thus implicate either this SNP or a distinct possibly nearby variant in high linkage-disequilibrium with rs324650. The rs324650 SNP was associated with an allelic effect on M_2-receptor binding (V_T) in healthy humans such that the T-allele was associated with higher V_T than the A-allele (figure 1). This effect accounted for 20% (partial eta^2=0.20) of the total variance in V_T. This is similar to the 28% contribution that variance in the HTR2A gene coding for the 5-HT2A receptor accounted for 5-HTT binding ([^{11}C]DASB PET) in the thalamus (67) in a recent study of a similar design. The rs324650 SNP also was associated with an interaction between V_T and diagnosis such that while BD subjects with either AA or AT genotypes did not differ from their respective control subgroups, BD subjects with the TT-genotype showed 27 to 37% reductions in V_T relative to TT controls across brain regions (figure 2). This difference among the T-homozygote's appeared to account for the abnorma reduction in [^{18}F]FP-TZTP V_T found previously in the entire BD sample relative to the healthy control sample [1]. This effect accounted for 27% (partial eta^2=0.27) of the total variance in V_T. *Post hoc* analyses suggested that within the BD sample the TT-genotype was associated with a more severe illness course, as manifested by lower socioeconomic function, poorer spatial recognition memory and a greater likelihood of having attempted suicide.

The effects of genetic variation within *CHRM2* on V_T were evident in brain regions where [^{18}F]FP-TZTP binding is relatively selective for M_2-receptors (2). The *in vitro* affinity of [^{18}F]FP-TZTP is highest for M_2-receptors (K_i=2.2 nmol/l), lower for M_1-receptors (K_i=7.4 nmol/l) and negligible for other muscarinic receptors (K_i≥80 nmol/l). Studies in muscarinic-receptor knock-out mice have shown that [^{18}F]FP-TZTP is relatively selective for M_2-receptors in most brain tissues, excepting the amygdala and hippocampus, where 20% to 23% of binding is attributable to M_1-receptors. Thus to our knowledge this report that [^{18}F]FP-TZTP binding in the ACC is influenced by genetic variation in *CHRM2* constitutes the first direct link between a single altered nucleotide among a sequence coding for a receptor and radioligand binding to that receptor *in vivo* in the human brain.

Nevertheless, since [^{18}F]FP-TZTP is sensitive to intrasynaptic concentrations of acetylcholine, the effect of genetic variance in rs324650 on M_2-receptor binding conceivably may be attributable to differences either in M_2-receptor density or affinity, or in intrasynaptic ACh-concentrations. In some brain regions the M_2-receptor functions as an autoreceptor that exerts inhibitory regulation over acetylcholine release (68, 69), so genetic variation that affects autoreceptor function may influence neurotransmitter release.

Nevertheless, a several hundred percent change in endogenous acetylcholine concentrations would be required to produce the magnitude of difference in V_T found between T-homozygous BD subjects and T-homozygous controls (3), making it unlikely that the effect of genetic variation on V_T is accounted for by differences in neurotransmitter concentration alone. The effect of variation in or near rs324650 on M_2-receptor binding more likely reflects an influence of this polymorphism on the regulation of gene expression or splicing to an extent that alters M_2-receptor density, or on the G-protein coupling or intracellular trafficking of the receptor to the cell membrane to an extent that alters M_2-receptor affinity.

A recent *post-mortem* study using the antagonist radioligand [^{3}H]AFDX detected reduced $M_{2/4}$-receptor binding in the dorsolateral frontal cortex (BA46) of subjects with MDD and BD while no change was detected in rostral prefrontal (BA10) and parietal (BA46) cortices and ACC was not tested (70). Another *post-mortem* study focused on the ACC observed no significant difference in BD relative to controls again using the *antagonist* radioligand [^{3}H]AFDX (71). The latter studies are measuring the total (high- and low-affinity state) M_2 and M_4-receptor density (B_{max}) whereas the present study using a M_2-receptor agonist, [^{18}F]FP-TZTP is preferentially measuring the pool of high-affinity M_2-receptors present rather than the total pool (72)(discussed in Cannon et al., 2006) and therefore is more sensitive to the functional state of the M_2-receptor system. Moreover, the V_T parameter is proportional to the product of density and affinity. Thus consideration of our data within the context of these *post mortem* data would lead to the hypothesis that the effect of the BD diagnosis on V_T reflects affinity rather than density at least in the ACC.

Despite *CHRM2* having been cloned (73, 74) the regulatory regions involved in neuronal expression and the nature of their influence over promoter activity and splice variant generation are only partly understood. The SNP rs324650 resides in intron 5 of the *CHRM2* gene on chromosome 7, located within a transposable element, a short interspersed repeat (SINE). SINE repeats can participate in the process of reverse transcription, whereby they drive transcription of their own transposase and cause aberrant expression of linked genes (29, 75–79). The *CHRM2* gene expression also is regulated by elements within a large 5′ untranslated region (UTR) encoded by multiple exons and by intronic regions upstream of the neuronal-specific promoter 5′UTR (80). Regulators of *CHRM2* expression may potentially act through transcriptional regulation, altered translation, epigenetic factors, heterodimerization, indirect interaction with other genes and endogenous regulators of receptor density, any of which may influence V_T.

Notably one *post-mortem* study assessed the influence of *CHRM2* SNPs rs324650, rs2061174 or rs324640 on gene expression in the superior and inferior parietal lobe and found no significant effect in a sample of 50 individuals (29). The *in vivo* measure of V_T is sensitive to a several factors (M_2 receptor density, affinity, endogenous neurotransmitter concentrations) that may have not been reflected by mRNA concentrations. Nevertheless, our data further suggest that the sensitivity of future *post mortem* studies of the effects of the rs324650 polymorphism on *CHRM2* expression may be enhanced by specific assessment of limbic structures such as the anterior cingulate cortex, amygdala and hippocampus. Genetic variation in CHRM2 does not appear to uniformly affect all brain regions. Possible explanations include currently unidentified region specific factors possibly including

epigenetic or other spatially localized modulators of transcription, translation and/or expression. Several studies support this by documenting tissue specific control of gene expression by cis-acting SNPs (81).

Since the M_2-receptor plays a major role in the regulation of acetylcholine release, genetic variation within *CHRM2* that alters the function of this autoreceptor could in turn alter cholinergic neurotransmission, and thus exert far-reaching effects on a variety of emotional and cognitive domains. Consistent with this expectation, associations have been reported previously between the M_2R gene and depression, IQ, alcoholism and Alzheimer's Disease. Wang et al. (2004) and Jones et al. (2004) reported associations between two SNPs within intron 4 (upstream of the coding sequence) and major depressive episodes arising within the context of alcohol dependence(4, 18). Downstream SNPs reportedly influenced the risk for alcohol dependence (independently of depression) and electrophysiological event-related oscillations (18). Moreover, a polymorphism in the 3′ UTR of *CHRM2* has been associated with the vulnerability for developing MDD in women (5) and with general intelligence (31). However, negative studies for an association between *CHRM2* and MDD have also been reported (7). The latter may be consistent with the lack of relationship detected between genotype, M_2-receptor binding and the MDD group.

With respect to the *CHRM2* SNP rs324650, in healthy humans this polymorphism reportedly influences personality traits including agreeableness, conscientiousness and openness (82). This latter correlation was noteworthy, since lower openness scores have been associated with a greater risk for developing depression (82) and, within the context of BD, suicidal ideation (83). Luo et al (2007) associated greater openness scores with the T-allele of rs324650 relative to the A-allele (p=0.029 unpublished data). Both the CHRM2 gene (28–32) and BD (84–87) are associated with altered cognitive function, and reduced protection against suicide and disability or reduced resilience (88). Therefore, we examined the relationship between genotype of rs324650 and suicide attempts, cognition and socioeconomic status (SES). BD subjects with the TT variant at rs324650 were more likely to have attempted suicide (table 2) and had lower SES scores and poorer spatial recognition memory. Nevertheless, the T-allele was not associated significantly with the likelihood of receiving the BD diagnosis, and in alcoholism with secondary depression a haplotype that included the T-allele of rs324650 appeared under-transmitted to affected individuals (4). Thus our data suggest the preliminary hypothesis that the TT-genotype of rs324650 or a polymorphism in high LD with rs324650 is associated with a more severe or disabling phenotype of BD, as characterized by a higher risk of attempting suicide, poorer social-occupational function and greater cognitive impairment, without clearly altering the vulnerability for developing depression. Taken together these deficits in the BD group who are homozygous for the T-allele may reflect poorer cognitive reserve, hypothesized to confer protection against more severe symptoms in neuropsychiatric disorders (88, 89). However, due to the small sample size studied these associations should be considered preliminary and warrant investigation in a larger sample.

Although we did not detect a significant association or interaction with V_T or diagnosis involving the *CHRM2* SNPs rs7810473, rs1824024, rs2061174, rs2350786 or rs8191992, these negative results may have reflected power limitations due to our small samples. It is

also the case that we will not have captured some proportion of common variation and a large range of rare alleles in the present approach. We capture 49% of the common (5%) alleles at rsq>0.3 across the coding region +/− 34 kb, or 57% of common alleles across the coding region =/− 10 kb. Of the SNPs we did investigate rs8191992 previously was associated with depression among females (31). This SNP is in LD with rs324650 (r^2=0.607)(18), a relationship which we confirmed independently in the CEPH sample from the International HapMap Project (version 21, accessed 2/2009; r^2=0.561, D′=0.78).

The relationship detected between *CHRM2* and BD but not MDD add to the body of knowledge regarding genetic overlap between the two depressive disorders. A number of markers appear to confer risk for both BD and MDD such as the gene coding for the alpha-1C subunit of the L-type voltage-gated calcium channel (CACNA1A)(90) and some that appear to be selective for BD but not MDD such as the neuregulin-1 gene (NRG1)(91). The present data suggest that muscarinic cholinergic neurotransmission may be more affected by *CHRM2* in BD than in MDD. Indeed it is not clear based on the current BD sample whether these findings extend to BD with psychosis or other subtypes not represented. Our data for the rs324650 T-homozygotes show an interaction with diagnosis such that the BD-TT subjects showed significantly lower V_T than the HC-TT subjects. In contrast, the TT subjects from the MDD group showed V_T values that were intermediate between, and not significantly different from, those of the HC-TT subjects and the BD-TT subjects. Our findings thus would appear to support a hypothesis that some, but not all MDD subjects may have a common genetic background with BD cases.

The secondary analyses detected an association between intelligence and the *CHRM2* SNP rs2061174. This SNP previously was associated with performance intelligence, along with the *CHRM2* SNPs rs2350786, rs8191992, rs2061174, rs324640 and rs324650 (28, 32). In addition, multiple SNPs spanning several LD blocks within the *CHRM2* gene (intron 4–5 and intron 5–6) have been associated with performance IQ. Given the evidence that variation in *CHRM2* plays a role in general cognitive performance, we cannot exclude the possibility that the apparent effect of the rs324650 TT variant on the severity of bipolar illness may be mediated by a more general effect on intelligence.

Compatible with this hypothesis, spatial recognition memory in BD individuals possessing the TT-genotype was impaired relative to A-carriers. Muscarinic cholinergic function previously has been implicated in other types of memory formation as well, including inhibitory avoidance formation (92) and consolidation of memory for salient events (93). Thus the interaction between BD and variation in the *CHRM2* gene may be associated more specifically with the cognitive deficits observed with BD (84, 86, 87, 94, 95). In contrast, in MDD poorer performance on the delayed match to sample task was evident in subjects with the AA-genotype relative to T-carriers (rs324650).

In BD, increasing cholinergic transmission via administration of muscarinic-receptor agonists or acetylcholinesterase-inhibitors exacerbates depressive symptoms in both illnesses and reduces manic symptoms in BD. In addition, neurophysiological responses to muscarinic-receptor agonist challenge are exaggerated both in currently-depressed and currently-remitted MDD or BD-subjects relative to controls (46). The muscarinic-

cholinergic system generally has been shown to play roles in evaluating and learning the salience of sensory stimuli(47), suggesting that disturbances of muscarinic-function may alter the perception of emotionally-valenced events (48). It might be hypothesized that such disturbances (1, 4, 5, 50) underlie the mood dysregulation associated with BD as a result of aberrant regulation brought about by SINE repeats within the *CHRM2* gene.

In summary, we detected an allelic effect of the *CHRM2* SNP rs324650 on M_2-receptor binding *in vivo* in healthy humans. In addition, we found an interaction between this SNP, M_2-binding and bipolar depression, in which the TT-genotype of rs324650 was associated with abnormally decreased M_2-receptor binding in T-homozygotes with BD [1]. The mechanism underlying the contrasting effects of the rs324650 SNP in bipolar depressives versus healthy controls remains unclear, but conceivably may reflect an interaction between this SNP and another genetic or environmental factor associated with bipolar disorder. If confirmed in a larger sample, these preliminary data hold the potential to identify a subgroup of bipolar disordered cases in which aberrant M_2-receptor expression or function plays a major role in pathogenesis.

Acknowledgments

We acknowledge Peter Herscovitch, M.D. and the Clinical Center PET Department for providing the radioligand and for technical assistance, Michele Drevets, R.N. and Joan Williams, R.N., for evaluation and recruitment of the research subjects, the staff of the NIH Clinical Center, Allison Nugent, Stephen Fromm, Kelly Anastasi and Laurentina Cizza for assistance in data management, and Dave Luckenbaugh, Ph.D. for advice regarding the statistical analysis of the PET data.

Funding: This study was supported by the intramural research program of the NIH/NIMH and a National Alliance for Research on Schizophrenia and Depression Young Investigator Award.

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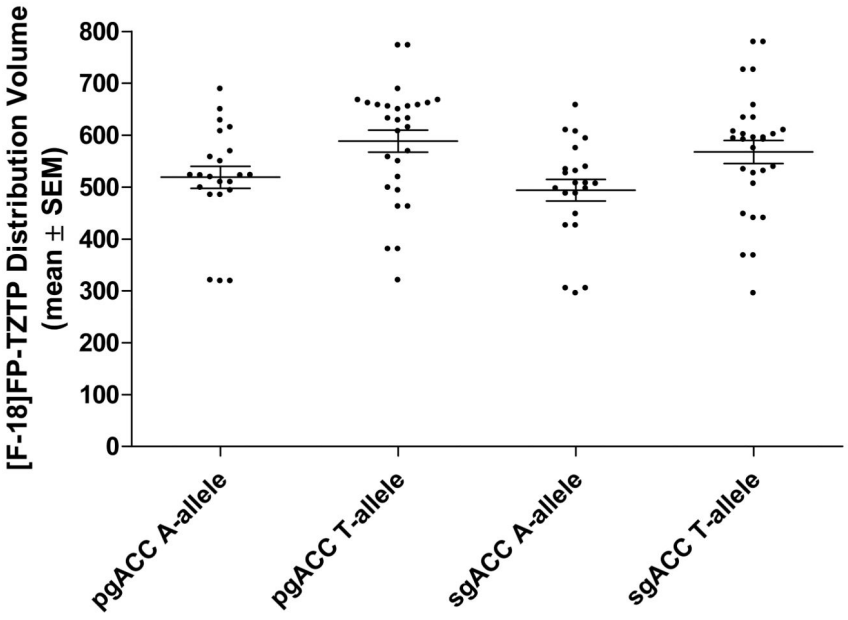

Figure 1. Association between regional distribution volume values (V_T) and genetic variation in the single nucleotide polymorphism at marker rs324650 on the muscarinic 2 receptor gene, *CHRM2* for healthy controls

After applying the false discovery rate correction for multiple testing the results in the pregenual anterior cingulate corticex (pgACC) is significant ($p_{corrected}<0.05$).

Abbreviations: pgACC pregenual anterior cingulate cortex, sgACC subgenual prefrontal cortex.

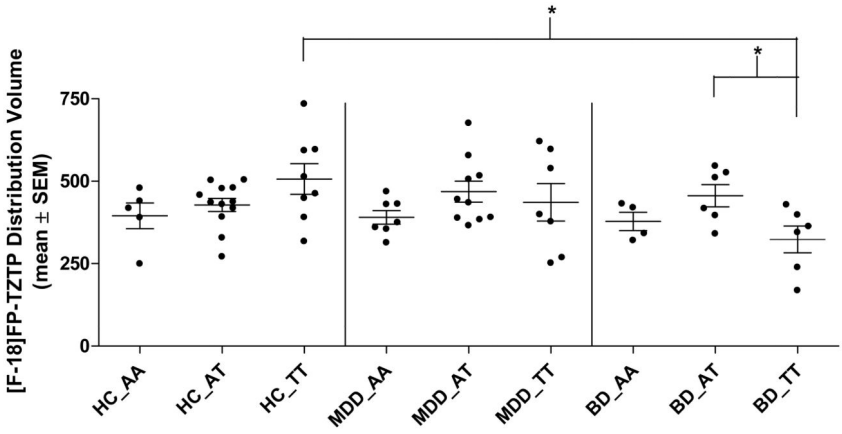

Figure 2. Reduced hippocampal [^{18}F]FP-TZTP V_T in subjects with BD and the TT-genotype of rs324650 in the *CHRM2* gene

A significant interaction was detected between rs324650 genotype and group in the hippocampus (F=5.42, p=0.01) that is accounted for by reduced [^{18}F]FP-TZTP V_T in BD subjects homozygous for the non-ancestral T-allele of rs324650 relative to heterozygotic BD-subjects (T=2.50, p=0.03) and relative to controls of the same genotype (T=2.74, p=0.019).

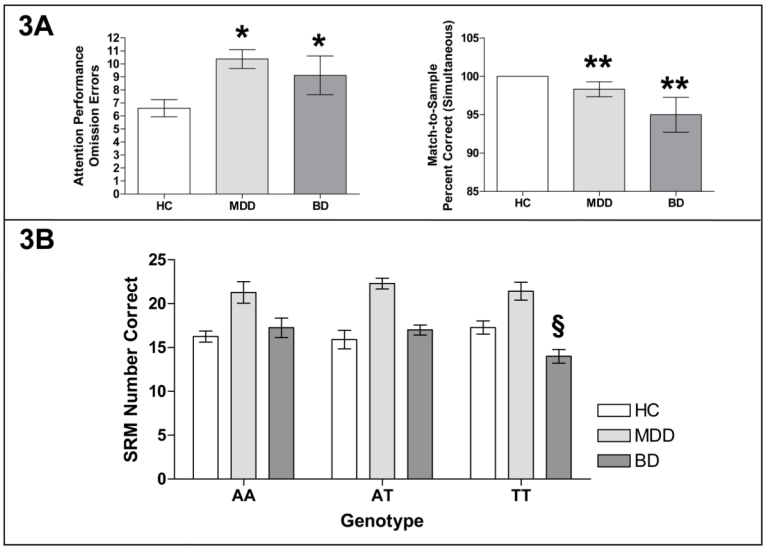

Figure 3. Cognitive performance A) impairment in BD and MDD groups relative to healthy controls, B) impairment in BD subjects homozygous for the T-allele versus A-carriers for rs324650

A. BD and MDD groups showed impairment in attention and memory evidenced by a greater number of errors of omission (*, RVIP, F=2.48, p=0.009), and reduced percentage correct responses (**, DMS, F=3.447, p=0.039) relative to controls, respectively. The HC group has no error bars because they are performing at 100%. **B.** In BD, spatial recognition memory performance is impaired in subjects homozygous for the T-allele versus A-carriers (§, T=3.36, p=0.005).

Table 1

Genotype frequencies for the *CHRM2* single nucleotide polymorphisms investigated

Genotype frequency for each of the six SNPs examined did not differ significantly across groups.

***CHRM2* SNP**	**HC**	**MDD**	**BD**	**Spearman's Chi-Square(p)**
rs7810473				
Genotype				
AA	8 (35%)	4 (27%)	9 (56%)	
AT	11 (48%)	9 (60%)	3 (19%)	
TT	4 (17%)	2 (13%)	4 (25%)	
Total	23	15	16	5.86 (0.21)
rs1824024				
Genotype				
GG	10 (40%)	16 (67%)	7 (44%)	
GT	14 (56%)	6 (25%)	6 (38%)	
TT	1 (4%)	2 (8%)	3 (19%)	
Total	25	24	16	7.23 (0.12)
rs2061174				
Genotype				
CC	5 (20%)	9 (38%)	2 (13%)	
CT	15 (60%)	5 (21%)	8 (50%)	
TT	5 (20%)	10 (42%)	6 (38%)	
Total	25	24	16	9.34 (0.05)
rs2350786				
Genotype				
AA	6 (24%)	9 (38%)	8 (50%)	
AG	15 (60%)	7 (29%)	6 (38%)	
GG	4 (16%)	8 (33%)	2 (13%)	
Total	25	24	16	9.83 (0.13)
rs324650				
AA	5 (20%)	7 (29%)	4 (25%)	
AT	12 (48%)	10 (42%)	6 (38%)	
TT	8 (32%)	7 (29%)	6 (38%)	
Total	25	24	16	0.89 (0.93)
rs8191992				
AA	6 (24%)	4 (17%)	6 (38%)	
AT	11 (44%)	8 (33%)	4 (25%)	
TT	8 (32%)	12 (50%)	6 (38%)	
Total	25	24	16	3.77 (0.44)

Table 2

Chi-square for clinical variables significantly more frequent in the SNP rs324650 TT genotype versus A-carriers among BD subjects. Post-hoc exploratory – p< 0.05

One participant with BD was adopted and no family history was available.

Clinical Variable	Absence	Presence	Chi-Square	
			Z	P
Suicide Attempt				
A-carriers	7	3		
TT group	1	5	4.27	0.039
First Degree Relative with BD				
A-carriers	5	5		
TT group	0	5	3.75	0.053