INVITED REVIEW

Biological phenotypes associated with individuals at high risk for developing alcohol-related disorders: Part 1

SHIRLEY Y. HILL

Department of Psychiatry, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA

Abstract

This article reviews the results of studies concerning particular classes of biological phenotypes that may have relevance for alcohol dependence. Broadly defined, these classes include brain neurotransmitter systems and neuroelectric potentials. Evidence is presented concerning genotypic variation in alcoholics and high-risk relatives suggesting that the etiology of alcoholism and other addictive diseases is mediated in part through sub-optimal neurotransmitter functioning. Research opportunities are offered with respect to specific candidate genes that have been cloned from these neurotransmitter systems that could be most fully utilized in family-based genetic analyses. Additional evidence is offered, suggesting that characteristics of particular neuroelectric potentials (e.g. the amplitude of the P300 component of the event-related potential) may provide another dimension of potential markers that could be used to identify children at risk. Finally, methodological considerations specific to high risk studies are discussed. Among these are the need to include a plan for studying more severe cases of alcohol dependence that are relatively uncomplicated by other major psychiatric disorders. Plans for long-term follow-up of children at highest risk for developing the disorder should also be included. Multiple domains of inquiry should not be viewed as "unfocused" but rather as an economical means for utilizing highly characterized samples of individuals meeting rigorous research criteria.

I. Introduction

The rationale for searching for biological phenotypes associated with individuals at high risk for developing alcohol-related problems is considerable. First, the classic twin, adoption and family studies initiated in the 1960s that demonstrated a significant genetic contribution to alcoholism risk have now been supplemented by large-scale behavioral genetic inquiries. Recently, more sophisticated statistical modeling techniques have been utilized to evaluate the relative contribution of genes and environment to alcohol dependence risk, confirming that the genetic contribution is considerable.[1,2] Kendler and colleagues studied six major psychiatric disorders in women, finding that alcoholism was substantially more heritable

than any of the disorders studied. A recent segregation analysis of families of male alcoholics also gave evidence for genetic factors.[2] In that study, families were ascertained through double probands, a methodology which tends to increase the recurrence risk to members of these families. Most family studies select families on the basis of a single affected case. Thus, an alcoholic proband is utilized to identify other family members. The double proband methodology selects families on the basis of two alcoholic siblings. Requiring the presence of a pair of alcoholic siblings tends to result in mulriple affected cases and increases the odds that the family selected will have greater genetic mediation of the disorder.

The primary purpose of this review of selected neurobiological markers and candidate neurotransmitter genes is to provide a framework for new research that could have the most significant impact with respect to understanding better the etiology of alcohol dependence, thereby suggesting better treatments and offering more informed methods of prevention. In keeping with the overall goal of the symposium held by NIAAA to identify gaps in our current understanding of genetic factors influencing alcohol abuse and alcohol dependence this review is not exhaustive, but rather selective with respect to those areas where the potential payoff of future studies might be the greatest. A secondary purpose of this review is to discuss methodological limitations currently utilized in high-risk study designs and to suggest ways that greater information could be gained from these studies. Because ongoing research specifically addressing particular candidate genes in alcoholics or their family members is sometimes limited, relevant studies will be reviewed for particular markers where more abundant data are available with respect to other psychiatric disorders. This review will discuss: (1) clinical studies involving genotyping of candidate genes regulating neurotransmitter function, and (2) studies of genetically mediated electrophysiological measures (EEG and event-related potentials [ERP]) that have been shown to systematically vary in high-risk individuals from that seen in low-risk individuals.

II. General methodological considerations
Background
A number of promising biological markers for alcoholism vulnerability have been identified, including variations in a number of neurotransmitter systems (e.g. GABA, endogenous opioids, dopamine and 5-HT). Also, considerable attention has been focused on brain potentials (EEG and event-related potentials). The following review will address the extant literature concerning these particular markers, highlighting those markers that have been found to show variation in either human studies of alcohol dependent persons or their high-risk relatives or in laboratory animals.

Qualitative versus quantitative criteria for measuring "risk"
Early studies that attempted to contrast persons with and without familial alcoholism risk utilized the Family History Positive (FHP)/Family History Negative (FHN) designation. Using this dichotomous strategy, some studies designated individuals as FHP based on the presence of a single alcoholic relative. An innovation in this process was the introduction of selection criteria, assuring a greater loading of alcoholic cases within families; namely, the use of the "double proband" methodology.[3]

The need to sample multiple domains contributing to alcoholism
Studies which are limited to investigations of single domains, although informative, may have less potential to explain major portions of the variance in outcome among high-risk individuals. Clearly, combining information concerning such diverse areas of inquiry as temperament and information processing characteristics will have greater likelihood of explaining more of the variance in outcome among high-risk populations than either alone.

Search for genes and neurobehavioral markers with developmental trajectories
It is highly unlikely that important genes for alcohol dependence will be found that are not age-specific. Gene expression clearly varies in development. Behavioral markers relevant in childhood (e.g. attention deficit hyperactivity disorder; P300 amplitude) may not be relevant in adulthood and vice versa. Accordingly, it may be useful to focus attention on proto-oncogenes (e.g. c-Fos, c-Myc) which are thought to play a role in the mediation of neural activity on gene expression.

III. Neurotransmitters and risk for alcoholism

Background

The following review will cover GABA, endogenous opioids, dopamine receptors (D2 and D4) and the serotonin receptor polymorphisms (5-HT1, 5-HT2, 5-HT3) and the serotonin transporter polymorphism (5-HTT).

Methodological considerations

Search for alcohol dependence genes versus psychopathology genes. The presence of heterogeneity is highly problematic for uncovering important genetic linkages between specific markers and a complex disorder such as alcohol dependence. While removing comorbidity from among alcoholic probands and family members may be labor intensive, the payoff in uncovering more specific genes for alcohol dependence is well worth the effort.[4]

Linkage versus association studies. Some research groups have concluded prematurely that one technique is better than another. In reality, it depends on the question one is asking and the feasibility of obtaining the type of individuals/families one needs to perform either an association or linkage study. For example, Risch & Merikangas[5] advocate conducting family-based association studies of candidate genes using the transmission disequilibrium test (TDT). It is important that we not conclude that current linkage analysis methods are not capable of detecting most genes underlying complex diseases.[6] Current linkage methods can detect genes with major effects. However, if one expects to find genes of minimal to modest effect, a family-based design with multiple sibs is the most ideal. Analysis of discordant sibs is especially useful because of problems encountered with the alcoholism phenotype being prone to phenocopies.[7] Recently a technique has been developed that is especially cost-effective, in that it minimizes the number of sib-pairs needed by combining extremely concordant with extremely discordant sib-pairs.[8]

Candidate genes versus genome scans. Again, the tendency has been to choose one methodology over another. For those groups with large DNA collections, it may be useful to engage in both approaches.

Endogenous opioids

Background. There is evidence that the endogenous opioids are involved in alcohol consumption inasmuch as activation or blockage of this system by pharmacological agents reduces or enhances alcohol consumption.[9] Both animal and human studies have shown that treatment with opioid antagonists such as naloxone[10] and naltrexone[11] can reduce alcohol consumption. The system is complex, involving various ligands, opioid receptors and opioid receptor subtypes, each of which subserve different behavioral and biological functions.[12] While naltrexone and naloxone block mu receptors, they also block kappa and delta-opioid receptors, suggesting that the effects are somewhat non-specific and, perhaps, effective through modification of the general activity of the system. Attempts have been made to identify the specific receptors involved in alcohol intake in animals.[10,13,14] This information has been applied to clinical studies, but only to a limited degree.

Results of studies involving genotyping for opioid variation. The OPRM1 locus has been scanned for polymorphisms by two groups[15,16] and six variants were found. Bergen *et al.*[15] directly sequenced OPRM1 and discovered four variants, while Berrettini *et al.*[16] scanned exon 1 of the OPRM1 locus using SSCP and discovered two variants. Bergen and colleagues[15] studied genetic variation in US and Finnish Caucasians and Southwest American Indians finding no association between any of the four variants of the OPRM1 locus and either drug or alcohol dependence. Berrettini *et al.*[16] studied 55 cocaine/opioid abusers and 51 controls finding no significant differences between the two polymorphisms identified in the mu receptor, although a trend towards a higher frequency of the single base pair change (C to T) in nucleotide 229. Sib-pair analysis based on 270 sib-pairs genotyped in our laboratory for the IVS2 + 691 C/G variant, one of the more common variants (60% of American Caucasians) identified by Bergen *et al.*,[15] revealed no significant differences between alcoholic and non-alcoholic members of high density families.[17]

Research opportunities. Although the results of both association and linkage studies have been negative to date, the evidence from both animal and clinical studies suggests the need for further study, especially the kappa and delta-opioid

receptor polymorphisms. Clinical studies would appear to be beneficial in understanding the etiology but could also point to better treatments. Naltrexone is an effective pharmacotherapy for alcoholism.[11] However, genotyping of alcoholics who do and do not respond to naltrexone might be beneficial in further understanding the mechanism of action.

Serotonin receptors

Background. There is ample reason to consider genotypic differences in serotonergic neurotransmission as having implications for phenotypic variation in alcohol dependence vulnerability. Evidence currently exists suggesting that some alcohol-dependent individuals may have lowered central serotonin (5-hydroxytryptamine, [5-HT]) neurotransmission (See[18] for review). Two lines of evidence from animal studies further suggest the importance of serotonergic regulation in the drive to consume alcohol. These are: (1) studies designed to measure the effects of alcohol intake on serotonergic neurotransmission, and (2) pharmacological manipulations that facilitate, or alternatively block, 5-HT neurotransmission. The general trend observed is that increases in serotonergic functioning decrease ethanol intake, whereas decreased serotonergic functioning increases ethanol intake.[18,19]

Complicating the search for 5-HT genes that could have major importance for alcohol dependence variability is the fact that 5-HT neurotransmission has wide-ranging implications for behavior. Among the effects of alterations in 5-HT neurotransmission are production of obsessions and compulsions,[20,21] anxiety,[22] depressed mood[23] and in the etiology of eating disorders, including anorexia nervosa[24] and bulimia.[25] An impressive literature also exists attesting to the role of serotonin in violent and impulsive behavior, including suicidal behavior.[26,27] Also, it would be naive to assume that any neurotransmitter has its effect on behavior in isolation from other neurotransmitters. For example, ethanol ingestion produces transient increases in serotonergic functioning that activates the mesolimbic dopaminergic reward system. Nevertheless, a search for candidate genes from among those regulating serotonergic function might be an excellent starting point for increased understanding of genetic vulnerability in alcoholism.

Cloning has revealed at least 14 mammalian 5-HT receptors, some of which have been characterized by pharmacological and physiological methods.[28,29] Seven distinct subfamilies of receptors exist. The 5-HT1, 5-HT2 and 5-HT5 subfamilies currently consist of five, three and two subtypes, respectively. For the 5-HT3, 5-HT4, 5-HT6 and 5-HT7 receptors, currently only one subtype has been identified. The present review will include those receptors for which there is either animal or human data suggesting the importance of the receptor, or studies suggesting substantial variation in other psychiatric disorders (e.g. affective disorder) commonly found as comorbid conditions with alcohol dependence.

Review of studies

(a) The 5-HT1 family. Two subtypes from this receptor family, 5-HT1A and 5-HT1D$_\beta$, have promise for uncovering genetic variants related to alcohol dependence.

(i) 5-HT1A. Several groups have investigated the role of 5-HT1A in psychiatric disorders including Tourette's syndrome, obsessive compulsive disorder,[30] bipolar affective illness,[31] alcohol dependence[32] and anxiety[33] although with largely negative results.

(ii) 5-HT1D$_\beta$. The 5-HT1D$_\beta$ receptor has potential importance in the investigation of receptor genes controlling development of alcohol dependence based on animal data.[34] Specifically, Crabbe and colleagues have tested null mutant mice that lack the 5-HT1D$_\beta$ receptor gene for alcohol consumption. These mice had been observed previously to display greater aggression than controls.[35] When these mice are tested for alcohol consumption they are found to drink twice as much alcohol as wild-type mice ingesting solutions of up to 20% concentration (a concentration typically rejected by mice and rats). To date, few clinical investigations have been made utilizing this receptor in clinical studies although one study of schizophrenic pedigrees was negative.[36] However, preliminary results are available from the NIH intramural program where a sib-pair analysis of alcoholics and non-alcoholics from family data showed a trend toward significance.[37] Analysis of this locus in sib-pairs from high density for alcoholism pedigrees in our laboratory[38] was negative.

(b) The 5-HT2 family. With respect to neuropsychiatric disorders, probably the most extensively studied subtypes of the 5-HT2 family are the 5-HT2A and 5-HT2C receptors.

(i) 5-HT2A. The 5-HT2A receptor has been explored with respect to mood disorders,[39-41] and schizophrenia.[42] A positive association for schizophrenia and 5-HT2A has been reported in a Japanese sample[43] although it has not been confirmed by others.[42]

(ii) 5-HT2C. The 5-HT2C receptor has shown promising results in animals studies.[44] Specifically, inbred lines of Preferring (P) and Non-preferring (NP) rats have been developed which consume significantly different amounts of alcohol. Evidence for 5-HT2C being involved in alcohol preference comes from the fact that the P animals show a greater density of 5-HT2C receptors in the hippocampus than do NP rats. Also, one clinical study suggests the utility of this marker. George *et al.*[45] have shown evidence that alcoholics may have reduced sensitivity of 5-HT2C receptors compared to controls. Pursuit of this receptor in high-risk studies would appear to hold promise.

(c) The 5-HT3 receptor. This receptor system has been implicated in a number of psychiatric illnesses including schizophrenia, anxiety disorders and substance dependence.[46] Although there are already 14 mammalian serotonin receptors, with the potential for many more to be identified through newer molecular biological techniques, the 5-HT3 receptor is unique among the 5-HT receptors because it is linked to an ion channel. While the distribution of this receptor in the central nervous system (CNS) is low compared to other 5-HT receptors, there is reason to believe that this receptor may be worthy of future genetic investigations in alcoholic or high-risk samples. This receptor is localized to presynaptic regions. Behavioral, electrophysiological and biochemical studies suggest that the 5-HT3 receptor regulates the release of acetylcholine, dopamine, norepinephrine, cholecystokinin and serotonin. Of particular importance to the substance dependence disorders is the fact that direct evidence has been provided showing that 5-HT3 receptors influence dopaminergic activity.[47-49] Specifically, these investigations have found that 5-HT3 agonists increase the release of dopamine in the nucleus accumbens and that 5-HT3 antagonists block agonist-induced increases in dopamine levels.

Establishing a link between 5-HT3 receptors and release of dopamine in the nucleus accumbens appears to have implications for 5-HT3 as a serotonin receptor capable of influencing drug and/or alcohol dependence. A well-known hypothesis of drug-induced reward proposes that all drugs of abuse increase mesolimbic dopamine activity.[50] Pharmacological treatments designed to decrease mesolimbic dopamine activity are thought to exert behavioral effects by concomitantly decreasing the rewarding effects of the abused drug. Most studies have been conducted examining the indirect effects of 5-HT3 antagonists on the reinforcing effects of amphetamine, cocaine, nicotine and morphine. Unlike these abused drugs, ethanol can directly alter the function of the 5-HT3 receptor and result in elevated mesolimbic dopamine levels (see [51] for review). The possible importance of the 5-HT3 receptor to alcohol dependence is underscored by human work in which the 5-HT3 antagonist ondansetron attenuated several subjective effects of ethanol and the desire to drink.[52,53]

(d) The serotonin transporter. The important role of the serotonin transporter has guided the development of specific antidepressant medications. With this has also come a better understanding of the 5-HT transporter (5-HTT). Following release of 5-HT into the synaptic cleft, the transporter that has been localized to the presynaptic neuronal membranes, begins its work of clearing 5-HT from the synaptic cleft. Transport of 5-HT appears to be sensitive to nanomolar concentrations of most antidepressants (see[54] for review). Thus, genetic studies of the 5-HT transporter are timely for uncovering possible relationships between 5-HT functioning and alcohol dependence. The human serotonin transporter protein is encoded by a single gene (SLC6A4) located on chromosome 17q11.1–17q12.[54-56] Evidence is mixed with respect to an abnormality in the coding region of the gene in affective disorder with one study showing no evidence,[57] and another finding significant differences.[58] Also, variations in 5-HTT have been associated with anxiety symptoms.[59] Currently, this gene has not been investigated in alcoholics or high-risk samples.

Research opportunities. Pursuit of the 5-HT1A receptor with respect to alcohol dependence may be of value because of the well-known role which 5-HT1A receptors appear to play in anxiety.[33] Genotyping of individuals from high-risk families could provide an opportunity for determining if variants of this receptor are associated with susceptibility to alcohol dependence. Additionally, the highly significant results found in the animal studies[35] suggest the importance of further studies of the 5-HT1D$_\beta$ receptor polymorphism in high-risk families. Further work with respect to the 5-HT3 receptor would appear to be warranted in view of the evidence showing the direct effect of 5-HT3 on dopaminergic activity. Limiting the search for 5-HT3 variability in affected and unaffected individuals is the absence of an identified polymorphism with well-known allele frequencies in the human 5-HT3 gene. Finally, pursuit of genetic variation in 5-HT transporter among alcoholics and controls or within families of alcoholics would appear to hold promise, particularly in view of the reported variation in affective disorders and anxiety symptoms in association with variants of the 5-HTT gene.

The GABA receptors

Background. GABA is the most widely distributed inhibitory neurotransmitter in the central nervous system.[60] GABA has been shown to regulate both presynaptic and postsynaptic neuronal activity.[61] At the molecular level, the GABA receptor is a complex ligand-gated chloride channel with multiple binding sites for compounds that modulate the influx of chloride ions.

Several lines of evidence suggest that GABA plays a fundamental role in mediating the effects of alcohol.[62] The discovery that an inverse benzodiazepine agonist (RO154513) could antagonize the intoxicating effects of alcohol has provided considerable incentive to investigate further the role of GABA receptors in psychopharmacological effects of alcohol. Because alcohol and other sedative drugs (benzodiazepines, barbiturates) all stimulate GABA receptor Cl-transport, it has been suggested that GABA alteration may explain the common psychopharmacological effects of these drugs. The recent discovery of loci on mouse chromosomes 1,4 and 11 that contain genes that influence alcohol withdrawal severity[63] suggests that GABA receptors may have implications for risk for alcohol dependence.

Review of studies involving genotyping GABA polymorphisms. Although GABA appears to play a major role in the reinforcing effects of alcohol, studies of possible genetic variation in GABA receptors among high-risk populations are rare. Schuckit *et al.*[64] studied 41 men selected for low and high response to alcohol and genotyped them for the GABA$_{A\alpha6}$ along with 5-HTT, 5-HT$_{2C}$ and 5-HT$_{2A}$. An association between alcoholism and the presence of a lowered response to alcohol and the GABA polymorphism was found. The Pro/Ser heterozygotes were significantly more likely to be alcoholic than were the Pro/Pro homozygotes.

Research opportunities. The preliminary findings for the GABAA receptor suggesting an association with alcoholism and lowered response to the effects of ethanol suggest the need for further study. Genotyping for other neurotransmitter polymorphisms along with GABAA, as was performed in the Schuckit *et al.* study[64] can be instructive.

The dopamine DRD2 receptor

Background. 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.[65,66] These findings, along with observations that administration of a dopamine receptor antagonist reduces both lever-pressing for alcohol and home cage intake in rats,[67–69] suggest the importance of dopaminergic activity and the acute rewarding effects of alcohol. Although the GABAA 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.[60]

The dopamine D2 gene on 11q22–23 was first described by Grandy and colleagues.[70] A two-allele TaqI A RFLP with a heterozygosity of 0.30 was detected initially at this locus.[71] Because the heterozygosity was not optimal for uncovering linkage between D2 and human disorders, Hauge and colleagues[72] searched for additional polymorphisms within or close to the D2 gene. As a result, two additional polymorphisms, the TaqI B

RFLP and the microsatellite C polymorphism, were later described.[72] 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, although disequilibrium was not found previously for either the TaqI A or B and the C polymorphism.[72]

Review of studies

(a) Population-based association studies. The initial report of a population-based association between alcoholism and the TaqI A D2 dopamine receptor polymorphism[73] 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.[74–83] However, positive population-based associations[84–86] have also been reported.

(b) Linkage studies. 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.*[74] 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[85] also failed to find linkage disequilibrium using a larger sample of 17 nuclear families. Previous attempts to find linkage in our laboratory[84] 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.

Cook and colleagues[87] conducted a nonparametric linkage analysis of the TaqI A in a sample of seven British families. A highly significant effect was found for the D2 locus and 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 10 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 sites and involving 105 families found no evidence for D2 receptor variation from the linkage analyses performed.[88] 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 test [AFBAC]) with results based on these analyses reported to be non-significant.[89] However, a reanalysis of that dataset utilizing alternative multipoint non-parametric linkage analyses implicated two regions containing candidate genes.[90] The reanalysis by Curtis *et al.*[90] found a MALOD (maximum lod score obtained for any of the transmission models) significant at 0.02 for the D2 TaqI A polymorphism. Due to the conservative nature of the program GENEHUNTER utilized in the COGA analysis,[88] this region had been missed previously. Also, reduced power to detect within-family association was present in the TDT tests performed by Edenberg and colleagues[89] because of the requirement that both parents be genotyped and not homozygous.

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, we undertook a study to test for the presence of linkage to the D2 TaqI A and C markers within 54 high-risk families utilizing non-parametric techniques. While definitive evidence for linkage between the broader alcoholism phenotype (presence of definite alcoholism by Feighner criteria and DSM-IV) and D2 was not seen, our 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 utilized.[91] The alcoholism phenotype requiring the presence of physical dependence symptoms resulted in evidence favoring linkage for the D2 polymorphism, TaqI A. Additionally, when the analyses involving alcoholics with ASPD were restricted to families identified through a pair of

female alcoholics, both 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 non-alcoholics, 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 non-alcoholics resulted in an explained variance of about 30% for the TaqI A polymorphism and 21% for the C polymorphism. Thus, while the results of that study[91] 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.

Methodological considerations. Recently, we have pointed out that when population-based association studies are conducted, the nature of the control group will influence whether significant population-based associations are found.[84,92] Screening to remove alcoholism and other psychiatric disorders from the control group has the potential to alter the results obtained. 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}.[93] Results of this analysis suggest that the D2 receptor may warrant further consideration with respect to alcohol dependence, although 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).

One of the criticisms raised frequently regarding positive D2 findings is that a mutation within the protein-coding region of the D2 receptor has not been found. The human D2 receptor gene contains at least 8 exons and spans over 50 kilobases (kb). The protein-coding region is separated from the promoter region by an intron that is over 25 kb. 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.*[94] 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.[95] 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.[96] As pointed out by these authors, these mutations can be located several hundred kilobases from an affected gene; yet these mutations 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.[96] The rearrangements identified occur between 4 and 400 or more kb from the affected gene and resulted in either activation or repression of gene expression.

Another criticism that has been raised concerning the positive D2 findings is whether the identified variants confer differing receptor numbers and/or binding properties in brain tissue. Noble and colleagues,[97] in a study involving both alcoholics and non-alcoholics, 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.*[98] Using [^{3}H] reclopride 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.[99] These investigators used positron emission tomography (PET) to study D2 receptor density in the striatum. Pohjalainen and colleagues[99] determined D2 receptor binding density (B_{max}), affinity (K_d) and availability (B_{max}/K_d) 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.

Research opportunities. Although some work has been carried out to identify possible mutations,[94] a search for alterations in genomic sequence of the promoter region of the D2 receptor has not been performed. Further work in areas that have not been scanned for mutations might prove important. Moreover, recent attempts to confirm functional differences in the pharmacokinetic properties of D2 receptors in individuals carrying the putative susceptibility alleles which have been positive[98,99] are important break-throughs in the controversy that continues to surround the role of the D2 receptor in alcoholism. Future attempts to genotype high-risk families for within-family association or linkage studies would appear to hold promise, avoiding some of the pitfalls of population-based association studies.

The dopamine D4 receptor polymorphism

Background. The D4 receptor gene is of interest because it contains an unusually polymorphic 16 amino acid repeat region. Moreover, the length of the D4 exon III-repeat sequences has been shown to affect the binding of ligands to the receptor.[100,101] These physiological differences in binding have been observed between the most common short receptor containing four repeats and the most frequently occurring long receptor containing seven repeats.[100,101]

Review of studies

(a) Association studies of D4. 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 United States,[102] one from a Finnish sample[103] and one based on three Taiwanese populations[104] have found no evidence for a genetic association between alcoholism and specific allele frequencies of the D4 locus. However, three positive associations have been reported, one in a Canadian population,[105] one in Israel[106] utilizing an opioid dependent cohort and one in Japan.[107]

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,[107] 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 co-factors for vulnerability were explored, a personality trait (Novelty-Seeking), which has frequently been found to be elevated in substance abusing samples,[108] was found to be associated with specific alleles of D4 (represented chiefly by the seven-repeat) in a sample of opioid dependent individuals.[106]

Variation in the novelty-seeking temperament and the presence of variants of both the D2 and D4 receptor polymorphisms have been reported,[109,110] although not replicated in all studies (see for example,[111]). Recently, the D2 and D4 receptors have been implicated in Negative Affectivity,[112] a temperament trait that is associated with substance dependence, especially cigarette smoking.[113] Thus, a common temperament trait co-varying with axis I disorders may be present among the D2 A1 carriers.

(b) Linkage studies of D4. In our laboratory, evidence favoring linkage was found ($p < 0.04$)

for the D4 VNTR and a more severe form of alcohol dependence (Feighner criteria alcoholism and the presence of one or more alcohol dependence symptoms was required). However, one haplotype relative risk study involving analysis of families of 29 alcoholics in which both parents were typed reported negative results.[102]

Research opportunities. Linkage analyses utilizing both candidate gene[87,91] and genome-wide strategies[90] appear to provide evidence that the D2 TaqI A polymorphism should be pursued further with attention being paid to the type of alcoholism that is being studied. There is evidence that both D2 and D4 are more likely to be informative with respect to the more severe alcoholism phenotypes in which physical dependence symptoms are prominent.[91] This suggests that future studies should pursue alternative alcoholism phenotypes with respect to this receptor polymorphism. Finally, relationships between temperament and both D2 and D4 have been reported in some studies though not all (see Hill *et al.*[112] for review). Characteristics of temperament more common among alcoholics (e.g. Sensation-Seeking, Negative Affectivity) would appear to hold promise for genotyping in families of alcoholics as was recently performed.[112]

IV. Event-related potentials (ERP) and electroencephalograms (EEG)

The P300 component of the event-related potential

Background. There is a long-standing and extensive literature discussing the cognitive impairment seen in chronic alcoholics, starting with the classic work of Victor and colleagues,[114] who described the neuropathological changes seen on autopsy of alcohol-dependent people. These subcortical changes were correlated with both memory and attentional deficits. The notion that cognitive changes as reflected in scores on neuropsychological tests administered to alcoholics might be a pre-morbid condition associated with the etiology rather than simply the consequences of alcohol use was conceptually appealing.[115]

The P300 component of the event-related potential (ERP) has received considerable attention as a possible neurophysiological risk marker for the development of alcoholism. P300 is a scalp-positive wave that peaks approximately

300 msec after an informative event. The possibility that the P300 component of the event-related potential may have etiological significance for alcoholism has been discussed for over a decade.[3,116,117] Nevertheless, it appears that an important question to ask is whether P300 reduction sometimes seen in adult alcoholics, usually older alcoholics, is a cause or consequence of drinking? Unraveling this puzzle is especially difficult because there are a multitude of factors affecting the emergent waveform of which P300 is but one component.

(a) P300 amplitude in adult alcoholics. Whether adult alcoholics without neuropathological changes due to chronic alcohol abuse differ in P300 amplitude from nonalcoholic controls is a debated question. Differences between alcoholics and non-alcoholics have not been found in all studies,[116,118-121] although reduced P300 has been reported for middle-aged abstinent male alcoholics when compared with control subjects.[122-124] On the other hand, adult female alcoholics were reported to display reduced P300 amplitude in comparison to age-matched normal controls.[125] This discrepancy between not finding P300 reduction in adult male alcoholics while seeing reduction in adult female alcoholics was puzzling, and provided the impetus for comparing adult male and female alcoholics in another study in our laboratory. Using both an auditory and visual odd-ball stimulus paradigm to investigate the amplitude of the P300 component, we found that neither male or female adult alcoholics had lower P300 compared to controls when co-morbid depression is taken into account.[126] These results confirm our recent observation that the reduced P300 seen in high-risk children/ adolescents relative to low-risk ones normalizes by adulthood in both females and males.[127]

(b) P300 in high-risk children—relationship to development. Despite the inconsistencies in the literature concerning P300 as a marker of risk in adulthood, a number of laboratories now have been able to document differences in P300 characteristics between high and low-risk children.[117,128-132] Not all high-risk (FHP; family history positive) children show reduced P300 but then, of course, not all high-risk children develop alcohol problems. Approximately one-third of high-risk boys and one-fifth of the girls have been found to display P300 amplitude reduction.[130]

Because the P300 component is one index of cognitive capacity, both the amplitude and latency of P300 have been studied among subjects thought to differ in some neurocognitive, behavioral or maturational dimension. For example, P300 amplitude and latency change with the developmental stages of childhood[133] and according to the varying rates of maturation of the auditory and visual modalities during childhood.[134] In fact, it has been suggested that the neural generators of P300 may differ for each modality.[135,136] More recent research addressing P300 as a risk marker in offspring from high density for alcoholism pedigrees has addressed important considerations including age, gender, drinking history and modality of stimulus presentation, uncovering some important information concerning the parameters under which P300 amplitude may be used as a risk marker.[130,131,127] Most recently a latent growth curve analysis revealed that high-risk children/adolescents relative to low-risk ones show a developmental delay in achieving their age appropriate, young adulthood, P300 amplitude.[127]

(c) P300: relationship to clinical outcome. Recently two follow-up studies have found increased rates of substance abuse among FHP children who showed reduction of P300 at baseline.[132,137] Berman and colleagues[132] found that those subjects who had the lowest P300 values when they were evaluated at age 12 had significantly increased rates of substance dependence when reevaluated at 16 years of age. Hill and colleagues[137] completed a follow-up of a pilot sample of high and low-risk children (FHP/FHN) initially tested for ERP characteristics in 1985, who were retested and evaluated clinically after approximately 8 years. The ERP testing was repeated with the same paradigm, and continued reduction of the P300 amplitude was apparent. Significantly more of the high-risk children met criteria for substance dependence. Moreover, the high-risk children, who were 18 years old at the time of retesting had significantly lower P300 amplitude. A longitudinal follow-up up currently in progress of a much larger sample of over 200 children with repeated measurement of the event-related potential including P300 (many have been tested over a 7-year period at annual intervals) will ultimately enable us to determine the relationship between the developmental course of P300 during childhood and the substance

dependence outcome. However, latent growth analysis of data obtained to date (635 assessments) indicates a developmental delay in the P300 trajectories of high-risk children in comparison to low-risk controls.[127]

(d) Heritability of P300 amplitude. A considerable body of evidence exists suggesting that brain neuroelectrical activity, including ERPs, is heritable. Greater similarity in ERP waves is observed between first-degree relatives than unrelated individuals, with the greatest similarity observed in monozygotic twins.[118,138,139] Data on alcoholic families subjected to segregation analysis to test the inheritance patterns for the P300 component suggests the presence of a major gene controlling the familial similarity in P300 amplitude.[140] More recently a path anlysis of P300 data from three generation families of alcoholics and controls ($N = 535$) revealed differing patterns of transmission in high and low-risk families.[141] Other laboratories have provided evidence for moderate heritability of P300 amplitude.[142,143]

In summary, specific components of the ERP waveforms appear heritable, suggesting that P300 amplitude is transmitted from parent to offspring with some of the transmission being genetically mediated. Because offspring from families with alcoholism may inherit a tendency to develop alcoholism and a tendency to have a lower P300 amplitude, P300 may be one index of genetic vulnerability to alcohol dependence. Both P300 amplitude and alcoholism susceptibility may, in turn, be related to differences in one or more neurotransmitters. Recently, a genetic association between P300 and the dopamine D2 receptor polymorphism was found in our laboratory.[144] While further research is needed to validate this neurobiological marker of risk, the evidence to date points to its usefulness as a childhood predictor of substance use and dependence in adolescence and young adulthood.

EEG activity as a marker of risk. Studies of EEG activity in high-risk populations have been based largely on the premise that individuals with a genetic predisposition to alcohol dependence respond differently to the pharmacological effects of alcohol. Two different theories have been proposed. The first, proposed by Schuckit (see[145] for review), is based on the notion that high-risk individuals have a low innate sensitivity to alcohol. Accordingly, such individuals will need to

drink larger quantities of alcohol to obtain the same subjective effect. This theory is based on the premise that the differences between family history-positive and family history-negative individuals are not due to differences in acquired tolerance, resulting from high-risk individuals tending to drink more alcohol than low-risk ones. Rather, this theory assumes that given equal acquired tolerance (usually because of obtaining a careful drinking history), the high-risk individual will show a lesser EEG response to the same dose of alcohol. Based on this theory, one would predict that EEG characteristics of high-risk individuals might differ, particularly following the consumption of alcohol. The other prominent theory concerning post-ethanol effects on EEG, proposed by Volavka and colleagues,[146] is essentially a tension-reduction model. Using this model one would predict that EEGs of individuals at high risk for the development of alcoholism would show higher frequency and lower amplitude (with more beta activity) before alcohol consumption but an enhanced level of increased slow alpha activity compared to their lower-risk counterparts.

The results of EEG studies done to date have been somewhat inconsistent. This is due partly to the fact that investigators studying EEG have analyzed different electrode positions and varying frequency bands. Sometimes the alpha band has been considered as homogeneous from the standpoint of analysis,[147] while others have looked at both slow and fast alpha.[148,149] Additionally, samples have varied in how family history of alcoholism has been defined as has the level of alcohol consumption of the included groups. These appear to be important considerations since Volavka et al.[146] found that the higher the density of alcoholism in the families of the subjects studied, the greater the decrease in alpha following alcohol consumption. Similarly, even when normal subjects have been studied, in the absence of stratification by family history, the amounts of alcohol consumed vary considerably from study to study. For example, although two studies[148,150] administered approximately similar doses of alcohol to similar aged young men without a personal or family history of alcohol dependence or psychiatric problems, conclusions about the effects of alcohol on EEG are inconclusive. For example, Ehlers and colleagues[148] studied young men who averaged 3.8 drinks per occasion when they drank. Cohen et al.[150]

studied normal young men who drank 2.3 drinks per occasion. While both the Ehlers and Cohen laboratories found increased power in slow frequency alpha in response to alcohol administration, the Ehlers laboratory[148] found differences in theta that was not found by Cohen and colleagues.[150]

Studies specifically addressing possible risk group differences at baseline have been negative, while those assessing EEG following alcohol administration have generally been positive. Two studies have reported baseline differences, however. Gabrielli and colleagues[151] found family history-positive subjects showing higher amounts of fast activity in the baseline condition. Similarly, Ehlers & Schuckit[149] have reported greater fast frequency alpha (9–12 Hz) at baseline in FHP subjects relative to FHN.

The response to alcohol in family history-positive subjects has been characterized generally by either augmentation or reduction, depending on the frequency band that is being influenced. For example, Ehlers & Schuckit[152] found FHP individuals had higher energy in the beta band (12–20 Hz) following alcohol ingestion. Cohen et al.[153] found alcohol administration increased slow alpha (7.5–10 Hz), and more so in FHP than FHN individuals. Bauer & Hesselbrock[154] found that FHP individuals had more fast alpha at baseline which, following alcohol administration, declined to the level seen in FHN individuals. Ehlers & Schuckit[149] also noted more fast-frequency alpha at baseline among FHP men. However, only the FHN men showed a reduction in fast-frequency alpha following alcohol administration. Finally, the important work of Volavka and colleagues[146] should be mentioned. Data were available for individuals followed 10 years following their participation in a study designed to assess the effects of alcohol on EEG in FHP and FHN men. Two findings are of note. First, the decrease in alpha following administration of alcohol when these individuals were 19 years old predicted membership in the alcohol dependence group 10 years later. Also, the response to alcohol administration for beta activity differed for those later destined to become alcohol-dependent.

In conclusion, several tools for uncovering biological variation between individuals who vary in their liability to develop alcohol dependence are available. These include recent advances in molecular genetic techniques that allow for rapid through-put genotyping, allowing for scanning

the entire genome for possible genetic variation between high- and low-risk individuals. This, coupled with pursuit of promising candidate genes chosen because of their demonstrated importance physiologically to elements of particular neurotransmitter systems (e.g. receptors, transporters), would appear to provide opportunities for new discoveries in the alcoholism field that would have implications for the development of new pharmacotherapies for alcohol dependence. Moreover, identification of these "risk" genes would make it possible to provide more informed understanding of the etiology of alcohol dependence and provide recommendations for prevention, particularly with respect to the more severe forms of alcohol dependence most likely to have greater genetic mediation.

Finally, the study of functional differences in brain activity is now possible because of advances in both neuroimaging (e.g. SPECT, MRI) and in sophisticated methodologies for uncovering brain electrophysiology (e.g. topographic mapping of the ERP). Technical advances in both molecular genetics and in neurophysiology will undoubtedly be productive in high-risk studies if they are combined with careful consideration of the methodological concerns addressed here and in the other paper presented.[155]

Acknowledgments

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