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ORIGINAL PAPER

A Cholinergic Receptor Gene (*CHRM2*) Affects Event-related Oscillations

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Abstract We report genetic linkage and association findings which implicate the gene encoding the muscarinic acetylcholine receptor M2 (*CHRM2*) in the modulation of a scalp-recorded electrophysiological phenotype. The P3 (P300) response was evoked using a three-stimulus visual oddball paradigm and a phenotype that relates to the energy in the theta band (4–5 Hz) was analyzed. Studies have shown that similar electrophysiological measures represent cognitive correlates of attention, working memory, and response selection; a role has been suggested for the ascending cholinergic pathway in the same functions. The results of our genetic association tests, combined with knowledge regarding the presence of presynaptic cholinergic M2 autoreceptors in the basal forebrain, indicate that the cognitive processes required by the experiment may in part be mediated by inhibitory neural networks. These findings underscore the utility of electrophysiology and neurogenetics in the understanding of cognitive function and the study of brain-related disorders.

Keywords Cholinergic · Acetylcholine · EEG · Event-related · Oscillation · Inhibition

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Introduction

Event-related potentials (ERPs) provide a simple non-invasive method to explore the characteristics of sensory processes and higher cognitive function (Donchin 1979). Possibly the best-studied ERP is the P3 (or P300) component. This positive electric potential deflection is elicited approximately 300–500 ms following the occurrence of infrequent stimuli during an oddball experiment paradigm. The P3 is known to be highly heritable (O'Connor et al. 1994; Katsanis et al. 1997; Van Beijsterveldt and Van Baal 2002) and to provide quantitative endophenotypes for some complex psychiatric and neurologic disorders (Hesselbrock et al. 2001; Porjesz et al. 2005). Therefore, finding the specific genes which modulate the P3 and other electrophysiological measures is a relevant and important endeavor. Family based genetic studies offer an increasingly viable method to elucidate the neurochemical and neuroanatomical substrates of electric potentials. Genetic data

will help constrain the physiological and psychological origins of the P3 and other neuroelectric phenomena. In addition EEG data offer valuable utility as quantitative endophenotypes of some complex psychiatric and neurologic disorders; these measures are also of great consequence since they represent traits more proximal to gene function than typical diagnostic or cognitive measures (Almasy 2003; Gottesman and Gould 2003).

Evidence indicates that variation in the P3 component amplitude may be of great importance to a number of psychiatric and neurologic disorders. For instance, a large number of studies have reported significantly reduced P3 amplitudes in abstinent alcoholic subjects when compared to non-alcoholic controls (e.g., Porjesz and Begleiter 1998) and in high-risk offspring of alcoholics when compared to matched low-risk control offspring (e.g., Begleiter 1984). Additionally, low P3 amplitudes are present in various disinhibitory conditions such as substance abuse (Herning 1996; Biggins et al. 1997; Brigham et al. 1997; Anokhin et al. 2000; Iacono et al. 2003), antisocial personality (Hesselbrock et al. 1993; Costa et al. 2000), conduct disorder (Iacono et al. 2002) and attention deficit hyperactivity disorder (Klorman 1991; van der Stelt et al. 2001). Low P3 amplitude has been observed in schizophrenia for the auditory modality in addition to a number of other disorders (c.f., Polich and Herbst 2000). There is also evidence to suggest that individuals with low P3 amplitude manifest significantly higher incidences of externalizing disorders and disinhibitory traits when compared to the high P3 amplitude individuals (Hill and Shen 2002). Therefore, the P3 component has significant clinical interest and may act as an endophenotype of a number of disinhibitory conditions (Porjesz et al. 2005).

The ERP task most commonly used to elicit the P3 is the ''oddball'' task, in which rare ''oddball'' stimuli (targets) are embedded in a series of frequent non-target stimuli (standards). If the subject is asked to attend or respond to the rare target stimulus, the P3's recorded to these task-relevant targets are maximum posteriorly on the scalp (over parietal regions) and are designated as P3b components. If the subject is not asked to attend to the rare stimuli, P3's recorded to these unattended rare stimuli in a repetitive background have a more frontal distribution and are designated as P3a. In the data analyzed in this work, the P3 ERP component was elicited using a visual oddball task in which the subjects were requested to attend to a rare stimulus and therefore P3 refers to the P3b component.

Currently two competing theories have been developed to explain the cognitive processes related to the P3 component. The first theory has proposed that the P3 reflects the allocation of attentional resources and the context updating processes of working memory (Donchin and Coles 1988; Polich and Herbst 2000). The amplitude of the P3 is then viewed as an index of context updating with larger amplitudes elicited by an increased amount of engaged attentional resources and/or processing capacity used to update the working memory. The latency of the P3 peak is influenced by complexity of the task and therefore reflects mental processing speed (Magliero et al. 1984). Verleger (1988) proposed the ''cognitive closure'' theory as an alternative to context-updating. Cognitive closure (or termination of a mental process) occurs after the detection of a target stimulus and the P3 thus reflects the activity of memory trace remodeling that occurs during the post-detection process.

There has been controversy in the literature regarding P3 generation and the significance of P3 amplitude. One view assumes that P3 generation is solely the result of excitatory neural activity. An alternative view is provided by the 'threshold regulation theory' which suggests that the amplitude of the P3 component also reflects inhibitory processes over widespread cortical regions in which activation of relevant neurons is accompanied by inhibition of the remainder of the neuronal network (Elbert and Rockstroh 1987). Therefore, the amplitude of the P3 may in part be taken to reflect CNS inhibition (the larger the P3, the more the inhibition) (Desmedt 1980; Birbaumer et al. 1990).

The neuroanatomical origins of the P3 are also an active area of current research. Although the P3 is observed to have maximal amplitude over parietal areas of the scalp, studies with depth electrodes in humans indicate that the neural origins of P3 involve frontal cortex as well as the amygdala and hippocampus (McCarthy et al. 1989; Baudena et al. 1995; Halgren et al. 1995a, b; Brazdil et al. 1999). More recent functional magnetic resonance (fMRI) studies support these findings and implicate the anterior cingulate area of the frontal cortex as critical for P3 generation (Menon et al. 1997; Kiehl and Liddle 2001; Ardekani et al. 2002; Rangaswamy et al. 2004). The neurochemical origins of ERPs are known to correspond to the release of neurotransmitters which induce excitatory and inhibitory postsynaptic potentials. Triggering of the P3 is believed to result from glutamatergic neurotransmission in the temporoparietal junction, the parietal cortical regions and the medial temporal lobe and are modulated by cholinergic and GABAergic neurotransmission (Frodl-Bauch et al. 1999).

Use of ERP components, such as the P3, as phenotypes of cognition and brain processing has the potential to identify the relevant modulatory genes. The most likely candidate genes are those involved in the coding and regulation of the brain's neurotransmitter and neuroreceptor systems. Identification of such genes will provide invaluable information which can be used to elucidate the neurochemical and neuroanatomical origins of ERP

components and in turn help unravel the underlying cognitive processes. Additionally, since it is known that brain function is likely to be involved in a genetic predisposition to develop alcoholism and other psychiatric disorders, these ERP components may serve as quantitative biological markers and could be used to identify susceptibility genes for developing these disorders. Hence, neuroelectric phenomena provide a rich source of potentially useful endophenotypes for genetics since they represent important correlates of human information processing and cognition (Gottesman and Gould 2003; Porjesz et al. 2005). The search for genes which modulate the P300 component is ongoing. Linkage analysis of P300 amplitude data from the Collaborative Study on the Genetics of Alcoholism (COGA) project has revealed significant linkage on a number of chromosomes using the visual oddball task (chromosomes 2, 5, 6, 13 and 17) (Begleiter et al. 1998; Porjesz et al. 2002) and using a semantic priming task (chromosomes 4 and 5) (Almasy et al. 2001). Genetic association has been reported between the amplitude of the P300 component elicited using a visual task in high risk children and a marker locus in a dopamine D2 receptor locus on chromosome 11 (Taq1 A RFLP near the DRD2 receptor locus) (Hill et al. 1998). However, negative associations have been reported for the same polymorphism with P300 amplitude using data from normal young female subjects (Lin et al. 2001) and patients diagnosed with depression (Chen et al. 2002). Replication failures such as these may be due to a variety of factors, including: ethnicity differences; age of the subjects; psychiatric state and differences in the experimental design.

Recent research suggests that ERPs may be formed through the superposition of multiple event-related oscillations (EROs) and are not simply the result of unitary transient phenomena (Basar 1980; Makeig et al. 2002; Gruber et al. 2005), although this view is still open to debate (Yeung et al. 2004; Kirschfeld 2005; Makinen et al. 2005). The P3 response is found to be primarily composed of superimposed delta (1–3 Hz) and theta (4–7 Hz) frequency band energy with delta energy more concentrated in the posterior region and theta more frontocentral (Basar-Eroglu et al. 1992; Yordanova and Kolev 1996; Basar et al. 1999; Karakas et al. 2000). Since the P3 ERP component is comprised of multiple source generators it seems appropriate to directly study these underlying P3 ''wave'' oscillations. Time–frequency distribution analysis methods offer an attractive means of studying such non-stationary EROs and one such method has been adopted here as an alternative measure of the P3 ERP amplitude.

Using ERO phenotype data underlying the visual evoked P3 component Jones et al. (2004) reported significant linkage on the 'q' arm of chromosome 7 using theta band (4–7 Hz) EROs. The cholinergic muscarinic receptor gene (*CHRM2*) was identified as underlying the observed linkage peak and linkage disequilibrium (LD) analysis revealed significant association between CHRM2 single nucleotide polymorphisms (SNPs) and both delta (1–3 Hz) and theta (4–7 Hz) band ERO data (Jones et al. 2004). The cholinergic M2 receptor gene belongs to a family of muscarinic acetylcholine G-protein coupled receptors (mAchRs) with five known subtypes (M1–M5). M1, M3 and M4 receptors are found in the cerebral cortex and hippocampus, M2 receptors are more concentrated in the basal forebrain region, while M5 receptors are found in small quantities throughout the brain. The specific actions in the brain of these subtypes are varied: for example the M1, M3 and M4 receptors are found to be mostly postsynaptic and facilitate cholinergic neurotransmission; in contrast, the M2 receptors in the forebrain and hippocampus are mainly presynaptic autoreceptors that act to control acetylcholine release from forebrain cholinergic terminals (Mash et al. 1985; Quirion et al. 1995; Douglas et al. 2001; Zhou et al. 2001; Douglas et al. 2002; Zhang et al. 2002).

In this work we extend the findings reported in Jones et al. (2004) through the analysis of a larger number of *CHRM2* SNPs (16 as compared to 4), by using additional association analysis methodologies and by making use of a refined theta band ERO phenotype extracted using the same subject population. In particular, we have refined the theta band ERO measure to better characterize evoked post-stimulus theta band oscillations, which are observed to have a fronto-central scalp distribution (c.f., Fig. 1), by using an improved time–frequency analysis window (300–500 ms time window and 4–5 Hz frequency window). This genetic analysis focused on the midline theta band oscillatory subcomponents of the P3 potential at three electrode sites (frontal: Fz; central: Cz; and posterior: Pz) and we present comparable linkage results to the Jones et al. (2004) study on chromosome 7q using the theta ERO data from the frontal and central midline electrodes (Fz and Cz). Results presented here from a population-based measured genotype association analysis confirms and strengthens the previously reported association findings between *CHRM2* SNPs in the 5′ UTR region of the gene with the frontal theta band ERO phenotype. Finally, we report results of a pedigree-based test of association of the frontal theta phenotype with a haplotype created using four consecutive SNPs which indicate significant population-based association (surrounding exon 4). The findings presented here and in the previous paper (Jones et al. 2004) are the first to link cholinergic brain pathways with theta band oscillations underlying the P3 wave using genetic analyses; as such they represent an important step in the understanding of the P3 phenomena.

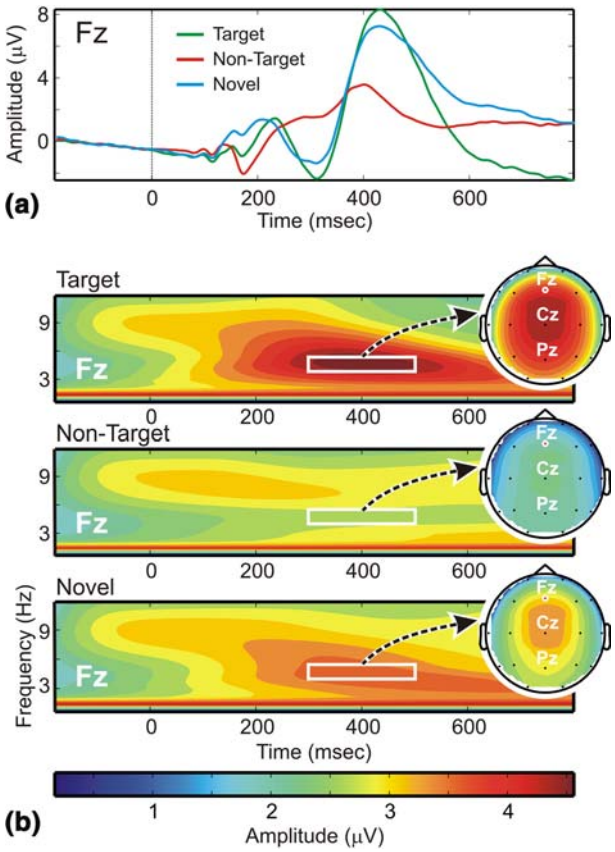

Fig. 1 Illustration of the theta band ERO phenotype used in the genetic analysis. The EEG data are obtained during an event-related visual oddball task with three conditions: target, non-target and novel. The grand average event related response for the three conditions is depicted in (**a**) for the Fz (frontal midline) electrode. Individual trials were decomposed using the S-transform and the instantaneous amplitudes averaged across individuals. Mean values within a time–frequency region of interest (4–5 Hz and 300–500 ms) were adopted as phenotypes for analysis. Time–frequency plots of the grand average S-transform amplitudes for each of the conditions are given in (**b**) for the Fz electrode. Inset head plots depict the relative strengths of the windowed data at the respective electrode positions. Data from approximately 1300 individuals were included in these grand averaged data

Methods

Subjects

Subjects included in this study were recruited and tested as part of the multisite COGA, a large national study implemented with the purpose of identifying genetic loci linked with the predisposition to develop alcoholism. Data from six COGA sites were included in the analysis: SUNY Downstate Medical Center, New York; University of Connecticut Health Science Center; Indiana University School of Medicine; University of Iowa School of Medicine; University of California School of Medicine, San Diego; and Washington University School of Medicine, St Louis. Ascertainment and assessment procedures have been outlined previously (Begleiter et al. 1995).

The families used in the analyses are taken from COGA dense alcoholic families. Multiplex families with alcoholism were initially recruited from alcoholic probands who were in treatment facilities. Psychiatric diagnoses on all family members were obtained by administering a polydiagnostic instrument designed by COGA (semi-Structured Assessment for the Genetics of Alcoholism, SSAGA) (Bucholz et al. 1994). All probands met DSM-IIIR criteria for alcohol dependence and Feighner definite criteria (COGA criteria). In addition to the proband, the study required two additional first-degree relatives who were alcohol dependent by the same COGA criteria on direct interview. Individuals with co-morbidity were included. Family members completed a neuropsychological battery and family history questionnaire, blood was collected for various biochemical assays and DNA, and EEGs/ERPs were recorded.

The total sample included in this genetic analysis consisted of 251 families with 1312 individuals ranging in age from 16 to 75 years (containing 574 individuals diagnosed as alcohol dependent). A Caucasian-only sub-sample comprising 209 families and 1049 individuals was used in the genetic association analysis (containing 462 individuals diagnosed as alcohol dependent). Blood was obtained for DNA extraction and estimation of microsatellite marker data and SNPs for use in general pedigree linkage and association analysis.

EEG Data Recording

Electrophysiological recordings were carried out using a fitted electrode cap containing 19 channels arranged according to the international 10–20 system (Jasper 1958). An electrode placed on the subjects' nose served as the reference and one on the forehead as a ground. Electrical activity was amplified 10 K (Sensorium EPA-2 Electrophysiology Amplifiers) and recorded over a bandwidth of 0.02–50.0 Hz at a sampling rate of 256 Hz. The visual oddball paradigm employed by COGA was described in a study on intersite data collection consistency (Cohen et al. 1994). Three types of visual stimuli were presented: target (the letter X), non-target (squares), and novel (a different colored geometric figure on each trial). The probabilities of occurrence of the trials were 0.125 for the target trials, 0.75 for non-target trials and 0.125 for novel trials. Each stimulus shape subtended a visual angle of 2.5°. Stimulus duration was 60 ms, and the interstimulus interval was 1.6 s. Subjects were requested to respond to the target stimulus by pressing a button with the left or right index finger as quickly as possible. Trials with baseline corrected amplitudes greater than 73 μV were marked as artifact

contaminated. The experiment terminated automatically when a minimum of 25 target stimuli, 150 non-target stimuli, and 25 novel stimuli artifact free trials had been acquired. No significant performance differences (numbers of correct and incorrect trials) were observed between individuals diagnosed as alcohol dependent and those diagnosed as not dependent. Typical ERP analysis involves averaging of the single trials according to trial type (target, non-target and novel) and peak-picking of component amplitudes after baseline correction and other processing procedures. Here, however, we analyze single trial data using time–frequency representations (TFRs) as described in the next section.

ERO Amplitude Estimation

To obtain estimates of localized power of the generally non-stationary evoked potential time series we use the S-transform TFR method (Stockwell et al. 1996). The S-transform is a generalization of the Gabor transform and an extension to the continuous wavelet transform. The S-transform generates a TFR of a signal by integrating the signal at each time point with a series of windowed harmonics of various frequencies as follows:

$$ST(f,\tau) = \int_{-\infty}^{\infty} h(t)\frac{|f|}{\sqrt{2\pi}}\mathrm{e}^{-\frac{(\tau-t)^2 f^2}{2}}\mathrm{e}^{-\mathrm{i}2\pi f t}\,\mathrm{d}t,$$

where $h(t)$is the signal, f is frequency, τ is a translation parameter, the first exponential is the window function, and the second exponential is the harmonic function. The S-transform TFR is computed by shifting the window function down the signal in time across a range of frequencies. The window function is Gaussian with $1/f^2$ variance and scales in width according to the examined frequency. This inverse dependence of the width of the Gaussian window with frequency provides the frequency-dependent resolution.

The electrophysiological phenotypes used in the analysis were derived using single trial visual oddball event related data from the target, non-target and novel experimental conditions. The instantaneous amplitudes of the S-transform TFR were averaged across single trials, per individual, to obtain an estimate of event related total amplitude response (stimulus onset phase locked plus non-phase locked oscillations). The total amplitude response enhances events that occur in a similar time range as related to the stimulus onset, and irrespective of their phase relations. Mean values were calculated from the TFR for use as phenotypes within time–frequency regions of interest (TFROI's) specified by frequency band ranges and time intervals (Lachaux et al. 2003). This study focused on evoked oscillation TFROI corresponding to the lower theta (4–5 Hz) frequency band and the 300–500 ms time window range. This TFROI was established by examination of target condition grand-mean TFR amplitudes and selecting a region bounding an observed stimulus evoked increase in theta band energy (c.f. Fig. 1b) which relates to a subcomponent of the P300 event related potential. In a similar way to the P300 ERP amplitude the theta band ERO phenotype showed a significant age effect with amplitude decreasing at a rate of 0.05μV/year (over the 16–75 year age range); therefore, age was included as a covariate in the genetic analyses.

Genotyping

Genotyping was performed at Washington University and Indiana University (Reich et al. 1998). Publicly available databases (NCBI) were used to identify SNPs within and nearby the *CHRM2* gene. PCR primers were selected using the MacVector 6.5.3 program (Oxford Molecular Group, Inc.) to give 200–500 bp genomic fragments containing the SNP. All of the SNP genotyping was performed using a Pyrosequencing method (Pyrosequencing AB). Standard PCR procedures were followed to generate PCR products. The sequence of the PCR product was entered into the Pyrosequencing Primer Design program to select sequencing primers for the SNP assay.

Linkage Analysis

Linkage analysis was performed using the variance component linkage package SOLAR (Almasy and Blangero 1998). SOLAR uses a variance–covariance matrix for each pedigree which depends on the predicted proportion of genes shared identity-by-descent (IBD) at a hypothesized quantitative trait locus, QTL; this proportion is also dependent on the proportion shared IBD at genotyped microsatellite markers and on the type of relative pair. Maximum likelihood estimates for the variance component parameters were obtained, and a LOD score computed as log 10 of the likelihood ratio comparing two models: a model where the additive genetic variance for the QTL is estimated versus a model where this variance is constrained to be zero (no linkage). Variance component linkage analyses were carried out at 1 cM intervals across all chromosomes. Exact IBD matrices were calculated using Genehunter (Kruglyak et al. 1996). The analyses were performed using the t-distribution rather than the multivariate normal distribution, since the t-distribution is less susceptible to distributional violations caused by slight kurtosis observed in the phenotype data (Blangero et al. 2001). Gender, gender-specific age, age, and age-squared data were incorporated in the analysis as covariates and retained when a likelihood ratio test was deemed

significant ($P < 0.1$) for these covariates. The initial genome-wide linkage scan of ERO phenotypes was performed employing the entire COGA dataset available with electrophysiological data (1312 individuals from 251 families).

Measured Genotype Association Analysis

Measured genotype association analyses directly test for correlations between phenotype and genotype and are based on identity-by-state (IBS) allele sharing. The additive measured genotype model assumes that each dose of the variant allele has an equal effect on a trait mean and places the heterozygote mean halfway between the homozygotes, thereby allowing a test of the trait mean variation with genotype. This is a conventional measured genotype association approach (Boerwinkle et al. 1986) which tests for differences in the trait mean by genotype, taking into account the relatedness of the individuals, and is therefore susceptible to false positives caused by population stratification. To curtail possible population stratification issues the measured genotype association analysis was constrained to a Caucasian-only subset of the dataset (1049 individuals from 209 families).

The Quantitative Pedigree Disequilibrium Test (QPDT)

To further test the evidence of association between the CHRM2 gene SNPs and the ERO phenotypes, the QPDTPHASE option of the UNPHASED suite of programs was utilized (Dudbridge 2003). This method is an implementation of the quantitative trait pedigree disequilibrium test (PDT) (Monks and Kaplan 2000) with extensions to deal with haplotypes and missing data. Despite being a less powerful test than the population-based measured genotype association method it is less susceptible to false positives arising from population stratification. The PDT is a generalization of the transmission disequilibrium test (TDT) to large pedigrees by defining a measure of LD for each triad and discordant sib-pair and averaging to obtain a measure for each pedigree. The quantitative PDT therefore provides measures of association of quantitative phenotype measures within a pedigree conditional on the genotypes of related family members. Two statistics are provided by the QPDTPHASE program: the SUM and the AVG statistics. The SUM statistic gives greater weight to larger pedigrees than the AVG statistic, which weights all pedigrees equally.

Issues of Power in the Genetic Analyses

This is the largest family study of its type for genetic research on electrophysiological parameters. However, linkage studies of quantitative risk factors require very large sample sizes. As such, our power to detect linkage is good for loci of large effect but weak for loci of relatively small effect. This means that we are likely to detect the most influential loci through our linkage analyses but also that we are likely to have missed some loci of interest. We have better power to detect loci of smaller effect through association analyses, provided one or more of our genotyped markers is in LD with a functional variant. Given this, we may detect different loci using linkage and association methods depending on the effect size of the underlying locus and the strength of disequilibrium between genotyped markers and functional variants. A variant of large effect may be detected through linkage and not by association due to lack of disequilibrium with the genotyped markers. A variant of small effect, on the other hand, could provide much stronger signals in an association analysis than in a linkage analysis.

Results

Figure 1 demonstrates the temporal and spatial nature of post-stimulus evoked oscillations during a three-stimulus visual oddball paradigm (at the frontal midline electrode Fz). The paradigm consists of rarely occurring target and novel stimuli as well as frequently occurring non-target stimuli. Visual inspection of the data reveals the presence of fronto-centrally distributed evoked theta activity during the target and novel conditions but not during the non-target condition (Fig. 1); therefore, genetic analyses were performed using the midline theta (4–5 Hz) ERO subcomponents of the P3. A genome-wide linkage screen resulted in a significant linkage peak on chromosome 7q near marker D7S509 for the target condition ERO phenotype derived from the central midline electrode Cz (LOD = 3.6) and the frontal midline electrode Fz (LOD = 3.16). These linkage peaks are depicted in Fig. 2. The *CHRM2* gene is located directly under the linkage peak and is a strong biological candidate gene. We have previously reported significant association results for a single SNP in the *CHRM2* gene using a similar theta ERO phenotype in the same dataset (Jones et al. 2004); however, as mentioned in the introduction, here we show stronger evidence of association using many additional SNPs.

It is worth noting that we have concentrated solely on the *CHRM2* gene and do not analyze the other cholinergic muscarinic genes on the genome (M1, M3, M4 and M5) since our analysis is driven by the observed linkage findings and is not a strict candidate gene approach. This gene finding strategy is recommended since it narrows down the number of candidate genes to study (in our case to one gene), which in turn alleviates the multiple comparison problem. Also, linkage analysis is not subject to population

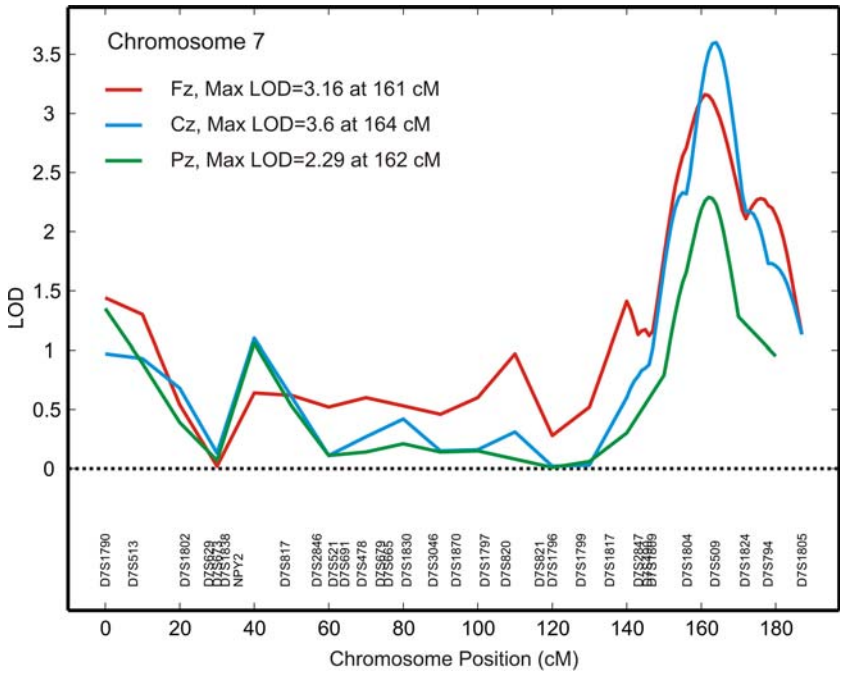

Fig. 2 LOD score plots of linkage results on chromosome 7. Results depicted for the target case theta band ERO phenotype calculated using the full COGA dataset and for the Fz, Cz, and Pz electrodes (frontal, central and parietal midline locations). Exact IBD matrices were calculated using Genehunter; genome-wide LOD scores were estimated via variance component linkage analysis using SOLAR. The full COGA dataset (with available EEG data) consists of 1312 individuals in 251 families

stratification issues which may adversely affect population-based association strategies (Vink and Boomsma 2002).

The relative positions of the 16 genotyped *CHRM2* SNPs and the gene structure are depicted in Fig. 3. A measured genotype test of association of the *CHRM2* SNPs was used to test association between the phenotype and an additive genotype model. Since this type of test can be subject to population stratification issues, the dataset was reduced to the largest ethnic subpopulation: Caucasian. Table 1 outlines the results of the association analyses which indicate association between several SNPs flanking exon 4 of the *CHRM2* gene and target condition EROs derived from the Fz and Cz electrodes. Although no significant or suggestive linkage findings were observed for the non-target and novel condition data we also tested these data for association with the *CHRM2* SNPs as a secondary analysis; no significant associations were observed with these data.

Pair-wise estimates of disequilibrium (Devlin et al. 1996; Abecasis and Cookson 2000) between the *CHRM2* SNPs are illustrated in Fig. 4. In general three regions of high LD ($|D'| > 0.75$) can be identified (Fig. 4a) with one group extending from rs324640 to rs324656 (10 kb downstream of the 3′-UTR), another group encompassing SNPs from rs2350780 to rs2350786, and a third LD group including the SNPs rs1424558, rs1424574 and rs1424569. Measures of statistical association between the SNPs afforded by the δ^2 measure suggest that the middle group of SNPs form a haplotype block (Fig. 4b). The SNPs showing the strongest association with the ERO phenotype (Table 1) exhibit similar allele frequencies and are in strong LD with one another. The results of a pedigree based

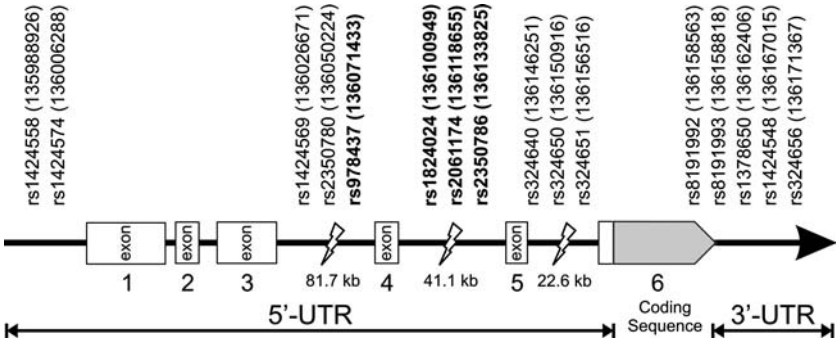

Fig. 3 Location of SNPs within and flanking the *CHRM2* gene on chromosome 7q. The gray boxed region represents the coding sequence of the gene. The values given in brackets are the base-pair locations of each SNP as given by the NCBI human genome map (build 35). All SNPs were analyzed using an additive model measured genotype association test with the theta band ERO phenotype. SNPs highlighted in bold were further analyzed using a haplotype pedigree disequilibrium test

Table 1 *CHRM2* SNP measured genotype association analysis results for three midline electrode sites

Additive measured genotype SNP model	(allele frequencies)	Fz	Cz	Pz
rs1424558	(C:0.36/G:0.64)	–	–	–
rs1424574	(C:0.15/T:0.85)	0.04	0.04	–
rs1424569	(A:0.48/G:0.52)	–	–	–
rs2350780	(A:0.64/G:0.36)	0.01	0.05	–
rs978437	(A:0.68/G:0.32)	**0.0003***	0.008	0.04
rs1824024	(G:0.33/T:0.67)	**0.00009****	**0.001***	0.01
rs2061174	(C:0.34/T:0.66)	0.003	0.02	–
rs2350786	(A:0.29/G:0.71)	0.002	0.002	0.02
rs324640	(C:0.45/T:0.55)	–	–	–
rs324650	(A:0.44/T:0.56)	–	–	–
rs324651	(G:0.88/T:0.12)	–	–	–
rs8191992	(A:0.41/T:0.59)	–	–	–
rs8191993	(C:0.70/G:0.30)	–	0.008	0.02
rs1378650	(C:0.58/T:0.42)	–	0.03	–
rs1424548	(A:0.38/G:0.62)	0.04	0.02	–
rs324656	(C:0.36/T:0.64)	–	–	–

Significant genetic association *P*-values (uncorrected for multiple tests) are provided for Caucasian-only visual oddball target condition theta band data and the *CHRM2* gene SNPs. These results are obtained using a population based additive measured genotype SNP model in which AA SNP genotypes are coded as −1, Aa genotypes are coded as 0 and aa genotypes are coded as 1. Age, gender, age-squared and age by gender were included in the model as fixed effects on the phenotype trait mean. *P*-values highlighted in bold are significant after application of a Bonferroni correction for multiple tests (*$P < 0.05$; **$P < 0.01$). The Caucasian-only dataset (with available EEG data) consists of 1049 individuals in 209 families. Association tests were performed using data from the frontal midline (Fz), central midline (Cz) and posterior midline (Pz) electrodes

test of association using a haplotype created with these high LD and significant SNPs is summarized in Table 2. These analyses reveal that the most common haploptye is associated with reduced theta activity, while the second most common haplotype results in increased evoked theta.

Discussion

Using electrophysiological measures we have demonstrated genetic association of target condition evoked theta EROs with SNPs from the regulatory regions of the cholinergic muscarinic M2 receptor gene (*CHRM2*). Common variants in this gene are associated with differences in these measures. However, these SNPs are located within introns of the *CHRM2* gene and are not associated any exonic variation in the gene. This suggests that the differences in theta EROs result from changes in *CHRM2* gene regulation rather than protein sequence. In this regard, we note that the most highly associated SNPs lie within a 30 kb region flanking exon 4, an alternatively spliced exon. The significance of the extensive alternative splicing of the 5′ UTR of the *CHRM2* gene remains unclear but may underlie the variation in theta EROs reported in this paper.

Behavioral and pharmacological evidence indicates that the cholinergic system is important for the generation of the P3. Administration of drugs known to influence the cholinergic system in humans such as carbachol (cholinergic agonist), atropine and scopolamine (anticholinergic agents) have been shown to modify P3 amplitude (Mohs and Davies 1985; Hammond et al. 1987; Meador et al. 1987; Dierks et al. 1994; Potter et al. 2000). Disorders which exhibit a modified P3 response such as Alzheimer's disease, Parkinson's disease and schizophrenia (Polich and Herbst 2001) are known to manifest abnormalities in the cholinergic system (Perry and Perry 1995). Also, it has been hypothesized that impairment to the cholinergic system may result in the reduced ability to detect, select, discriminate and process relevant stimuli and the eventual decline of memory functions characteristic of diseases such as Alzheimer's (Sarter and Bruno 1999). It is expected, therefore, that variation in measures characterizing the P3, which are mediated by attentional processing, working memory, decision making and response selection, may reflect the influence of the cholinergic system.

Neural oscillatory responses have been attributed to various cognitive processes in the literature. Delta responses are considered to mediate signal detection and decision-making (Basar et al. 1999; Schurmann et al. 2001), while theta rhythms have been attributed with attention, recognition memory, and episodic retrieval (Klimesch et al. 1997; Doppelmayr et al. 1998, 2000; Basar et al. 2001; Klimesch et al. 2001). The theta component of the P3 response may be of particular relevance in relation to the cholinergic system in light of recent neurophysiological data acquired from experiments on rat brains. In vitro studies have suggested that the presence of a cholinergic agonist in the rat hippocampus induces oscillations in the delta, and theta frequency range (Fellous and Sejnowski 2000). In vitro administration of high concentrations of the muscarinic agonist carbachol in the rat hippocampus has been shown to induce short episodes of theta oscillations (Fellous and Sejnowski 2000). Theta band oscillations have also been induced in rat neocortical slices by use of cholinergic agonists (Lukatch and MacIver 1997). Results from these studies fit the pharmacological and physiological model of an ascending cholinergic pathway originating in the brain stem which modulates oscillations likely to be implicated with arousal, sensorimotor processing, learning and memory. In addition, scalp and intracranially recorded human theta rhythms have been observed during verbal and spatial memory tasks, and it has been suggested that theta synchronization across different brain regions is characteristic of high level cognitive 'top-down' processes (von Stein and Sarnthein 2000).

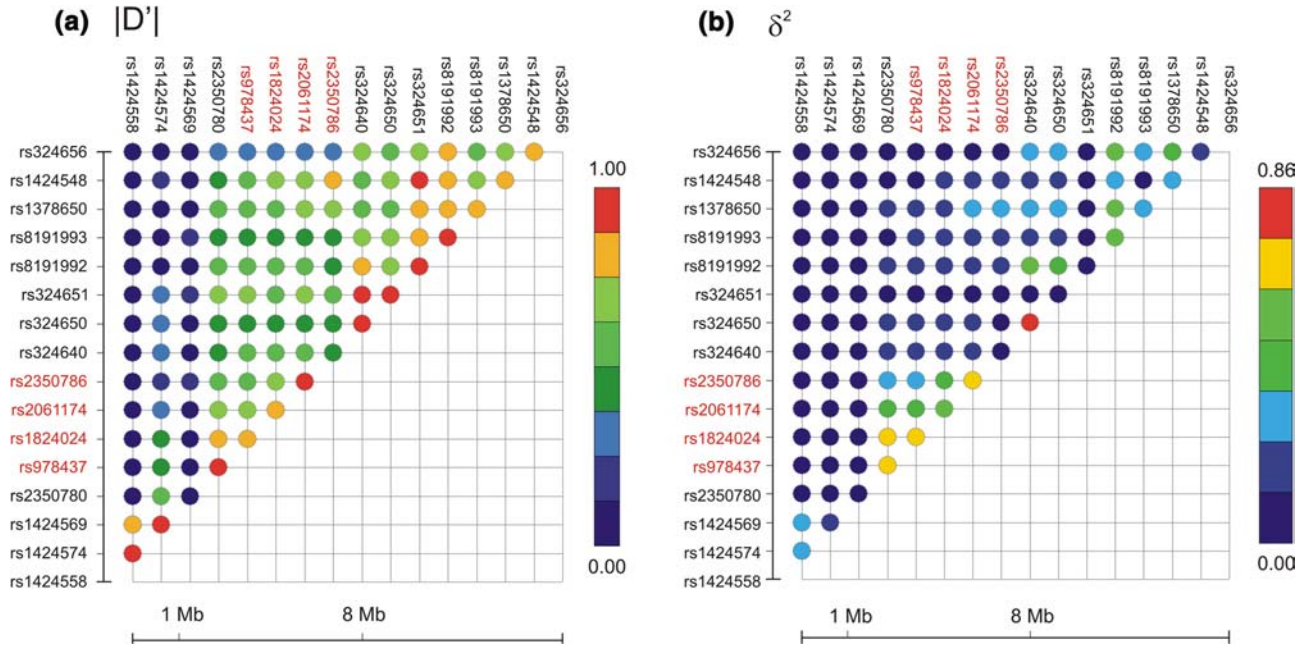

Fig. 4 Pattern of pairwise LD within and flanking the *CHRM2* gene. The colored circles represent the estimated value of pairwise LD between the corresponding two SNPs using (**a**) Lewontin's |D′| and (**b**) δ^2 measures of LD. Lewontin's |D′| is a popular measure of LD which includes rates of recombination, but which can be upwardly biased when measured using small sample sizes. The δ^2 LD measure represents a statistical association between the SNPs and can be more useful in dividing closely spaced SNPs into blocks; however δ^2 is not related to the recombination fraction. SNP names in red denote SNPs used in a haplotype analysis of association with the theta band ERO phenotype

The predominantly presynaptic nature of the cholinergic M2 receptors in the basal forebrain indicates that M2 receptors may be involved in the inhibitory mediation of many high order cognitive functions through the inhibition of the presynaptic release of acetylcholine. The notion that M2 autoreceptor modulation of acetylcholine is important for cognitive function is supported by recent genetic association studies which have reported significant findings between cholinergic M2 receptor gene SNPs with an alcoholism and depression diagnosis (Wang et al. 2004). A finding that has been replicated using an independent case–control sample (Luo et al. 2005). Also, a recent study of M2 receptor knock-out mice performing maze tests indicates that these mice manifest deficits in behavioral flexibility and working memory (Seeger et al. 2004). In addition, it has been found that pharmacological blocking of M2 autoreceptors in the prefrontal cortex of the mouse results in increased release of acetylcholine which also coincides with EEG activation in the prefrontal cortex and modulation of EEG slow waves and spindles (Douglas et al. 2002). The origin of acetylcholine in the mouse prefrontal cortex is found to mostly arise from basal forebrain cholinergic neurons (Kitt et al. 1994). These data provide support for the idea that muscarinic M2 autoreceptor inhibitory modulation of acetylcholine release influences cortical excitability by promoting relevant

Table 2 *CHRM2* SNP haplotype analysis

rs978437, rs1824024, rs2061174, rs2350786	Founder haplotype frequencies	Target AVG QPDT		Target SUM QPDT	
		Z	*P*-value	*Z*	*P*-value
A-T-T-G	0.66	−2.932	**0.003***	−2.958	**0.003***
G-G-C-A	0.16	3.032	**0.0025***	3.023	**0.0025***
G-G-T-G	0.06	0.996	0.319	−0.731	0.465
Frequencies less than 3%	0.12	−0.229	0.819	−0.278	0.781

Results obtained using a family-based QPDT of association with the Caucasian-only theta band target condition data derived from the Fz electrode (frontal midline location). Results are obtained using the AVG statistic which gives equal weight to all the pedigrees in the analysis regardless of pedigree size and the SUM statistic which gives a greater weight in the analysis to larger sized pedigrees. Global AVG test χ^2 12.5, $\boldsymbol{P = 0.0019}$**; Global SUM test χ^2 12.3, $\boldsymbol{P = 0.0021}$**. *P*-values highlighted in bold are significant after application of a Bonferroni correction for multiple tests (*$P < 0.05$; **$P < 0.01$)

cholinergic and glutamatergic neurotransmission, and that this modulation is relevant to the facilitation of cognitive processes (Quirion et al. 1995).

Our genetic association findings suggest that evoked theta oscillations are in part mediated by cholinergic M2 autoreceptors in the basal forebrain, a region known to be involved in high-order cortical functions. It is feasible that M2 modulation of acetylcholine release has a role in inhibiting cortical subsystems which are irrelevant to the processing of the target condition and thereby facilitating the promotion of the relevant systems. Therefore, we hypothesize that the genetic findings presented here may be interpreted in terms of the threshold regulation theory of P3 generation (Elbert and Rockstroh 1987) in which cholinergic M2 receptor modulation of the acetylcholine system provides the necessary inhibition of irrelevant subsystems in order to achieve the proper target condition response. Hence, these findings support a role for CNS inhibition in the production of the P3 response. Also, the significant genetic linkage and association results were found using measures derived from target condition data, and importantly, equivalent findings were not obtained using data from the novel and non-target conditions. The target condition requires additional processing functions over the non-target and novel conditions. Given the strong evidence linking human theta rhythms with working memory performance (Klimesch 1997; Doppelmayr et al. 1998, 2000; Klimesch et al. 2001) it is tempting to propose that the increased working memory demands needed to complete the target condition require increased suppression of the irrelevant brain networks which is mediated by cholinergic M2 receptors in the basal forebrain. It should be noted, however, that the major operating oscillations, such as theta, may be context sensitive and change their functional roles depending on the nature of the experiment (Basar et al. 2001). Therefore, the findings presented here may not only be specific to the task condition but also to the experiment design and demands.

The use of neuropsychiatric genetic data, such as those outlined in this article, in combination with neurochemical and neuroanatomical information, have the potential to unravel the complex interplay of the subsystems which are relevant to the generation of brain oscillations evoked under differing cognitive conditions. We expect, therefore, that the identification of genes which regulate our mental processes will be of enormous benefit to the field of psychiatric genetics and the study of psychiatric and neurological disorders and concomitant dysfunctions. Recent studies have indicated that the predisposition towards alcoholism, major depression, drug dependence and affective disorders is influenced by variations in the same region of the *CHRM2* gene as influencing the ERO phenotype presented here (Wang et al. 2004; Luo et al. 2005). These data support the notion that carefully chosen brain oscillations may be adopted as endophenotypes in psychiatric genetics since they can reflect the liability of many psychiatric disorders. As with all genetic studies of complex phenotypes caution must be advised until the findings are supported and replicated using an independent data sample.

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