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Endophenotypes Successfully Lead to Gene Identification: Results from the Collaborative Study on the Genetics of Alcoholism

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The use of endophenotypes has been proposed as a strategy to aid gene identification efforts for complex phenotypes [Gottesman, I. I., and Shields J. (1972). *Schizophrenia and Genetics: A Twin Study Vantage Point*. London: Academic]. As part of the Collaborative Study of the Genetics of Alcoholism (COGA) project, we have analyzed electrophysiological endophenotypes, in addition to clinical diagnoses, as part of our effort to identify genes involved in the predisposition to alcohol dependence. In this paper we summarize published results from linkage and association analyses of two chromosomal regions in which the use of endophenotypes has successfully led to the identification of genes associated with alcohol dependence [*GABRA2* (Edenberg *et al*., (2004). *Am. J. Hum. Genet*. **74**:705–714) and *CHRM2* (Wang *et al*., (2004). *Hum. Mol. Genet*. **13**:1903–1911)]. Our experience in the COGA project has been that the analysis of endophenotypes provides several advantages over diagnostic phenotypes, including the strength and localization of the linkage signal. Our results provide an illustration of the successful use of endophenotypes to identify genes involved in the predisposition to a complex psychiatric phenotype, a strategy originally proposed by Gottesman and Shields in 1972.

KEY WORDS: Alcohol dependence; association; EEG; endophenotype; genetics; linkage.

INTRODUCTION

Identifying genes involved in complex human behaviors and clinical disorders has proven difficult. Early enthusiasm (Egeland *et al*., 1987; Gershon *et al*., 1988) was quickly curtailed (Kelsoe *et al*., 1989), as it became apparent that the strategy successfully employed to identify genes for many single-gene disorders would not be nearly as useful for identifying most of the genetic variation contributing to complex phenotypes. Many strategies have been adopted in gene identification efforts to deal with the complexities introduced by studying disorders that are believed to have multifactorial, polygenic origins, rather than simple Mendelian patterns of inheritance. One strategy that has been proposed is the use of endophenotypes.

Psychiatric diagnoses were formulated for the purpose of clinical classification and communication between care providers. Diagnoses are based on observable symptoms and there is considerable

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heterogeneity within any given diagnosis. Because there is likely a complex cascade of events between the genetic underpinnings of a disorder and the eventual manifestation of symptoms, it is not clear that clinical diagnoses are the best phenotype for use in genetic analyses. A more optimal phenotype for genetic analyses might be an intermediary measure of neuropsychiatric functioning that is involved in the pathway between genotype and the outcome of interest. These intermediary phenotypes have been termed endophenotypes. The concept of endophenotypes was first applied to psychiatric disorders by Gottesman and Shields (Gottesman and Shields, 1972), and a recent review provides an excellent overview of the rationale for using endophenotypes in gene identification efforts in psychiatry (Gottesman and Gould, 2003). Several criteria have been delineated for a biological marker to represent an appropriate endophenotype for genetic analyses (Gottesman and Gould, 2003). The marker should be associated with the illness. In addition, it should be found in higher rates in the unaffected relatives of affected individuals than in the general population. The marker should be heritable, and there should be a genetic correlation between the trait and disorder, indicating that shared genes are contributing to the observed relationship (de Geus and Boomsma, 2001).

COGA is a multidisciplinary collaborative project with the goal of identifying genes involved in alcohol dependence and related phenotypes, such as quantitative indices of alcohol use and other psychiatric disorders, such as depression, that are commonly comorbid with alcohol dependence. Although alcohol dependence clearly has a genetic component, with heritabilities in the range of 50–60% for both men and women (Heath, 1995; McGue, 1999), it embodies many of the complexities inherent to gene identification efforts for most psychiatric disorders. Many genes are thought to be involved, each one likely only contributing a small effect. The environment clearly plays an important role in the development of drinking patterns and dependence symptoms (Rose *et al*., 2001b; Rose *et al*., 2003). There is evidence of gene-environment interaction associated with alcohol dependence (Dick *et al*., 2001; Heath *et al*., 1989; Koopmans *et al*., 1999; Rose *et al*., 2001a). Finally, there is substantial heterogeneity among alcohol dependent individuals (Cloninger, 1987; Finn *et al*., 1997).

One strategy that the COGA project has employed to attempt to deal with these complexities is the use of electrophysiological endophenotypes as a complement to clinical diagnoses for use in genetic analyses. There is a substantial body of literature suggesting that electrophysiological measures represent relevant endophenotypes for alcohol dependence. It has been proposed that the genetic predisposition to alcohol dependence may involve central nervous system (CNS) disinhibition/hyperexcitability (Begleiter and Porjesz, 1999), and electrophysiological abnormalities may reflect this CNS disinhibition. Abnormalities in alcoholics and their family members are found both in the human electroencephalogram (EEG), as well as evoked EEG rhythms, or event-related potentials (ERPs), such as the P300 response. The electrophysiological endophenotypes used in linkage analyses described in this paper focus on the beta frequency band of the human EEG, and the delta and theta frequency band evoked oscillations, which are the primary constituents of the P300 evoked component elicited during cognitive processing of stimuli (Basar *et al*., 1999; Yordanova and Kolev, 1996). Several lines of evidence suggest that these traits may represent useful endophenotypes indexing familial risk for alcohol dependence. The beta frequency band of EEG is highly heritable, with heritability estimated at 86% (van Beijsterveldt *et al*., 1996). Thus, this endophenotype is more highly heritable than alcohol dependence diagnoses themselves (McGue, 1999). In the COGA sample, increased beta power in all three bands of resting EEG has been observed in alcohol dependent individuals, as compared to controls (Rangaswamy *et al*., 2002, 2003). An increase in beta power has also been observed in the offspring of male alcoholics, further suggesting this may be a marker of an inherited predisposition to alcohol dependence (Rangaswamy *et al*., 2004b). Several studies have demonstrated that a reduced P300 amplitude is associated with the risk for alcoholism (e.g., see (Begleiter *et al*., 1984; Carlson *et al*., 2004; Reese and Polich, 2003)). Recent evidence indicates reduced theta and delta oscillations in alcohol dependent individuals and individuals at risk (For reviews, see (Porjesz and Begleiter, 2003; Porjesz *et al*., 2004; Porjesz *et al*., in press)). Differences in P300 correspond to activation differences in the bilateral inferior parietal lobule and the bilateral inferior frontal gyrus on functional magnetic resonance imaging tests (fMRI), signifying that a dysfunctional frontoparietal circuit may be responsible for the reduced P300 found in subjects at high risk for alcoholism (Rangaswamy *et al*., 2004a). Taken together, these results suggest that electrophysiological endophenotypes may

represent biological markers of the genetic predisposition to alcohol dependence and may have utility in genetic analyses. Another useful quality is that these endophenotypes are quantitative traits, potentially allowing for more powerful tests of linkage.

The COGA analytic strategy has been to use both electrophysiological endophenotypes (Almasy *et al*., 2001; Begleiter *et al*., 1998; Ghosh *et al*., 2003) and clinical diagnostic phenotypes (Foroud *et al*., 2000; Reich *et al*., 1998), as well as novel alcohol-related phenotypes (e.g., maximum number of drinks in a 24 hour period (Saccone *et al*., 2000), alcohol factor scores (Dick *et al*., 2002), and an alcohol symptom severity phenotype (Foroud *et al*., 1998)) in genetic analyses. The study design employed by COGA has been to ascertain large families, densely affected with alcohol dependence; to conduct non-parametric linkage analyses to identify chromosomal regions likely to contain genes predisposing to alcohol dependence and related phenotypes; and to conduct family-based association analyses on candidate genes within those regions of interest to identify the specific genes involved. We have chosen initially to focus on regions in which the electrophysiological endophenotypes and clinical phenotypes show (loosely) converging evidence of linkage. This strategy has successfully led to the identification of several genes that show significant association with alcohol dependence and related psychiatric phenotypes. An overview of the results from two of the most promising chromosomal regions that we have investigated to date, on chromosomes 4 and 7, is presented here.

METHODS

Sample

Families were identified through probands in inpatient and outpatient alcohol dependence treatment centers at six sites across the United States: Indiana University, State University of New York Health Science Center, University of Connecticut, University of Iowa, University of California/San Diego, and Washington University, St. Louis. The institutional review boards of all participating institutions approved the study. Probands were invited to participate if they had a sufficiently large family (usually sibships >3 with parents available) with two or more members in any of the COGA catchment areas (Reich, 1996). A total of 1227 families of alcohol dependent probands were recruited for the first stage of the study. All individuals were administered the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) interview, which is a polydiagnostic instrument that assesses most major psychiatric disorders (Bucholz *et al*., 1994; Hesselbrock *et al*., 1999). Families that had at least two affected first degree relatives in addition to the proband (excluding probands who were the offspring of two affected parents) were invited to participate in the more intensive stage of the study. In these families, all first degree relatives of affected individuals and connecting family members were assessed, along with their mates, if the union had produced offspring. Second and third degree relatives in the families were assessed when they were considered to be informative for the genetic linkage studies. A total of 987 adult individuals from 105 extended families were included in the initial genotyped data set (Reich *et al*., 1998). A replication sample was ascertained and genotyped following identical procedures; it consisted of 1295 individuals from 157 extended families (Foroud *et al*., 2000). Thus, a total of 2282 individuals from 262 multiplex alcoholic families are available for genetic analyses. An average of 9 individuals were genotyped per family. Of the 2282 genotyped individuals, 895 met criteria for the COGA definition of alcoholism used for recruitment (DSMIIIR affection plus Feighner definite alcoholism); these individuals created 491 affected sibling pairs (n-1) (Foroud *et al*., 2000). The complete manual detailing COGA ascertainment is available on-line at http://zork.wustl.edu/niaaa/.

All individuals who participated in the intensive stage of the study underwent a more extensive protocol, in addition to the SSAGA interview. This stage involved obtaining blood for genetic analyses, and an electrophysiological protocol, including EEG and a battery of auditory and visual evoked potentials. EEG was recorded using the 19 channel montage, as specified according to the 10–20 international system. EEG data were collected in the awake, eyes-closed condition at a sampling rate of 256 Hz for 4.25 minutes (see (Porjesz *et al*., 2002) for additional details on the procedure). EROs were measured in response to a visual oddball paradigm in which three types of visual stimuli were presented: target, non-target, and novel. 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 (complete details on the procedure provided in (Jones *et al*., 2004)).

Genotyping

Genotyping for the COGA project is carried out in laboratories at Indiana University and Washington University, St. Louis, as described previously (Reich *et al*., 1998). Standard methods for pedigree checking and generating recombination based marker maps have been employed (Foroud *et al*., 2000). The initial genome scan of COGA pedigrees included 292 markers with an average intermarker distance of 13.8 cM (Reich *et al*., 1998). Regions with gaps >20 cM were typed more extensively, as were regions with suggestive linkage in the initial genomic scan. In addition, the marker maps have been rebuilt several times as physical position information became available from NCBI. Thus, the marker maps and positions differ slightly between many of the analyses reviewed here from papers published at different points in this on-going study.

For association analyses, SNPs were chosen across each candidate gene from public databases; we did not restrict ourselves to coding regions or exons, because allele frequencies for such SNPs are often low. In addition, we hypothesized that many SNPs involved in these complex phenotypes would be regulatory. We aimed for even spacing and full coverage of each of the genes being tested. Locations were in most cases determined from the annotations in the NCBI human genome assembly. SNP genotyping has been conducted using a Pyrosequencing method or using a modified single nucleotide extension reaction, with allele detection by mass spectrometry (Sequenom MassArray system; Sequenom, San Diego, CA).

Statistical Analyses

In general, nonparametric allele-sharing linkage methods for affected sibling pairs and extended pedigrees have been used in analyses of alcohol dependence in the COGA sample. The program ASPEX (Hinds and Risch, 1999) has been used extensively to conduct analyses on dichotomous traits (e.g., affection status) using affected sibling pairs. Many semi-quantitative traits have been analyzed using the Haseman-Elston routine implemented in MAPMAKER/SIBS, as this method only assumes normality of the residuals of the quantitative trait, rather than normality of the trait itself. This has been a particular concern in many analyses of phenotypes in COGA, where the trait distribution has been skewed (e.g., symptom counts). Variance components methods of analysis, largely implemented in the package SOLAR, developed by a COGA co-investigator (Almasy and Blangero, 1998), have been employed to analyze most of the quantitative endophenotypes. Analyses have been conducted using the *t*-distribution option in SOLAR, since it is less susceptible to distributional violations caused by slight kurtosis observed in many of the electrophysiological endophenotypes. In general, ascertainment correction has not been applied in the analyses reported here. In early analyses of the COGA electrophysiological traits, including an ascertainment correction had relatively little impact on the analyses (see Almasy *et al*., 1999); accordingly, most subsequent analyses have not employed ascertainment correction.

Some additional details about methods used in analyses are included in the presentation of individual studies, and we refer the reader to the original publications for full details. For a nice overview of different linkage methods appropriate for analyzing quantitative traits under different conditions see (Ferreira, 2004).

RESULTS

Chromosome 4

Linkage analyses of alcohol dependence diagnoses did not detect significant evidence of linkage on chromosome 4 in either the wave 1 (Reich *et al*., 1998) or wave 2 (Foroud *et al*., 2000) COGA samples using nonparametric methods for affected relative pairs. However, using related phenotypes and varying analytic methods, this chromosome repeatedly emerged with interesting linkage findings. Figure 1 shows results of linkage analyses on chromosome 4 for the phenotype "maximum number of drinks in a 24 hour period," after log-transformation and adjustment for gender (referred to as "max drinks" throughout the remainder of the paper) (Saccone *et al*., 2000). This variable shows a heritability of approximately 50% (A. Heath, unpublished data), and was recorded as part of the SSAGA interview with the question "What is the largest number of drinks you have ever had in a 24 hour period?". Responses were log-transformed to minimize the impact of extremely high reports that were likely inflated. Genome-wide linkage analyses were performed using MAPMAKER/SIBS multipoint Haseman-Elston (all pairs unweighted) for the wave 1, wave 2, and combined COGA samples. The most consistent, strongest evidence of linkage to this phenotype emerged on chromosome 4, with a maxi-

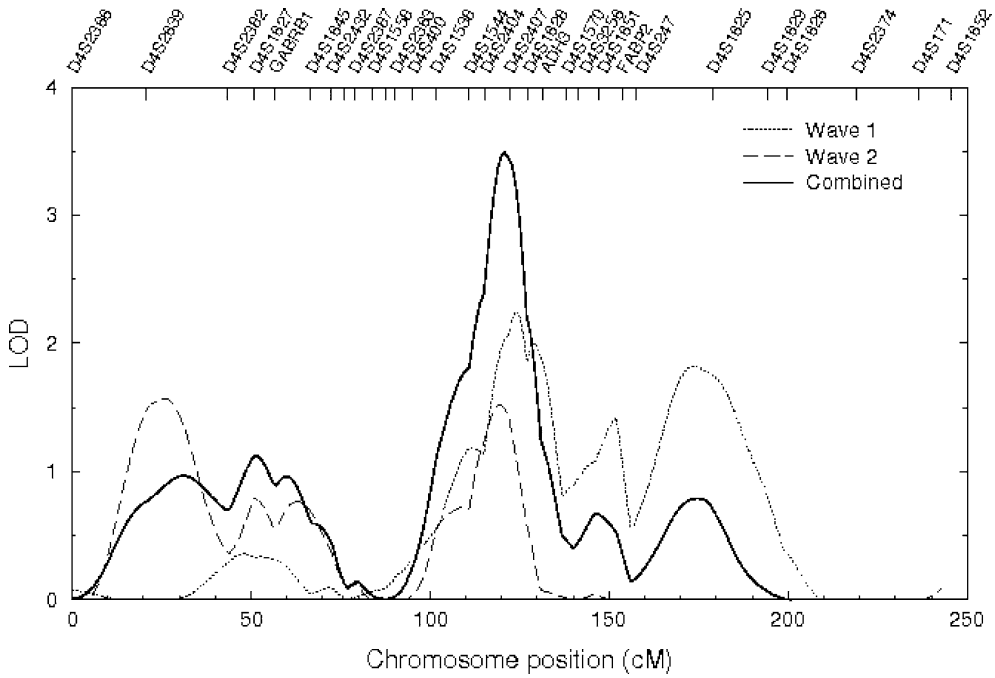

Fig. 1. Multipoint LOD scores for chromosome 4 for the phenotype "maximum number of drinks in a 24 hour period" (M), log-transformed and corrected for gender (Saccone *et al.*, 2000).

mum lod score of 3.5 in the combined sample at 121 cM near the marker D4S2407. Interestingly, analyses of the wave 1 data had shown evidence of increased allele-sharing among concordantly *unaffected* sibling pairs, and decreased allele-sharing among discordant sibling pairs, to a nearby region of chromosome 4, with a maximum multipoint lod score of 2.50 (Reich *et al.*, 1998).

Figure 2 shows the results of variance components linkage analyses performed using the program SOLAR (Almasy and Blangero, 1998) for alcoholism diagnoses using DSM-IV, ICD-10, and the COGA definition of alcoholism, which consisted of DSM-IIIR diagnoses plus Feighner criteria for alcoholism at the "definite" level (Williams *et al.*, 1999). Age and sex were used as covariates in all analyses. These analyses were performed on the Wave 1 sample of 105 pedigrees. The maximum lod score was 2.8, obtained with DSM-IV diagnoses of alcohol dependence at ~100 cM near the marker D4S1628. This is the same region of chromosome 4 that also showed linkage to max drinks. A joint multipoint linkage analysis of multivariate discrete and continuous traits was subsequently applied in a bivariate analysis of qualitative alcoholism diagnoses and quantitative event-related potentials. For these analyses, DSM-IV alcohol dependence diagnoses were analyzed jointly with the amplitude of the P300 component of the Cz event-related potential (Williams *et al.*, 1999). The linkage peak significantly increased, with a maximum lod score of 4.75.

The strongest evidence of linkage on chromosome 4 was found to the beta frequency band of the human EEG (Porjesz *et al.*, 2002). The sample included in the linkage analysis was drawn from 250 COGA families and consisted of 1553 individuals between the ages of 7 and 70. The EEG data for the beta 1 (12.5–16.0 Hz), beta 2 (16.5–20.0 Hz) and beta 3 (20.5–28.0 Hz) bands were analyzed using variance components linkage analyses, carried out in SOLAR (Almasy and Blangero, 1998). The strongest evidence of linkage was found on chromosome 4p. The peak lod score for all bands occurred at the marker GABRB1, with a maximum lod score of 5.01 obtained for the Beta 2 phenotype (Fig. 3). A combined linkage/linkage disequilibrium analysis was subsequently carried out in SOLAR to test for association between the beta 2 EEG phenotype and the GABRB1 microsatellite marker. Using this method, the lod score increased to 6.53, and significant evidence of linkage disequilibrium was found between beta 2 and the GABRB1 marker ($p = 0.004$), suggesting that a genetic variant influencing the beta 2 phenotype was in or near the GABRB1 microsatellite

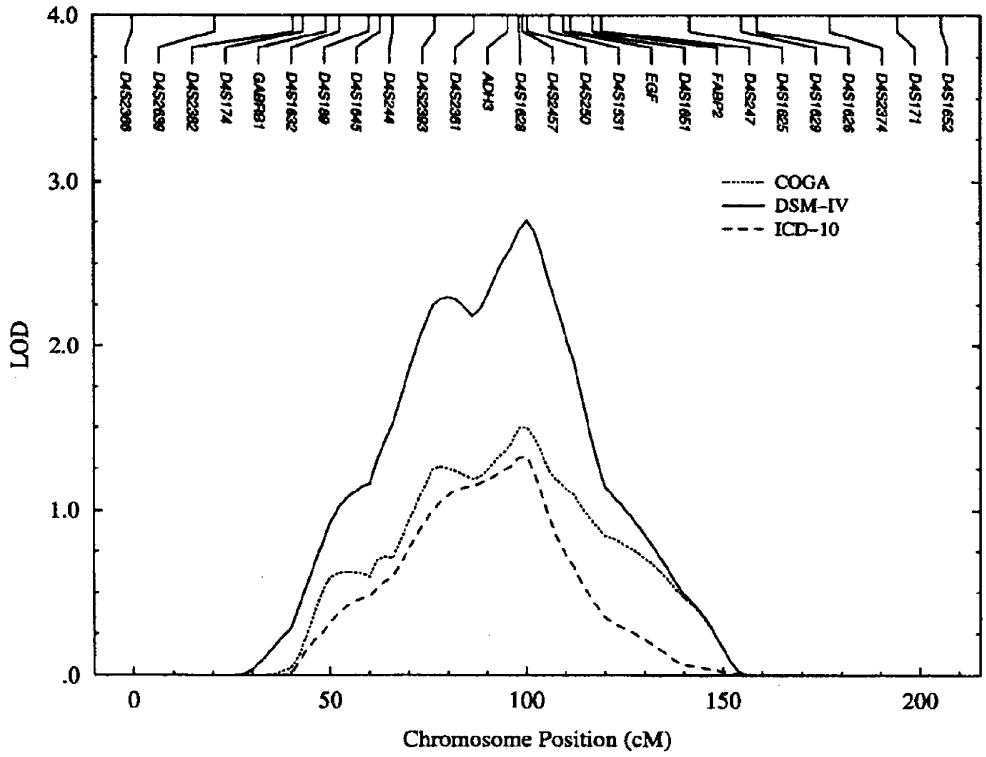

Fig. 2. LOD scores for chromosome 4 for alcohol dependence diagnoses using multiple diagnostic systems (Williams *et al.*, 1999).

marker (Porjesz *et al.*, 2002). Significant linkage with the beta 2 EEG trait was also detected on chromosome 4 using a novel nonparametric regression procedure (Ghosh *et al.*, 2003): two distinct peaks emerged, with the strongest region of linkage at the GABRB1 marker, and a slightly smaller peak near the ADH3 marker (the same region where the peak is located for the max drinks and dependence diagnosis analyses discussed above). Family-based association analyses of the microsatellite marker in GABRB1 also provided modest evidence of association with alcohol dependence in the COGA pedigrees (Song *et al.*, 2003). Analyses of alcohol dependence in an independent sample of Southwestern American Indians provided evidence of linkage to a marker very near the GABRB1 gene (Long *et al.*, 1998).

GABRB1 is located within a tight cluster of four $GABA_A$ receptor genes on chromosome 4p: *GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1*. Evidence from animal, human, and *in vitro* cell models suggests that γ aminobutyric acid (GABA), the major inhibitory neurotransmitter in the human central nervous system, is involved in many of the neurochemical pathways affecting alcohol use and related disorders. GABA is involved in several of the behavioral effects of alcohol, including motor incoordination, anxiolysis, sedation, withdrawal signs, and ethanol preference (Buck, 1996; Grobin *et al.*, 1998). $GABA_A$ receptor agonists tend to potentiate the behavioral effects of alcohol, while $GABA_A$ receptor antagonists attenuate these effects. $GABA_A$ receptors have been implicated in ethanol tolerance and dependence (Grobin *et al.*, 1998), and GABA is believed to play a role in central nervous system disinhibition related to the predisposition to alcoholism (Begleiter and Porjesz, 1999). $GABA_A$ receptors are thought to be involved in beta brain rhythms as measured by the EEG (Porjesz *et al.*, 2002). These studies, taken together with converging linkage evidence on chromosome 4, led us to believe that these genes provided strong candidates for their involvement in alcohol dependence and related (endo/) phenotypes.

To systematically explore potential association with these genes, we genotyped 69 single-nucleotide polymorphisms (SNPs) within the cluster of four $GABA_A$ receptor genes on chromosome 4 (Edenberg *et al.*, 2004). Linkage disequilibrium between the SNPs was determined using the program GOLD

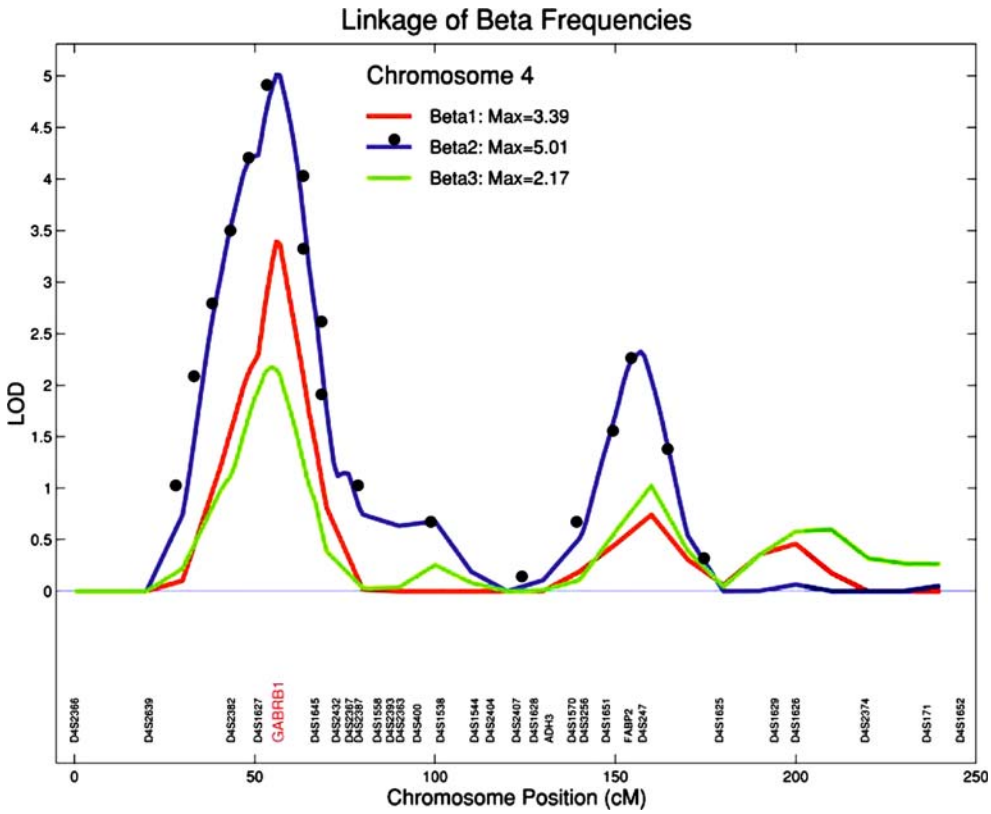

Fig. 3. LOD scores for EEG beta frequencies on chromosome 4 (Porjesz *et al.*, 2002).

(Abecasis and Cookson, 2000). LD was generally high within genes, and low between genes, allowing us to distinguish between the four tightly clustered $GABA_A$ receptor genes.

The Pedigree Disequilibrium test (PDT) (Martin *et al.*, 2000) was used to analyze association with alcohol dependence in the extended COGA pedigrees. The PDT utilizes data from all available trios in a family, as well as discordant sibships. Association analyses with the EEG Beta 2 phenotype were carried out using the measured genotype test implemented in SOLAR (Almasy and Blangero, 1998). Significant evidence of association with DSM-IV alcohol dependence was observed with 31 SNPs in *GABRA2*, but only 1 SNP in the surrounding $GABA_A$ receptor genes; significant evidence of association with EEG was observed with 25 *GABRA2* SNPs, but only 1 SNP in the flanking genes (Edenberg *et al.*, 2004). A haplotype comprised of three SNPs in *GABRA2* that showed significant evidence of association individually with both alcohol dependence and the EEG endophenotype, provided highly significant evidence of association with *GABRA2*. Additionally, systematic, sliding-window haplotype analyses found 43 consecutive three SNP haplotypes that were significant across *GABRA2*. Significant association between *GABRA2* and alcohol dependence has subsequently been replicated by two independent research groups (Covault *et al.*, 2004; Kranzler *et al.*, 2004; Xu *et al.*, 2004). We are currently investigating functional differences associated with genetic variation in *GABRA2*, in order to determine how this gene is involved in the predisposition to alcohol dependence.

Chromosome 7

In the initial Wave 1 COGA sample of 105 pedigrees, chromosome 7 provided the strongest evidence of linkage to alcohol dependence (Reich *et al.*, 1998). Using the COGA criteria for alcohol dependence, the maximum multipoint lod score on chromosome 7 was 3.49 near the marker D7S1793, using the SIBPHASE option of ASPEX (Hinds and Risch, 1999). Subsequently, an additional 10 markers were genotyped on chromosome 7. The multipoint

LOD score from sibling pair linkage analyses dropped to 2.0; however, the Wave 2 sample of COGA also showed modest, consistent evidence of linkage to chromosome 7 (Foroud *et al*., 2000). When the samples were combined for analysis, the maximum lod score shifted to a more distal marker, peaking at 2.9 near the marker D7S821 (Fig. 4).

There was also significant evidence of linkage to electrophysiological endophenotypes on chromosome 7. The event-related theta band of the P300 event related potential showed significant evidence of linkage on chromosome 7, with a maximum lod score of 3.5 between the markers D7S1837 and D7S509 (Fig. 5) (Jones *et al*., 2004). The linkage peak observed with the frontal theta band oscillation of the P300 ERP was located directly at a muscarinic cholinergic receptor gene, *CHRM2*. The muscarinic cholinergic receptors are G-protein coupled receptors that influence the effects of acetylcholine in the central and peripheral nervous system; therefore, they are expected to have a direct influence on the P300 generation (Frodl-Bauch *et al*., 1999). The muscarinic cholinergic receptors are also thought to be involved in cognition and memory (Comings *et al*., 2003). Recently, significant deficits in behavioral flexibility, working memory, and hippocampal plasticity were observed in *CHRM2* knockout mice (Seeger *et al*., 2004). These findings are relevant because ERP components have been associated with a variety of higher cognitive processes such as object recognition, motor planning, working memory, and inhibitory executive control (Ruchkin *et al*., 1995). Similarly, specific frequency rhythms of oscillatory responses have been attributed to underlie various cognitive processes, with delta involved in signal detection and decision-making (Basar *et al*., 1999) while theta is involved in conscious awareness, recognition memory, and episodic retrieval (e.g., Klimesch *et al*., 1994). These observations are consistent with a role of *CHRM2* for theta and delta event-related oscillations.

Accordingly, a small number of SNPs in and around the *CHRM2* gene were tested in the COGA sample. Significant linkage disequilibrium was observed with a SNP in *CHRM2* for the theta band endophenotype (Jones *et al*., 2004); inclusion of the SNP as a covariate in the linkage analyses produced an ~10% LOD score drop for the theta band phenotypes, suggesting that this SNP played some functional role in the endophenotype. In addition to our report of linkage disequilibrium between event-related theta band oscillations and *CHRM2*, an

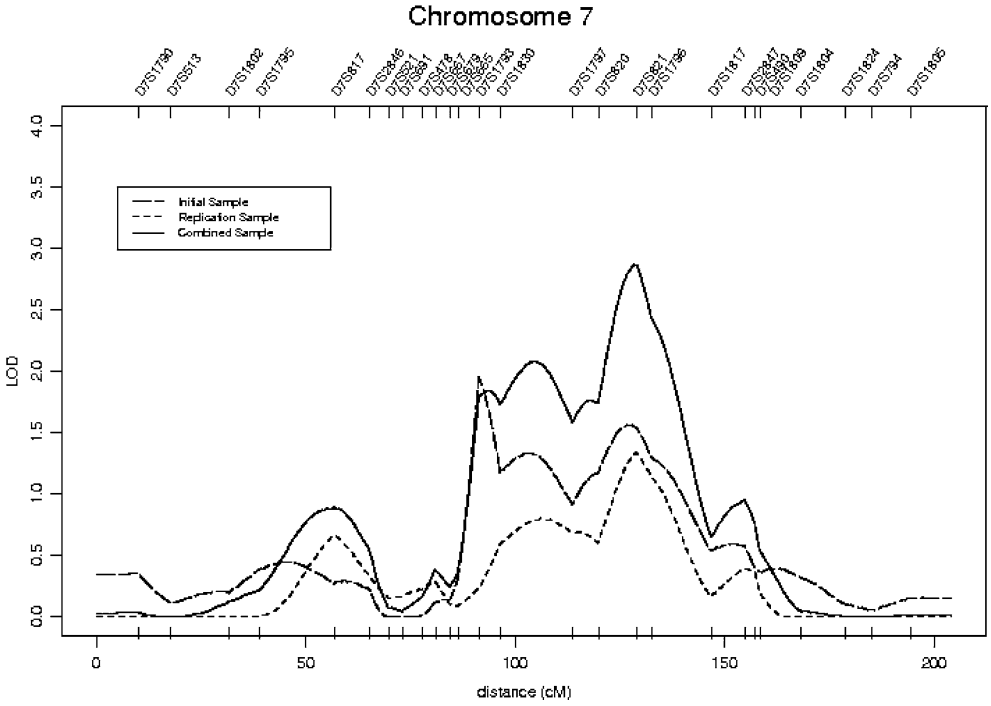

Fig. 4. LOD scores for the COGA definition of alcohol dependence on chromosome 7 (Foroud *et al*., 2000).

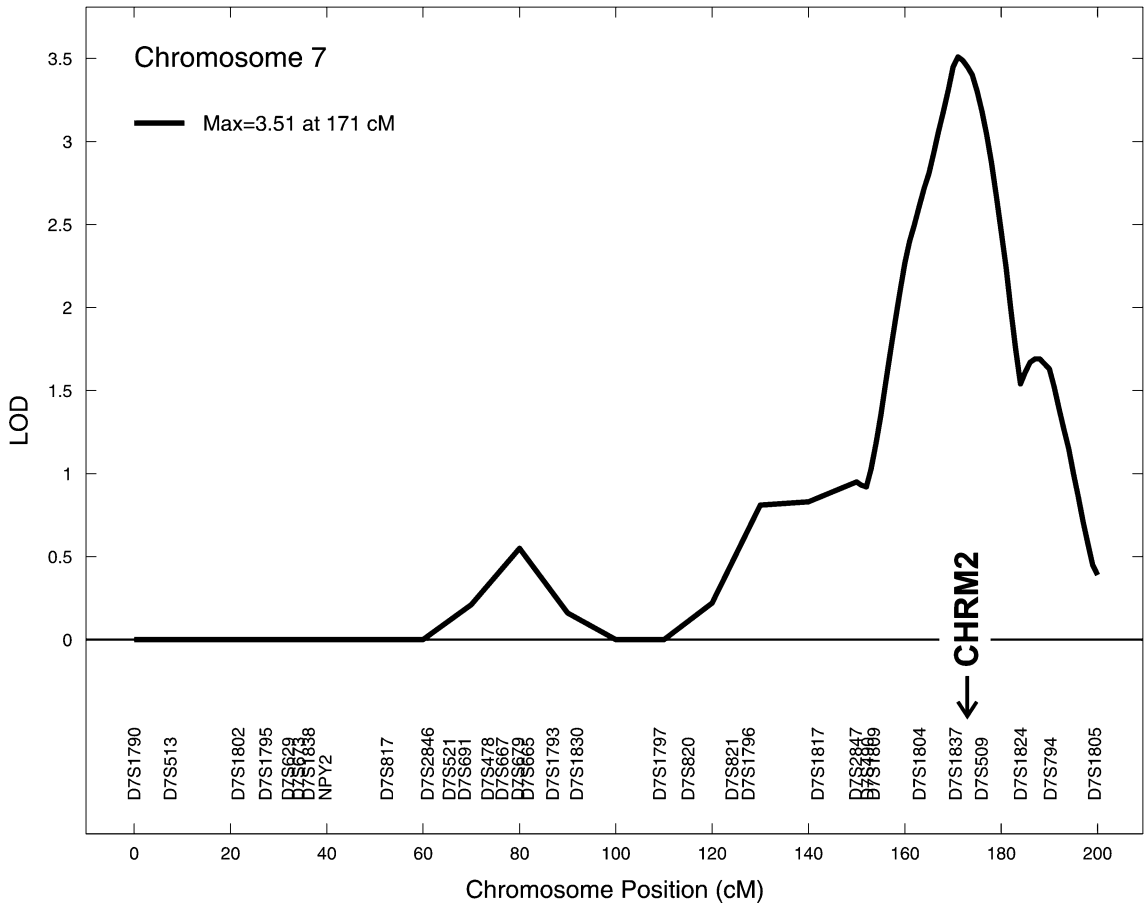

Fig. 5. LOD scores on chromosome 7 for the target case visual evoked oscillation phenotype. The phenotype data were derived using time-frequency analysis of the event- related potential (ERP) data. Mean values were calculated from time-frequency representations within a time-frequency region of interest corresponding to the P300 event (300–700 msec) and the theta frequency band (3–7 Hz) (Jones *et al.*, 2004).

independent study also reported an association between a polymorphism in *CHRM2* and major depression in women using a case-control association design (Comings *et al.*, 2002).

These results led us to further explore the evidence for linkage on chromosome 7 and association with the *CHRM2* gene, to both alcohol dependence and depression. We typed additional microsatellite markers on chromosome 7 and reran linkage analyses using affected sibling pair methods (using the SIBPHASE option in ASPEX) with the phenotypes COGA alcohol dependence, major depression, alcoholism *or* major depression (testing the possibility that the gene predisposed to either condition), and alcoholism *and* major depression (testing the possibility that the gene predisposed to the comorbid condition) (Fig. 6) (Wang *et al.*, 2004). The peak LOD score for alcohol dependence (488 sib pairs, IBD sharing = 56.5%) was 2.9 observed at D7S1799 (note that this was a newly added marker located adjacent to the marker showing the peak lod score with alcohol dependence in the previous analyses of the sample) (Foroud *et al.*, 2000). The peak lod score for depression (259 sib pairs, IBD sharing = 58.1%) was 2.3, located between D7S1799 and D7S1817. The highest lod score was obtained with the phenotype alcoholism or major depression (639 sib pairs, IBD sharing = 56.2%), resulting in a maximum lod score of 3.4. However, the strongest allele-sharing was observed with the comorbid phenotype alcoholism *and* depression (144 sib pairs, IBD sharing = 61%), with a peak lod score of 2.3. Because of the previous evidence of linkage disequilibrium with the ERP phenotype, we conducted family-based association analyses on 11 SNPs spanning a 70 kb region within and flanking the *CHRM2* gene (Wang *et al.*, 2004). There was significant evidence of association with multiple SNPs in *CHRM2* with both alcohol

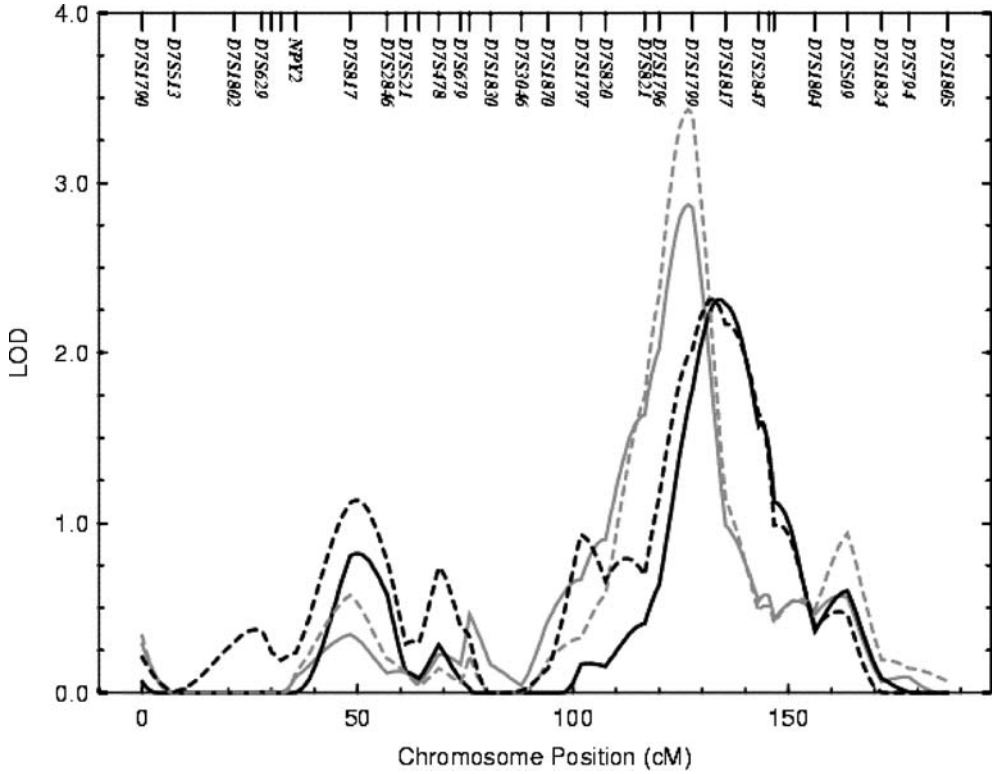

Fig. 6. LOD scores on chromosome 7 for the COGA definition of alcohol dependence (solid gray line), major depressive syndrome (solid black line), alcohol dependence and major depressive syndrome (dashed black line), and alcohol dependence or major depressive syndrome (dashed gray line) in the combined COGA sample (Wang *et al*., 2004).

dependence and major depression, and haplotype analyses were also significant with both phenotypes. Interestingly, there was a common protective haplotype observed for both phenotypes; however, the component alleles of the risk haplotypes differed for each disorder. These analyses provide evidence of both common and specific genetic effects on alcohol dependence and depression. Unlike the previous report, there was no evidence for sex-specific effects in the association results in our sample (Comings *et al*., 2002). Additional work is on-going to attempt to identify how *CHRM2* is involved in the risk pathways for these disorders.

DISCUSSION

We believe that these analyses demonstrate that the strategy adopted by the COGA project is successfully leading to gene identification. Other fields have a history of using quantitative risk factors to identify susceptibility genes for disease; for example, cholesterol levels, blood pressure, and body fat have been analyzed to identify genetic risk factors influencing cardiovascular disease (Falchi *et al*., 2004; Ma *et al*., 2004). The application of this strategy to the study of psychiatric disorders has been more recent. In addition to the alcohol dependence field, the use of endophenotypes has been employed in the study of genetic risk factors for schizophrenia (Clementz, 1998). A recent paper reported that the analysis of cognitive trait components of schizophrenia yielded higher lod scores than analyses of diagnosis (Paunio *et al*., 2004), similar to our experience comparing results from quantitative endophenotypes and diagnostic outcomes in the COGA sample.

In this paper, we have highlighted our systematic efforts focusing on two particular chromosomes where linkage to electrophysiological endophenotypes has led us to significant associations with clinical diagnoses. However, we think it is important to note that we are simultaneously employing complementary strategies, including testing for association with candidate genes selected based on hypotheses about biological processes believed to contribute to alcohol dependence. Although some of these

candidate genes are located in regions that show little evidence of linkage, we recognize that linkage studies are generally underpowered to detect genes of small effect and that association analyses may provide a more powerful test. Employing a hypothesis-driven approach based upon information about biological systems likely to be involved in alcohol dependence has also led us to genes that significantly influence alcohol dependence in our sample (Dick *et al.*, 2004). In addition, we are currently following up chromosomal regions that show linkage to either clinical phenotypes or endophenotypes (but not necessarily both). One disadvantage of working with endophenotypes is that the number of traits available for analysis can create problems associated with multiple testing. These can generally be addressed through simulation; however, it can be unclear how to interpret linkage/association signals specific to the endophenotype that show no correlation with linkage/association signals related to clinical diagnoses. These findings may be informative for advancing our understanding of the underlying processes involved in the electrophysiological signals; however, our most successful efforts to date in COGA have been on chromosomes showing evidence of linkage with both electrophysiological endophenotypes and clinical diagnoses.

The goal of this paper has been to collate and summarize a series of analyses conducted over the course of the COGA project that, we believe, raise a number of interesting points regarding the use of endophenotypes in gene-identification efforts. Our experience thus far has been that the linkage peaks observed with endophenotypes have been located directly over the genes subsequently found to be associated with alcohol dependence; alcohol dependence diagnoses have not yielded linkage peaks at the location of the (currently identified) genes. On both chromosome 4 and chromosome 7, although there was linkage to both clinical diagnoses and to electrophysiological endophenotypes, in neither case were the linkage peaks located at the same position. Two scenarios could create this situation: (1) the linkage evidence from electrophysiological endophenotypes and clinical diagnoses could result from different genes or (2) there could be random variation in the location of the linkage peak, although both linkage signals are detecting the same genetic effect. Simulation studies have demonstrated considerable variation in the linkage peaks observed for complex phenotypes with sample sizes comparable to that of COGA (Roberts *et al.*, 1999).

On chromosome 4 the peaks observed for alcohol dependence/alcohol-related phenotypes and EEG were ~50 cM apart. Analyses of the quantitative phenotypes (max drinks and EEG) clearly suggest two linkage peaks on chromosome 4: one near the $GABA_A$ receptor gene cluster and another near the ADH gene cluster. Alternatively, analyses of alcohol dependence diagnoses show only one very broad peak, with the one-lod interval stretching nearly 50 cM and the two-lod interval stretching nearly 100 cM! Importantly, we note that although *GABRA2* shows significant association with alcohol dependence, it was ultimately the strong linkage signal observed directly over the $GABA_A$ receptor gene cluster with the EEG endophenotypes, as well as strong evidence from the literature suggesting that GABA reception is involved in the predisposition to alcohol dependence, that led us to focus on the $GABA_A$ receptor genes on chromosome 4. The lod score observed with dependence diagnoses directly at the location of the $GABA_A$ receptor gene cluster was < 1. We are currently conducting association analyses with the ADH genes on chromosome 4, which are located very near to the linkage peaks observed for dependence diagnoses and for max drinks. We find significant association with genes in that region as well (Edenberg *et al.*, in preparation). It is possible that the wide region of linkage observed with the clinical diagnoses reflected the presence of multiple genes on chromosome 4 influencing alcohol dependence, in both the GABA and ADH gene regions. Only the quantitative phenotypes distinguished two distinct peaks in the region.

On chromosome 7 the linkage peaks for alcoholism and depression are located ~30 cM centromeric to the ERP linkage peak. The *CHRM2* gene is located directly under the linkage peak observed with the ERP phenotype. Although *CHRM2* showed significant association with both alcohol dependence and major depression, the gene lies outside of the 1 lod support interval of the linkage peaks observed with the diagnostic phenotypes. In general, our experience thus far is that the linkage peaks associated with the endophenotypes have been narrower and have been located directly over the gene subsequently found to be associated with both the endophenotype and with clinical diagnosis. We believe it is likely that there are additional genes on chromosome 7 that influence the development of alcohol dependence. Approximately 15 cM distal of the alcoholism peak (and 14 cM proximal to the *CHRM2* gene) lies a gene coding for the TAS2R16 taste receptor, identified as a bitter taste

receptor for beta glucopyranosides (such as salicin) (Bufe *et al*., 2002).

A functional polymorphism in this gene appears to increase susceptibility to develop alcohol dependence in the COGA sample (Hinrichs *et al*., 2005). We have tested SNPs in a number of additional candidate genes located more directly under the alcohol dependence/depression linkage peaks, although none have yielded the significant association results observed with *CHRM2* thus far. We are currently planning a systematic screen of SNPs located across the alcohol dependence peak on chromosome 7. We believe that this is necessary to more definitively determine whether there are other genes in the region contributing to the alcohol dependence linkage signal. The results from these analyses should help us determine whether *CHRM2* was the only gene contributing to the linkage observed with alcohol dependence and the localization was simply poor, or whether the discrepancy between the location of the linkage peaks for ERP and alcohol dependence was due to additional gene(s) being detected by the diagnostic phenotypes.

In conclusion, the decision to measure and include electrophysiological endophenotypes in addition to clinical diagnoses in the COGA project has played a critical role in our gene identification efforts. These endophenotypes have successfully led to the identification of *GABRA2* and *CHRM2* as genes associated with alcohol dependence. Alcohol dependence diagnoses yielded evidence of linkage on both chromosome 4 and chromosome 7, the chromosomes on which these genes are located, respectively; however, in neither case was the peak observed with the dependence diagnoses located at these genes. However, there were narrow, significant linkage peaks obtained with the electrophysiological endophenotypes directly at the location of these genes. In addition to the sharp resolution provided by the endophenotypes, the linkage signals have generally been stronger with these quantitative traits as compared to signals obtained from analyses of dependence diagnoses. As an example, on chromosome 4, the peak lod score obtained for alcohol dependence was 2.8. However, the EEG linkage peak maximized at 6.53, clearly surpassing the threshold for significance suggested by Lander and Kruglyak (Lander and Kruglyak, 1995). We have generally not found linkage peaks associated with dependence diagnoses that reach the level of significance suggested by Lander and Kruglyak (Lander and Kruglyak, 1995), even in chromosomal regions where genes have subsequently been identified. Thus, the use of endophenotypes has been advantageous in advancing our understanding of genetic contributions to alcohol dependence in a number of ways. In addition to being informative about the processes likely to be involved in the predisposition to alcohol dependence, they have proven very useful in aiding gene identification efforts for alcohol dependence.

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