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Linkage Studies of D2 and D4 Receptor Genes and Alcoholism

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The purpose of the present study was to evaluate two polymorphisms near the D2 receptor gene (TaqI A RFLP and C microsatellite) and a VNTR for D4. A nonparametric linkage (NPL) technique, SIBPAL, was used to test for the presence or absence of linkage in 54 multiplex alcoholic families. These families had been ascertained through two alcoholic proband siblings in order to increase the density of alcoholic cases within these pedigrees. Phenotypic definitions of alcoholism were manipulated in an effort to determine the impact of severity (signs of physical dependence, early age of onset, presence of antisocial personality disorder) on the likelihood of finding positive evidence for linkage. A regression analysis that simultaneously evaluated the allele sharing identical by descent for Feighner criteria alcoholism in affected, unaffected, and discordant sib pairs (SIBPAL) for two D2 polymorphisms and the D4 polymorphism gave no evidence for linkage. Phenotypes associated with greater alcoholism severity (presence of physical dependence symptoms, earlier onset, or comorbid antisocial personality disorder) revealed some evidence for linkage. The presence of one or more physical dependence symptoms in combination with Feighner criteria alcoholism provided some evidence favoring linkage (TaqI A and D4). Alcoholics with an earlier onset of alcoholism showed some evidence for linkage especially when the presence of physical dependence was required (e.g., morning drinking, wanted to stop drinking but could not, binges or benders, and evidence of withdrawal symptoms). Finally, alcoholics with antisocial personality disorder differed significantly in their allele sharing from nonalcoholics for both D2 polymorphisms. Am. J. Med. Genet. (Neuropsychiatr. Genet.) 88: 676–685, 1999. © 1999 Wiley-Liss, Inc.

INTRODUCTION

The dopamine D2 gene on 11q22-23 was first described by Grandy and colleagues [1989a]. A two allele TaqI A RFLP with a heterozygosity of 0.30 was initially detected at this locus [Grandy et al., 1989b]. Because the heterozygosity was not optimal for uncovering linkage between D2 and human disorders, Grandy and colleagues searched for additional polymorphisms within or close to the D2 gene. As a result, two additional polymorphisms, the TaqI B restrictive fragment length polymorphism (RFLP) and the microsatellite C polymorphism were later described [Hauge et al., 1991]. The TaqI B site is located 5′ of the first coding exon of the D2 receptor. The microsatellite C polymorphism has been localized to the intron separating exons 2 and 3 of the D2 gene. This polymorphism has a heterozygosity of 0.68. The TaqI A and B polymorphisms appear to be in strong disequilibrium though disequilibrium was not previously found for either the TaqI A or B and the C polymorphism [Hauge et al., 1991].

The initial report of a population-based association between alcoholism and the TaqI A D2 dopamine receptor polymorphism [Blum et al., 1990] generated considerable interest in D2. However, evidence for the presence of either association and/or linkage of the alcoholic phenotype to allelic variation in D2 remains highly controversial. Most of the studies performed to date have been population-based association studies. Many have failed to find an association [Bolos et al., 1990; Gelernter et al., 1991; Schwab et al., 1991; Goldman et al., 1992, 1993; Cook et al., 1992; Turner et al., 1992; Suarez et al., 1994].

Positive population-based associations [Neiswanger et al., 1995; Parsian et al., 1991; Pato et al., 1993] have been reported. Recently, we have pointed out that the nature of the control group, whether or not screened for alcoholism and other psychiatric disorders, will influence whether significant population-based associations are found [Neiswanger et al., 1995; Hill and Neis-

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wanger, 1997]. Supporting this contention that screening of controls alters the results of association studies is a recent analysis of all published studies to date concerning the D2 TaqI A polymorphism, excluding those studies with unscreened controls, which reveals χ^2 values of 21.8, with a P-value of 3.01×10^{-6} [Turner et al., 1997]. Results of this analysis suggest that the D2 receptor may warrant further consideration with respect to alcohol dependence, though careful attention must be paid to 1) sample size and power to detect differences, 2) possible ethnic differences between high- and low-risk groups, and 3) the nature of the control groups employed (with or without various types of psychopathology).

Although population-based association studies are abundant, only a few attempts have been made to uncover linkage or within-family associations between the D2 receptor polymorphisms and alcoholism. Bolos et al. [1990] reported an absence of both association and linkage between alcoholism and the D2 locus. However, only two families consisting of 14 individuals (eight alcoholic) were included in the parametric linkage analysis. Parsian and colleagues [1991] also failed to find linkage disequilibrium using a larger sample of 17 nuclear families. Previous attempts to find linkage in our laboratory [Neiswanger et al., 1995] were unsuccessful when 20 families of male alcoholics with a severe form of alcoholism were studied using parametric linkage techniques and when a within-family association analysis was performed.

Recently, Cook and colleagues [1996] conducted a nonparametric linkage analysis of sib pairs using the extended sib pair analysis (ESPA) method [Sandkuyl, 1989] in a sample of seven British families. Both the RFLP TaqI A and the microsatellite C polymorphism were investigated. The program permits the calculation of the classical identity-by-descent (IBD) analysis as well as the ESPA. Each locus was analyzed separately along with the haplotype formed from the A and C polymorphisms combined. These families were chosen in an attempt to replicate previous evidence for linkage obtained in a sample of 11 nonsystematically ascertained families of alcoholics. Standard IBD analysis revealed a highly significant effect of the D2 locus for a research diagnostic criteria (RDC) defined alcoholism phenotype. Combined analyses of 11 families revealed significant findings for both A and C allele data ($P = 0.044$ and $P = 0.011$, respectively) but not for the A/C haplotype. However, the excess allele sharing was explained by one fully informative large family with a sibship of ten individuals.

An opportunity for evaluating polymorphisms in or near the D2 receptor was provided by the Collaborative Study on the Genetics of Alcoholism. Results of sib-pair analyses (SIBPAL) and multipoint analyses (GENEHUNTER) from a genome-wide search based on data collected from six cites and involving 105 families found no evidence for D2 receptor variation from the linkage analyses performed [Reich et al., 1998]. A subset of the COGA data consisting of affected individuals where both parents were genotyped ($N = 264$) were also analyzed using other within-family techniques (Transmission Disequilibrium Test [TDT] and the Affected Family-Based Control [AFBAC] test) with results based on these analyses reported to be nonsignificant [Edenberg et al., 1998]. However, a reanalysis of that data set using alternative multipoint nonparametric linkage analyses implicated two regions containing candidate genes [Curtis et al., 1999]. Using the program MFLINK, the MALOD statistic was calculated [Curtis and Sham, 1995]. The MALOD is defined as the maximum lod score obtained for any of the transmission models considered (a range of different dominant and recessive models were evaluated). The reanalysis by Curtis et al. found a MALOD significant at 0.02 for the D2 TaqI A polymorphism. Due to the conservative nature of the program GENEHUNTER used in the COGA analysis [Reich et al., 1998], this region had previously been missed. Also, reduced power to detect within- family association was present in the TDT tests performed by Edenberg and colleagues [1998] because of the requirement that both parents be genotyped and not homozygous. Thus, linkage analyses using both candidate gene [Cook et al., 1996] and genome-wide strategies [Curtis et al., 1999] appear to provide evidence that the TaqI A polymorphism should be pursued further.

Some interest has been shown in the D4 receptor polymorphism with respect to alcohol dependence. The dopamine D4 receptor gene is in the same class as D2 but has somewhat different pharmacological properties. The D4 polymorphism, which is a tandem repeat (VNTR), has been explored with respect to alcoholism vulnerability in a number of diverse populations. Three studies, one from the U.S. [Parsian et al., 1997], one from a Finnish sample [Adamson et al., 1995], and one based on three Taiwanese populations [Chang et al., 1997], have found no association between alcoholism and specific allele frequencies of the D4 locus. However, three positive associations have been reported, one in a Canadian population [George et al., 1993], one in Israel [Kotler et al., 1997] using an opioid-dependent cohort, and one in Japan [Muramatsu et al., 1996].

In two of the positive studies, other known characteristics of vulnerability were explored with respect to D4 allelic variation. For example, a point mutation in the aldehyde dehydrogenase 2 gene (ALDH2) is considered a protective factor with respect to developing alcoholism. In the sample of 655 Japanese alcoholics studied by Muramatsu and colleagues [1996], 80 were found to carry the ALDH2 variant which should have conferred protection from alcoholism.

Interestingly, among the 80 alcoholics who carried the ALDH2, a protective variant, a significantly higher frequency of the five repeat allele of the 48-bp D4 repeat polymorphism was found. In the other positive study in which cofactors for vulnerability were explored, a personality trait (novelty seeking), which has frequently been found to be elevated in substance-abusing samples [Cloninger et al., 1995], was found to be associated with specific alleles of D4 (represented chiefly by the 7-repeat) in a sample of opioid-dependent individuals [Kotler et al., 1997].

Having found evidence for a population-based association in our laboratory for the D2 TaqI A polymorphism in alcoholics and screened controls from our

laboratory, the goal of the present study was to test for the presence of linkage to the D2 TaqI A and C markers within high-risk families using nonparametric techniques. A second goal was to investigate the D4 VNTR within the same set of families.

MATERIALS AND METHODS

The sib pairs used in the SIBPAL analysis were drawn from a series of high-risk pedigrees. The high-risk families were part of two larger studies of alcoholism, one concerning families of female alcoholic probands (Biological Risk Factors Family Study, BRFFS) and the other involving families of male alcoholic probands (Cognitive and Personality Factors Family Study, CPFFS). Demographic and clinical characteristics of the sample used for the present analyses may be seen in Table I. The number of individuals genotyped by gender and affection status (Feighner criteria) may be seen in Table II. The high-risk family studies, from which the present set of individuals was drawn, were designed to include direct interviews of all available first-degree relatives to determine both clinical status and variation on a number of neurobiological indicators of risk. These high-risk "target" families had been ascertained through a proband set comprised of two alcoholic sisters (BRFFS) or two alcoholic brothers (CPFFS), one of whom was in treatment for alcoholism at the time the family was ascertained. The presence or absence of alcoholism or other psychopathology was determined for the proband pair and most available first-degree relatives through face-to-face interviews (Diagnostic Interview Schedule [DIS]), allowing for DSM-III and Feighner criteria to be applied.

In some cases in which in-person interview information was not available, a minimum of two consistent reports of the relatives' status were required to assign a diagnosis. Because our study design included obtaining family history information from all relatives interviewed, multiple reports on all members of the family, both first- and second-degree members, were available. Thus, a valid family history diagnosis could be correctly assigned. Of the total set of 313 individuals used in the linkage analysis, only a small number of subjects were diagnosed by family history (N = 31). All but one of these was from the parental generation; most were deceased.

Definition of the Alcoholism Phenotype

All of the subjects included in the analysis were diagnosed for the presence of alcoholism using Feighner criteria [Feighner et al., 1972]. All met criteria for alcohol dependence by DSM-III-R, and at least DSM-III and IV criteria for alcohol abuse. A diagnosis of alcoholism by Feighner criteria requires the presence of at least one symptom in three out of four possible categories of problems. The four categories include 19 separate symptoms. The items used include those covering the alcohol-dependence syndrome (e.g., morning drinking, feeling one cannot stop) and occupational-social problems (e.g., others object, losing a job, driving problems, trouble with police). While the items included are quite similar to RDC items, the criteria for alcoholism by RDC are less stringent (three or more symptoms out of a list of 20) [Spitzer et al., 1978].

Other alcoholism subtypes were included in our analyses in order to assess the influence of varying definitions of alcoholism severity (early or late onset, and presence or absence of antisocial personality disorder [ASPD]) on the significance level of results obtained. The effect of changing the definition of unaffected was explored using the nonalcoholic designation provided by Feighner criteria (two or fewer symptom categories), as well as including only those nonalcoholic individuals with no categories.

TABLE I. Demographic Characteristics of the 269 Individuals Genotyped (Mean Age ± SD)*

	Affected sibs (N = 128)	Unaffected sibs (N = 72)	Parents (N = 69)
Age at interview	33.7 (6.5)	34.7 (9.0)	59.4 (8.2)
Age at first problem[a]			
Mean	18.8 (5.3)		32.2 (10.3)
Median	18		

*All individuals were Caucasian. Some individuals from the total sample (N = 313) did not have all three markers genotyped: TaqI A (N = 244); C polymorphism (N = 253); D4 (N = 267).
[a]Eighty-six affected sibs were used for "early" and "late onset" classification.

TABLE II. Sample Characteristics of Individuals Genotyped by Gender

	Males	Females
TaqI A		
Affected[a] sibs	84	34
Unaffected sibs	30	33
Parents	23	40
C Locus		
Affected sibs	90	23
Unaffected sibs	35	36
Parents	25	44
DRD4		
Affected sibs	88	38
Unaffected sibs	35	37
Parents	25	44

[a]By Feighner criteria: definite diagnosis or three or more categories of possible four.

Physical Dependence Phenotype

DSM-III-R and IV differ markedly in their conception of the importance of tolerance and physical dependence in the definition of alcohol dependence or alcoholism. Schuckit et al. [1998] have discussed the changing emphasis on the requirement that there be a pathological use of alcohol in combination with either tolerance or withdrawal in DSM-IV for alcohol dependence to be diagnosed. Using data collected on 3,395 DSM-III-R–diagnosed alcohol-dependent individuals, Schuckit and colleagues demonstrated that individuals with either tolerance and/or withdrawal had greater overall severity (more DSM-III-R symptoms endorsed, more alcohol-related life problems, larger maximum number of drinks in 24 hours) than did DSM-III-R–defined alcohol-dependent individuals without a physiological component. For this reason, it appeared appropriate to consider the presence and number of physical withdrawal signs as an alternate phenotype for analysis in the present report.

The alcohol dependence phenotype was treated as a categorical variable with respect to the four items from Feighner criteria specifically addressing physical dependence that had been obtained for all subjects (wanted to stop drinking but could not, morning drinking, frequent binges or benders, and presence of withdrawal symptoms). A "physical dependence" phenotype was created using the information from these questions in which a negative response (none of these) or a positive response (one or more symptoms) were contrasted in the statistical analysis. In order to maximize phenotypic disparity, nonalcoholics could not have symptoms in even one Feighner criteria category.

Early Age-of-Onset Phenotype

A number of studies have noted that the earlier the age of onset of alcohol dependence, the more severe the disorder appears to be [Buydens-Branchey et al., 1989; Turner et al., 1993]. Buydens-Branchey et al. [1989] reported that individuals who became alcoholic before their 20th birthday had a significantly higher incidence of paternal alcoholism and were twice as likely to be incarcerated for crimes involving physical violence. Turner et al. [1993] concluded that age of onset was an intervening variable in the development of alcoholism that is highly related to alcohol consequences and dependence. In the present study, the median split for the age of onset of the proband pairs was used to classify families into either "early" or "late" onset.

In order to classify the subjects on the basis of age of onset, it was necessary to first describe the distribution of onset for our subject group. Many studies have used <25 years and >25 years as a dichotomous split for assessing early and late onset as this age was found to be a useful split for defining Type I and Type II alcoholics [Parsian et al., 1991]. In the present sample the majority of cases had their first problem before the age of 25 (only three cases were later than 25) indicating that the alcoholics were most similar to the Type II alcoholics. Although the group as a whole had relatively early onset, a distribution of ages of onset was evident.

Accordingly, each affected individual was assigned an age of onset based on the occurrence of the first symptom in any of the three Feighner diagnostic categories. A mean age of onset for each affected proband pair was then determined and that age was used to classify families into early and late using a median split. Using this method, the median age of onset for male proband pairs was 18 years, while the age of onset for the female proband pairs from the study which identified families through the presence of at least two alcoholic sisters was 16 years. To check the validity of this approach, age of onset for each affected sib was used to generate a separate distribution. Comparing the two distributions, we find the median and quartile age of onset to be quite similar. Using the affected proband pair distribution, the quartiles were 15, 17.5, and 21 years, whereas using each affected individual's age of onset, the quartiles were 14, 17, and 21 years. Thus, the similarity in the distribution of age of onset using the two methods justified the use of the mean of the proband pair for classification of families.

Antisocial Personality Phenotype

The prominent association between alcoholism and ASPD has led to the proposal that a distinction be made between alcoholics with and without ASPD [Hesselbrock et al., 1984]. This proposal was based on the observation that alcoholics with ASPD began abusing alcohol at an earlier age and had a faster progression to severe alcoholism than did alcoholics without ASPD. Similar observations have been made by Liskow et al. [1991] finding ASPD alcoholics to have an earlier onset with more rapid course and an increased percentage of alcoholism symptoms. Also, presence or absence of ASPD within the alcoholic family was originally used to define Type I and II alcoholism [Cloninger et al., 1981]. Therefore, ASPD was of interest with respect to possible subtyping that would clarify the role of the dopamine polymorphisms studied. Accordingly, an analysis was performed comparing alcohol-dependent individuals with and without ASPD with respect to linkage to these polymorphisms.

Genotyping Methods

The D2 TaqI A1/A2 Alleles. The sibs and parents provided consent to draw blood by venipuncture. DNA was extracted from both immortalized cell lines and peripheral blood using minor modifications of the salting out method [Miller et al., 1988]. Genomic DNA (10 μg) was restricted overnight using a threefold excess of TaqI restriction endonuclease, according to the manufacturer's specifications. Samples were size fractionated by electrophoresis on 1.0% agarose gels and transferred overnight by capillary action [Southern, 1975] to MSI nylon transfer membranes (Magna NT; Micron Separations, Inc.). Filters were prehybridized 2–4 hrs at 42°C in 50% formamide, 5× Denhardt's solution, and 450 μg/ml sheared salmon sperm DNA. The TaqI A probe used in these analyses was the 1.7-kb BamHI fragment of HD2G1 provided by Dr. David Grandy (Vollum Institute, Oregon Health Sciences University). The probe was radiolabeled by random priming [Feinberg and Vogelstein, 1983] to a specific activity of $>10^9$ cpm/μg with [α-32P]dCTP. Hybridization reactions were carried out at 42°C for 24–72 hrs in 50% formamide, 6× SSC, 1× Denhardt's solution, 10% dextran sulfate, 0.5% SDS, and 800 μg/ml sheared salmon sperm DNA. Filters were washed extensively at a final stringency of 0.1× SSC at 65°C and exposed to Kodak XAR-5 film at –70°C for 3–7 days.

Typing of Microsatellite C Polymorphism. Using the sequence described by Hauge et al. [1991] primers 509(CAGGAGCACGTTTCTCATAC) and 419(CGAGGGCGGTGCGGTCAT) were used to amplify the microsatellite. We performed polymerase chain reaction (PCR) in a total volume of 12.5 μl containing 50 ηg of genomic DNA, 1 pmol of one primer 5′-end-labeled with ^{32}P, 5 pmol of the unlabeled primer, 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 200 μM dATP, dTTP, dGTP, and dCTP, and 0.5 unit of *Taq* polymerase (Promega). After an initial denaturation at 94°C for 3 min, amplification in a Perkin-Elmer thermal cycler was for 30 cycles of 30-sec denaturation at 94°C, 30-sec annealing at 58°C, and 30-sec

extension at 72°C. PCR products were resolved by electrophoresis on 6% denaturing polyacrylamide DNA sequencing gels and were detected by overnight autoradiography. Fragment sizes were measured relative to a size standard, a DNA sequence ladder derived from bacteriophage M13mp18.

Typing of VNTR Polymorphism for D4. Using the sequence described by Lichter et al. [1993] primers D4-3 (GCG ACT ACG TGG TCT ACT CG) and D4-42 (AGG ACC CTC ATG GCC TTG) were used to amplify the VNTR. PCR was performed in a total volume of 25 μl containing 100 ηg of genomic DNA, 10% DMSO, 200 μM dATP, dTTP, and dCTP, 100 μM dGTP and 100 μM deaza-dGTP, 1 μM of each primer, 1× *Taq* polymerase buffer (10 mM Tris-8.3, 50 mM KCl, 1 mM $MgCl_2$, 5 μM EDTA, 0.01% gelatin), and 3 units of DNA Taq polymerase (Promega). DNA was denatured at 99°C for 1 min prior to the addition of the other components. Using the Perkin-Elmer 9600 thermal cycler, 40 cycles of 95°C (20 sec), 54°C (20 sec), and 72°C (40 sec) were performed followed by a 4-min chase at 72°C. After amplification, loading dye was added and the total volume (25 μl) was loaded onto a 3.5% agarose gel. The gel was run for 2½ hr and then stained with ethidium bromide. Two size standards were used in order to determine number of repeats: Phi X174 HaeIII and Msp I digest of pBR322.

Statistical Methods

A total of 313 individuals was included in the analysis (see Tables I and II). Nonparametric sib-pair methods were used to test for linkage [Haseman and Elston, 1972] in full sibs using SIBPAL (version 3.1) from the Statistical Analysis for Genetic Epidemiology [SAGE, 1997] package. No half-sibs were included. The SIBPAL method for testing for linkage between a categorical variable (e.g., alcoholism versus no alcoholism by Feighner criteria) and a marker locus were used for most of the analyses performed. First, Feighner criteria alcoholics were contrasted with nonalcoholics (those individuals not meeting two or more symptom categories). Second, the Feighner criteria alcoholics with one or more symptoms of physical dependence were contrasted with nonalcoholics with zero Feighner criteria categories. Next, Feighner criteria alcoholics with early onset were contrasted with nonalcoholics as were the late-onset alcoholics using a categorical (affected/unaffected) phenotypic analysis. The effect of having physical dependence symptoms present along with Feighner criteria–diagnosed alcoholism was evaluated in the early- and late-onset groups. The proportion of alleles IBD was estimated for each marker for all possible affected sib-pairs, $n(n-1)/2$ affected sib pairs for n affected sibs, and compared to the null hypothesis of 0.50 using a one-sided *t*-test [Blackwelder and Elston, 1985]. Evidence for increased allele sharing among unaffected sib pairs was also evaluated. (In cases where parental genotypes are missing, allele frequency estimates are used to produce a weighted estimate of IBD). Evidence for linkage is simultaneously evaluated in the affected, unaffected, and discordant pairs using a regression analysis [Haseman and Elston, 1972]. This method regresses the squared trait difference against the estimated proportion of alleles IBD for each sib pair to detect a negative slope, which suggests linkage.

Next, the Feighner criteria alcoholics with ASPD were evaluated with respect to alcoholics without ASPD and nonalcoholics, using the quantitative trait option of SIBPAL. Members of all families were analyzed (those ascertained through either male or female alcoholic probands). Next, the analysis was performed restricting the data set to members of families identified through female alcoholic probands.

RESULTS

Maximizing the Phenotypic Distance: Feighner Criteria Phenotype With Evidence of Physical Dependence and Nonalcoholics With No Symptom Categories

The linear regression analyses result for the linkage between the Feighner criteria alcoholism phenotype (yes/no) and the TaqI A and C polymorphisms were not significant. The D4 VNTR polymorphism was also not significant using the categorical split between Feighner criteria alcoholism (three positive symptom categories) and absence of these three categories. However, when a positive diagnosis of alcoholism by Feighner criteria was combined with evidence of one or more physical dependence symptoms, results were significant for one of the D2 polymorphisms and the D4 polymorphism (Table III). It should be noted that persons judged to be nonalcoholic using Feighner criteria can have one or two symptom categories positive under that definition of alcoholism. Therefore, the nonalcoholic phenotype was further refined by requiring that the nonalcoholic group have not even one symptom category. As may be seen in Table III, sharpening the nonalcoholism phenotype by requiring no symptom categories be present, and requiring evidence of physical

TABLE III. Sib-Pair Analyses of 313 Individuals*

	DRD2-TaqI A			DRD2-C		DRD4		
	P	Informative pairs	r^2	*P*	Informative pairs	*P*	Informative pairs	r^2
Alcoholic[a] versus nonalcoholic[b]	0.031	(106)	5.23%	N.S.[c]	(112)	0.036	(130)	4.31%

*Alcoholics meeting Feighner criteria with physical dependence were contrasted with nonalcoholics with no Feighner problem areas.
[a]A diagnosis of alcoholism by Feighner criteria requires three symptom categories out of a possible four categories.
[b]A diagnosis of no alcoholism was made only if no Feighner criteria symptom categories were present.
[c]Not significant.

dependence for the alcoholic phenotype, resulted in a significant result for the TaqI A ($t = 1.89$, df = 65, $P = 0.031$) and the D4 ($t = 1.83$, df = 74, $P = 0.036$) polymorphisms.

Effect of Early Onset In Feighner Criteria Alcoholism

Because early onset of alcoholism appears to be a more severe alcoholism phenotype [Buydens-Branchey et al., 1989; Turner et al., 1993] with more alcohol-related consequences, it appeared useful to create early and late subtypes within the alcoholic group diagnosed by Feighner criteria. As may be seen in Table IV, requiring an early onset gave some evidence for linkage to the TaqI A ($t = 1.78$, df = 66, $P = 0.04$). Further requiring the presence of at least one sign of physical dependence among the early-onset group, Feighner-diagnosed alcoholics also gave evidence for linkage to the TaqI A ($t = 2.39$, df = 56, $P = 0.01$).

Alcoholism/ASPD Phenotype

The total data set included families ascertained through a male proband pair (N = 234) and families ascertained through female proband pairs (N = 79). ASPD had been free to vary by study design. Epidemiological survey data have reported higher rates of ASPD among alcoholic women than alcoholic men [Helzer et al., 1991]. In comparison with nonalcoholic women, Helzer and colleagues found the risk ratio for ASPD among alcoholic women was 29.6 times greater whereas alcoholic men were 12 times more likely than nonalcoholic men to have ASPD. With higher co-occurrence for ASPD among alcoholic women than alcoholic men, it was possible that ASPD might be more prominent among the families ascertained through pairs of alcoholic women than in families ascertained through male proband pairs. In order to examine the influence of ASPD on the linkage results, data were recoded to include three groups: 1) not alcoholic, 2) alcoholic by Feighner criteria but no ASPD, and 3) alcoholic with ASPD. Linkage analysis was performed using the quantitative trait option of SIBPAL to test the alcoholic (affected) phenotype with and without ASPD and the nonalcoholic (unaffected) phenotype. The analysis was repeated using the ASPD alcoholic (affected) phenotype. As may be recalled, when the entire set of families (relatives of both female and male proband alcoholics) was analyzed using the least severe definition of alcoholism (those meeting Feighner criteria only), no evidence for linkage was seen for any of the markers genotyped. However, using the entire data set and evaluating the alcoholics with and without ASPD in comparison to nonalcoholics, evidence for linkage with the TaqI A polymorphism was seen ($t = 2.07$, df = 119, $P = 0.02$; Table V). Results for the C and D4 polymorphisms using this phenotype were not significant.

Results were analyzed separately for families identified through female alcoholic probands (N = 79) where ASPD appears to be a prominent feature. When alcoholics with ASPD were evaluated with respect to alcoholics without ASPD and with nonalcoholics, significant results were seen for both D2 polymorphisms (TaqI A: $t = 3.54$, df = 29, $P = 0.0007$; and for C: $t = 1.96$, df = 14, $P = 0.035$; Table VI). Thus, ASPD appears to increase significance levels when linkage between alcoholism and the dopamine receptor polymorphisms are evaluated. This effect appears to be most salient in families ascertained through female alcoholic probands where the presence of ASPD is an especially prominent feature of the disorder.

DISCUSSION

The overall conclusion that can be drawn from this series of analyses is that definitive evidence for linkage between alcoholism and the D2 and D4 polymorphisms has not been found. However, the results do support evidence for linkage when more severe alcoholic phenotypes (presence of physical dependence, presence of ASPD, or membership in an early-onset family) are used. The alcoholism phenotype requiring the presence of physical dependence symptoms resulted in evidence favoring linkage for one D2 polymorphism, TaqI A, and the D4 polymorphism. Additionally, when the analyses involving alcoholics with ASPD were restricted to families identified through a pair of female alcoholics, both

TABLE IV. Sib-Pair Analyses of 305 Individuals*

	Feighner criteria alcoholic[a] versus nonalcoholic[b]			Feighner criteria alcoholic[a] with physical dependence[c] versus nonalcoholics[b]		
	P	Informative pairs	r^2	*P*	Informative pairs	r^2
Early-onset families						
TaqI A	0.040	(146)	4.57%	0.010	(114)	9.27%
C	N.S.[d]	(169)		N.S.	(123)	
DRD4	N.S.	(185)		N.S.	(143)	
Late-onset families						
TaqI A	N.S.	(132)		N.S.	(67)	
C	N.S.	(134)		N.S.	(67)	
DRD4	N.S.	(138)		N.S.	(70)	

*Effect of maximizing phenotypic disparity: Alcoholics with and without physical dependence were stratified by age of onset and contrasted with nonalcoholics.
[a]A diagnosis of alcoholism by Feighner criteria requires three symptom categories out of a possible four categories.
[b]A diagnosis of no alcoholism requires that symptoms be present in two or fewer categories.
[c]One or more physical dependence symptoms (zero to four were possible).
[d]Not significant.

TABLE V. Sib-Pair Analyses of 313 Individuals Selected Through Both Male and Female Proband Alcoholics*

	DRD2-TaqI A			DRD2-C			DRD4		
	P	df	r^2	*P*	df	r^2	*P*	df	r^2
Groups A, B, and C[a]	0.020	(119)	3.50%	N.S.[b]	(117)	—	N.S.	(131)	—

*Alcoholics meeting Feighner criteria with ASPD were evaluated with respect to alcoholics without ASPD and with nonalcoholics. Analyses were based on SIBPAL quantitative trait analyses.
[a]Group A, alcoholic by Feighner criteria (three symptom categories out of a possible four) and the presence of ASPD by DSM-III criteria; Group B, alcoholic by Feighner criteria but no ASPD; Group C, nonalcoholic requires that Feighner criteria symptoms be present in two or fewer categories.
[b]Not significant.

D2 polymorphisms were significant, the TaqI A being particularly significant with $P = 0.0007$. In view of the fact that alcoholism is a complex disease, undoubtedly influenced by multiple genes, we sought to determine the percentage of explained variance for each of the phenotypes. We found that the variance explained by the alcoholism phenotype was approximately 5% for the TaqI A polymorphism and about 4% for D4. However, when the early-onset alcoholics with physical dependence symptoms were considered and compared with nonalcoholics, the variance explained increased to approximately 9%. In the case of the families ascertained through female alcoholic probands, contrasting alcoholics with and without ASPD with nonalcoholics resulted in an explained variance of about 30% for the TaqI A polymorphism and 21% for the C polymorphism. Thus, while the present results do not provide definitive evidence for linkage they do suggest the importance of these polymorphisms in the more severe forms of alcohol dependence characterized by early onset, presence of physical dependence symptoms, ASPD comorbidity, and selection of the family through female alcoholic probands.

Previous reports have indicated the possible importance of alcoholism severity for one of the D2 polymorphisms tested (TaqI A1) [Noble and Blum, 1991; Blum et al., 1993; Kono et al., 1997; Parsian et al., 1991]. Noble and Blum reported a significant linear trend ($P = 0.0002$) relating the prevalence of the A1 allele and the degree of alcoholism severity seen in the alcoholic subjects studied. This was a reanalysis of the combined data collected in their laboratory and data presented by Bolos et al. [1990]. Also, Blum et al. [1993] reported increasing A1 frequency with severity in a new sample in which 21% of nonalcoholics, 34% of less-severe alcoholics, and 63% of severe alcoholics carried the A1 allele. Parsian and colleagues considered the A1 frequency and alcoholism severity using two methods. First, the Cloninger Type I and Type II subtypes were analyzed with no differences found. However, when the alcoholics were classified for severity on the basis of physical dependence symptoms, the more-severe alcoholics were significantly more likely to carry the A1 allele. Also, Kono and colleagues [1997], studying 100 Japanese alcoholics, found the frequency of the A1 allele to be significantly higher in early-onset alcoholics compared to controls.

Previous attempts to find linkage between an alcoholism phenotype and either the D2 or D4 polymorphisms have been negative. D2 has been studied using parametric linkage tests [Bolos et al., 1990; Neiswanger et al., 1995], and nonparametric linkage methods [Parsian et al., 1991; Cook et al., 1996; Reich et al., 1998] along with within-family association tests [Neiswanger et al., 1995; Edenberg et al., 1998]. Use of nonparametric methods provides a significant improvement over conclusions that can be reached from studies using only parametric techniques in which results are highly dependent on model assumptions and/or choice of parameters. For example, Bolos et al. [1990] used only parametric analysis of two families in which alcohol dependence was segregating. While our previous analysis employed both parametric linkage and within-family association analysis of 20 families [Neiswanger et al., 1995], the current study used a greatly expanded sample (54 families) and the power of the SIBPAL nonparametric analysis to further test for linkage. Thus, prior to the present study, six studies have directly assessed linkage or linkage disequilibrium to a D2 receptor polymorphism in families with largely negative results.

As noted previously, population-based association studies of the D2 receptor show highly variable outcomes with a large number of both positive and negative results. In an earlier report [Neiswanger et al., 1995], evidence was found for a population-based asso-

TABLE VI. Sib-Pair Analyses of Members of Families Selected Through a Pair of Female Proband Alcoholics*

	DRD2-TaqI A			DRD2-C			DRD4		
	P	df	r^2	*P*	df	r^2	*P*	df	r^2
Groups A, B, and C[a]	0.0007	(29)	30.12%	0.035	(14)	21.53%	N.S.[b]	(30)	—

*Alcoholics with ASPD were evaluated with respect to alcoholics without ASPD and with nonalcoholics (N = 79). Analyses were based on SIBPAL quantitative trait analyses.
[a]Group A, alcoholic by Feighner criteria (three symptom categories out of a possible four) and the presence of ASPD by DSM-III criteria; Group B, alcoholic by Feighner criteria but no ASPD; Group C, nonalcoholic requires that Feighner criteria symptoms be present in two or fewer categories.
[b]Not significant.

ciation for the TaqI A polymorphism and alcoholism. One interpretation of the significant population-based association found in our laboratory is that screened controls have lower frequencies of the TaqI A1 allele than either alcoholics or the general population. Specifically, if one uses an unscreened control population, then a significant amount of alcoholism, depression, and other common psychiatric disorders can be expected to occur within that control population. If the elevation in the A1 allele is not specific to alcoholism but is related to a latent trait highly predictive of alcoholism development, then that latent trait could be the foundation for other disorders as well. For example, the D2 receptor has been implicated in other phenotypes from among the substance-dependence disorders [Blum et al., 1996]. However, the observed relationship between the substance-dependence disorders and the A1 allele may be the result of underlying temperament differences. Variation in the novelty seeking temperament and the presence of variants of both the D2 and D4 receptor polymorphisms have been reported [Benjamin et al., 1996; Ebstein et al., 1996], though not replicated in all studies (see, for example, Pogue-Geile et al. [1998]). Recently, the D2 and D4 receptors have been implicated in negative affectivity [Hill et al., 1999], a temperament trait that is associated with substance dependence, especially cigarette smoking [Lerman et al., 1998]. Thus, a common temperament trait covarying with axis I disorders may be present among the D2 A1 carriers.

The present analysis found no evidence for linkage between the D2 and D4 polymorphisms and Feighner criteria alcoholism. However, phenotypes associated with greater alcoholism severity (presence of physical dependence symptoms, earlier onset, or comorbid ASPD) were evaluated and found to be generally more positive. The presence of one or more physical dependence symptoms in combination with Feighner criteria alcoholism revealed some evidence favoring linkage (TaqI A and D4). Also, alcoholics with an earlier onset of alcoholism showed some evidence for linkage especially when the presence of physical dependence was required (e.g., morning drinking, wanted to stop drinking but could not, binges or benders, and evidence of withdrawal symptoms). Finally, alcoholics with ASPD differed significantly from nonalcoholics for both D2 polymorphisms.

Results for the D4 VNTR and Feighner criteria alcoholism were positive when the presence of one or more alcohol dependence symptoms was required. Previously both positive and negative population-based association studies have been reported for D4 and alcoholism. One haplotype relative risk study has been reported for 29 alcoholics in which both parents were typed with negative results [Parsian et al., 1997].

In spite of the evidence favoring linkage uncovered by these analyses, albeit limited, the troublesome issue has to do with the fact that a mutation within the protein-coding region of the D2 receptor has not been found. The human D2 receptor gene contains at least eight exons and spans over 50 kilobases. The protein-coding region is separated from the promoter region by an intron that is over 25 kilobases. If the polymorphism for which linkage has been found resides in this large intron, how might there be justification for considering this an important finding functionally? The answer may be found in at least two considerations. First, Gejman et al. [1994] did not look in the 3′ or 5′ regions when looking for mutations. Variation in the genomic sequence of the promoter region of the D2 receptor gene could effect regulation or expression of the gene. A functional polymorphism in the 5′ promoter region of D2 has been identified (−141C INS/Del) which appears to affect susceptibility to schizophrenia [Arinami et al., 1997]. Also, "position effect" mutations have been described that are capable of altering gene expression through long-range effects in which they disrupt distal regulatory elements of a gene [Bedell et al., 1996]. As pointed out by these authors, these mutations can be located several hundred kilobases from the affected gene and have been causally associated with a number of human genetic diseases (e.g., campomelic dysplasia). Fourteen different human and mouse mutations thought to result in position effects have been identified [Bedell et al., 1996]. The rearrangements identified occur between 4 and 400 or more kilobases from the affected gene and resulted in either activation or repression of gene expression.

Another important issue with respect to the D2 findings is whether the identified variants confer differing receptor numbers and/or binding properties in brain tissue. Noble and Blum [1991], in a study involving both alcoholics and nonalcoholics, found that individuals who were homozygous for the A1 allele had lower densities of the dopamine D2 receptors in striatum than did individuals who were homozygous for the A2 allele. Recently these results have been strengthened by the findings of Thompson et al. [1997]. Using [^{3}H]raclopride to detect D2 ligand binding, autoradiography of the caudate, putamen, and nucleus accumbens was performed in tissue from normal middle-aged and elderly individuals without histories of substance abuse, neurological disorders, or psychopathology. Analysis of the data revealed that the presence of one or both A1 alleles was associated with reduced receptor binding throughout the striatum with statistically significant decreases being found in the ventral caudate and putamen. Further support for a relationship between variants of the TaqI A polymorphisms and pharmacokinetic properties of D2 receptors comes from a recent in vivo study of 54 healthy Finnish volunteers [Pohjalainen et al., 1998]. These investigators used positron emission tomography (PET) to study D2 receptor density in the striatum. Pohjalainen and colleagues determined D2 receptor binding density (Bmax), affinity (Kd) and availability (Bmax/Kd) in the volunteers using [^{11}C]raclopride to perform the PET studies. A statistically significant reduction in D2 receptor availability reflecting an alteration in receptor density was observed in the A1/A2 genotype group compared to the A2/A2 group. These results suggest that the A1 allele of the TaqI A polymorphism may be in linkage disequilibrium with a mutation in the promoter/regulatory gene element that affects dopamine D2 receptor expression.

While the association and linkage studies performed

in alcoholics to date may still be viewed as controversial, there is ample evidence from the animal literature to suggest that dopamine plays an important role in the appetite for alcohol. In general, enhancement of dopamine transmission in the nucleus accumbens increases ethanol-reinforced responding, whereas decreasing transmission decreases ethanol responding [Hodge et al., 1992, 1994]. These findings along with observations that administration of a dopamine receptor antagonist reduces both lever pressing for alcohol and home cage intake in rats [Pfeffer and Samson, 1985, 1986, 1988] suggest the importance of dopaminergic activity and the acute rewarding effects of alcohol. Although the GABA A receptor complex, along with opioid peptides, appears to operate in concert with dopamine in the ethanol "reward" circuit (midbrain-forebrain-extrapyramidal circuit), dopamine clearly has an important role [Koob, 1992].

Possibly the alteration in the rewarding effects of alcohol might be part of a generalized reduction or enhancement of reward related to inherited variation in D2 receptors as suggested by Noble and colleagues [1994]. However, dopamine is not the only reward transmitter, and dopaminergic neurons are not the final common path for all rewards. As Wise and Rompre [1989] have shown, there is only partial overlap in the distribution of dopaminergic fibers and the "reward fibers" seen upon mapping of animal brains using electrical stimulation techniques. Nevertheless, activation of the dopamine system appears to alter general arousal as reflected in locomotor activity, a role that may be necessary in the performance of motivated behavior. Considerations of the role of dopamine in motivated behavior may explain the observed relationship between P300 amplitude and allelic variation in the D2 receptor previously found in our laboratory [Hill et al., 1998].

In summary, while definitive evidence for linkage between the alcohol dependence phenotypes investigated and the dopamine D2 and D4 receptor polymorphisms has not been found, the results are sufficiently suggestive to warrant further work with these genotypes.

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