The Alcohol Clamp: Applications, Challenges, and New Directions – An RSA 2004 Symposium Summary

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This article summarizes the proceedings of a symposium organized and cochaired by Vijay Ramchandani and Sean O'Connor and presented at the 2004 Research Society on Alcoholism meeting in Vancouver, BC, Canada. The objectives of this symposium were: (1) to provide a rationale for the development and use of the alcohol clamp and the requirements for its use in alcohol challenge studies; (2) to highlight recent studies conducted using the alcohol clamp to identify sources of variation in the pharmacokinetics and pharmacodynamics of alcohol, as well as to address important research questions related to the relationship between the response to alcohol and the risk for alcoholism; and (3) to provide a perspective on progress, address limitations of the clamp, and identify new directions for alcohol challenge research. The symposium began with an introduction and overview of the alcohol clamp, by Vijay Ramchandani. This was followed by 4 presentations that highlighted recent studies conducted using the clamp including: (1) determination of the influence of alcohol dehydrogenase polymorphisms on alcohol elimination rates in a male Jewish population, by Yehuda Neumark; (2) examination of family history of alcoholism, recent drinking history, and levels and rates of administration as determinants of the response to alcohol and risk for alcoholism, by Sean O'Connor; (3) evaluation of the time course of ethanol intoxication on neuroendocrine function in humans, by Ulrich Zimmermann; and (4) a study of the effects of steady-state blood alcohol levels on auditory event-related potentials in rats, by Sandra Morzorati. Harriet de Wit summarized and discussed the research presented at the symposium and provided her perspective on future directions for research using the alcohol clamp.

Key Words: Alcohol Clamp, Alcohol Elimination Rate, ADH, Family History of Alcoholism, Neuroendocrine Effects, Auditory Event-Related Potentials.

T HE PHARMACOKINETIC AND pharmacodynamic effects of alcohol vary widely across individuals, in ways that may predict risk for developing alcohol-related problems. Individuals vary as much as 3- to 4 fold in systemic concentrations and metabolic rates after alcohol administration, and they vary 2- to 3 fold in subjective, cognitive, and physiologic responses to the drug. Many factors contribute to the variability, including the amount and rate of prior alcohol exposure, drinking history and food intake, differences in gastric emptying, liver volume, and blood flow. The variability in pharmacokinetics complicates the study of individual differences in the pharmacodynamic responses to alcohol, which may be especially relevant to risk for abuse. In particular, there is considerable intersubject variability in alcohol concentrations when the drug is consumed orally. Therefore, alternative methods for administering alcohol that reduce this variability are desirable to study genetic and environmental determinants of responses to alcohol. The alcohol clamp technique is a highly effective method for controlling variability related to absorption.

The alcohol clamp is a method of infusing alcohol to achieve and maintain a target breath alcohol level for a prolonged, predetermined duration of time, across subjects. Combined with physiologically based pharmacokinetic (PBPK) modeling, infusions can achieve fine control over the rate, magnitude and duration of exposure to alcohol The parameters of the PBPK model are tailored to each individual and used to compute individualized infusion profiles that achieve the same exposure ($\pm$ 5 mg%) in every individual. Thus, the alcohol clamping method allows us to assess the response to alcohol under precisely controlled conditions of exposure and provides a unique

platform to evaluate the effects of any number of determinants on the response, such as the magnitude, route, and rate of exposure to alcohol, gender, age, family history of alcoholism recent drinking history (RDH), and the effects of drugs on the response to alcohol. Alcohol response phenotypes obtained under the carefully controlled conditions of the alcohol clamp can be used to examine candidate genes that may be associated with the response to alcohol and which might underlie the risk for alcoholism.

Another major application of the clamp method follows from the ability to obtain a direct measure of the alcohol elimination rate (AER). During the alcohol clamp, when the breath alcohol concentration (BrAC) and the infusion rate are both at steady state, the infusion rate becomes a direct measure of the elimination rate of alcohol in grams per hour. This direct measurement of the AER allows the evaluation of various determinants of alcohol metabolism, including gender, age, ethnicity, and polymorphisms of the alcohol metabolizing enzymes, as well as the role of various factors such as food intake, lean body mass, liver blood flow, and menopause in explaining the variability in alcohol.

Physiologically based pharmacokinetic models scale well across species. Computing the infusion profile to achieve a steady brain exposure to alcohol for a rat is as simple as substituting a rat's cardiac rate, blood volume, total body water, and AER per unit body weight into the model used for a human. This line of research provides the same platform in animals and people, encouraging translational research. Thus, the alcohol clamp methodology provides a precise, accurate, reliable, and unique platform to study the pharmacology of alcohol and to help us better understand the sources of variance in the PK and PD of alcohol and their significance especially in relation to the risk of alcohol dependence and alcohol-related problems.

The objectives of this symposium were: (1) to provide a rationale for the development and use of the alcohol clamp and the requirements for its use in alcohol challenge studies; (2) to highlight recent studies conducted using the alcohol clamp to identify sources of variation in the pharmacokinetics and pharmacodynamics of alcohol, as well as to address important research questions related to the relationship between the response to alcohol and the risk for alcoholism; and (3) to provide a perspective on progress, address limitations of the clamp, and identify new directions for alcohol challenge research.

USE OF THE ALCOHOL CLAMP TO DETERMINE THE INFLUENCE OF ADH POLYMORPHISMS ON ALCOHOL ELIMINATION IN A MALE JEWISH POPULATION— *YEHUDA NEUMARK*

Several studies have now shown that inheritance of the ADH1B*2 (ADH1B*47His, formerly ADH2*2) allele is associated, in Jews, with reduced alcohol consumption (Carr et al., 2002; Luczak et al., 2002; Neumark et al.,

1998; Shea et al., 2001) and a lowered risk of alcohol dependence (Hasin et al., 2002), and that the ADH1B*2 allele is relatively common in Jewish populations in the United States and Israel (allele frequency 0.19–0.32). Since ADH is an enzyme with rate-limiting activity in the pathway for metabolizing alcohol, we hypothesized that inheriting the protective effect of the ADH1B*2 allele would be correlated with a greater AER, yielding lower and shorter brain exposures to alcohol, and a less pleasant drinking experience because of greater concentrations of acetaldehyde. To test the hypothesis, we recently examined the influence of ADH1B polymorphisms on the pharmacokinetics of ethanol metabolism in young adult Jewish males (Neumark et al., 2004). We did not anticipate any substantial AER differences across ADH1C genotypes given our earlier findings. These analyses were now extended using identified SNPs and haplotypes within the ADH1B gene.

To provide a direct measure of the AER (g/h), a BrAC clamping technique (O'Connor et al., 1998) was used. Application of the "Indiana clamp" allows for direct measurement of elimination, based on a steady infusion achieving a steady BrAC, thereby avoiding much of the background noise that may have prevented detection of the ADH signal in some earlier studies (e.g., Mizoi et al., 1994). In the Indiana BrAC clamp, the infusion rate of 6% (v/v) ethanol in Ringers lactate solution is adjusted so that the difference between online measurements of BrAC and a target concentration of ethanol is eliminated. In the steady state, after equilibration of ethanol in all body water compartments, the mass flow of infused ethanol precisely equals the AER if the BrAC is sustained at the target level. In addition, the infusion is managed so that the brain of each subject is exposed to the same time course of BrAC (Ramchandani et al., 1999). An infusion profile was precomputed by forcing a PBPK model of an individual's alcohol distribution and elimination to follow the desired time course of BrAC (linear ascension to a target BrAC of 50 mg% at 20 minutes, followed by a steady BrAC maintained within 5 mg% of the target until a steady-state basis for calculating the AER was established). This target concentration is a reasonable approximation of the mean BrAC during moderate social drinking and low enough that nausea would not pose a problem for most subjects. After attaining steady-state levels of both BrAC and infusion rates for a minimum of 45 minutes, the infusion was stopped and BrAC was measured every 15 minutes for determination of the descending limb slope (DLS; mg%/h). The DLS measures the disappearance rate of alcohol concentration calculated as the slope of the pseudo-linear portion of the descending limb of BrAC, by linear least-squares regression over at least 5 measurements.

Thirty-seven SNPs were identified across the ADH-cluster of 7 genes (Foroud et al., 2003). For this report, we focused on the 6 common SNPs (allele frequency >5%), found on the ADH1B gene. We used r^2 to assess linkage

disequilibrium (LD) to identify tagSNPs that capture the majority of common variation in the ADH1B gene (Carlson et al., 2004). A Bayesian approach, implemented through the software package PHASE, was used to infer haplotypes based on the chosen tagSNPs.

A total of 109 male, Jewish, physician-examined, healthy, university students participated in the study. The average age of the participants was 26.0 ± 2.5 years (range 19–33), and the majority (79%) were of Ashkenazic (European) origin. The ADH1B*2 allele was carried by 32% ($n = 35$) of subjects including 4.6% ($n = 5$) who were homozygous for the variant allele. The ADH1C*2 allele was found in 51 subjects (47%) of whom 11 were homozygous for this allele. Both of these genotype distributions were in accordance with Hardy-Weinberg equilibrium (ADH1B $\chi^2 = 0.68$, $p = 0.41$; ADH1C $\chi^2 = 0.98$, $p = 0.32$).

A significantly higher mean AER was observed among subjects who carried the ADH1B*2 allele (8.09 ± 1.4 g/h, $n = 34$) compared with ADH1B*1 homozygotes (7.14 ± 1.5 g/h, $n = 71$), $p = 0.003$. The mean AER increased monotonically with an increasing number of copies of the ADH1B*2 allele. In regression analysis, the ADH1B allele alone explained 8.5% of the AER variance. As expected, differences in AER observed across ADH1C genotype groups were not statistically significant ($p = 0.375$).

The mean value of the DLS was steepest among those carrying 2 copies of the ADH1B*2 allele (DLS mean-SD = 15.6 ± 4.9 mg%/h), compared with 13.6 ± 3.6 mg%/h among heterozygotes, and 13.08 ± 4.2 mg%/h among ADH1B*1 homozygotes. A test of repeated measures showed these differences to be of borderline significance ($p = 0.048$). This difference achieved statistical significance ($p = 0.015$) when these 3 genotype groups were collapsed into 2 groups—individuals with and without the ADH1B*2 allele. Virtually no difference in the mean slope of the descending limb was noted across ADH1C genotypes.

On ADH1B, all 6 SNPs were in low LD ($r^2 < 0.064$); each bin contained a single SNP. Thirteen haplotypes were thus defined based on 93 subjects using 5 tagSNPs (1 SNP was omitted because of a relatively large number of missing values). Six common ($>5\%$) haplotypes described 94% of the 186 chromosomes. Only 1 SNP (identified as rs1229984 and corresponding to the ADH1B*2 allele) was found in univariate analysis to be associated with AER in this sample. In this additive model, each additional variant allele of the ADH1B gene reduced AER by 0.76 g/h ($p = 0.003$). No significant independent effects for any of these SNPs were noted in the multivariate model. A single common haplotype (carried by 16.1% of individuals in the sample) showed a significant association with AER ($\beta = -0.955$, $p = 0.003$, $r^2 = 0.106$). The independent association of this haplotype with AER remained significant in multivariate analysis, using different haplotypes as the reference group.

We believe that application of the novel "Indiana clamp" enhances AER measurement accuracy, allowing for detection of differences that were previously undetectable. Evidence for variation in alcohol metabolism in different ADH genotypic groups provides further support for the role of physiological protective factors in alcohol drinking. Alongside social and cultural influences that have long thought to explain the reduced levels of alcohol drinking among Jews, inherited genetic background seems to a play a role. The relative contribution of genetic effects on alcohol consumption and metabolism will be enhanced as we continue to investigate additional loci within the ADH-cluster and elsewhere. Exploration of putative gene–gene and gene–environment interactions will further our understanding of the mechanisms involved in the metabolism of alcohol in this population. Enhanced measurement precision and increased informativity and utility of genetic markers may well lead to the early identification of high-risk individuals and the development of targeted prevention activities.

DETERMINANTS OF RESPONSE TO ALCOHOL AND RISK FOR ALCOHOLISM: FAMILY HISTORY OF ALCOHOLISM, RDH, AND LEVELS AND RATES OF ADMINISTRATION— *SEAN O'CONNOR*

The Indiana Alcohol Research Center continues to develop experimental methods for evaluating determinants of alcohol responses based on the combination of intravenous infusion of alcohol and PBPK modeling. These efforts follow from our assumption that oral administration of alcohol yields an unacceptably high degree of variability in the experimental exposure of brain to alcohol, measured by BrAC (mg%) as a function of time across subjects, and on the observation that, in the steady state, a subject's AER (grams of ethanol per hour) can be measured directly. The latter observation is true at face value and powerful in its implications for studies of drugs or conditions that can modify an individual's AER. We believed we needed to test our assumption about BrAC variability in response to oral dosing and tested 40 healthy young (21–30 years) social drinkers who agreed to forego alcohol for 2 days and fast since midnight the evening before weekday testing in the laboratory. Subjects arrived at 7:00 AM and ate a standardized 350-calorie breakfast at 7:30 AM. At 10:00 AM, after baseline administration of a battery of brain function measures, each consumed a mixture of 20% ethanol (v/v) in diet soda, in 4 equally divided doses administered 2 minutes apart. The cumulative dose was carefully calculated, on the basis of each subject's total body water, to be 1.0 g ethanol per liter, minimizing the effects of gender, age, and body size and composition on subsequent kinetic variation in the time course of BrAC. Figure 1 shows the BrAC versus time curve for 44 subjects and illustrates the substantial 3- to 4-fold variability in exposure following oral administration of alcohol.
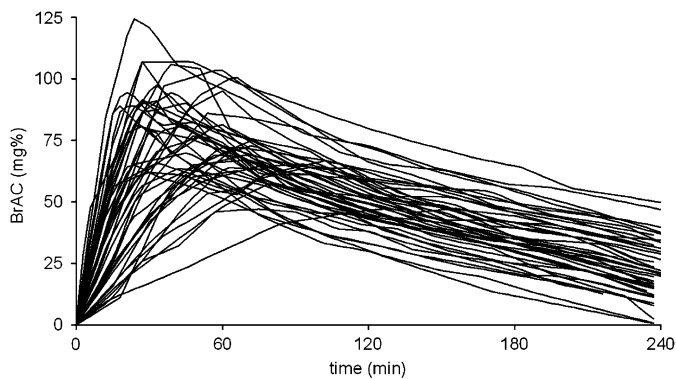
Fig. 1. Variability in the time course of breath alcohol concentration (BrAC) after oral administration of 1 g ethanol per L total body water, after controlling for as many of the variables that could be controlled in an outpatient setting, including dose, vehicle, concentration, food, activity, proximal drinking, time of day, lab setting, and technician. BrAC measurements were obtained frequently for the next 4 to 5 hours, and linear interpolations between measurements, plotted here, demonstrate what we believe to be the minimum variability in the subsequent time courses achievable with oral dosing ($n = 44$).
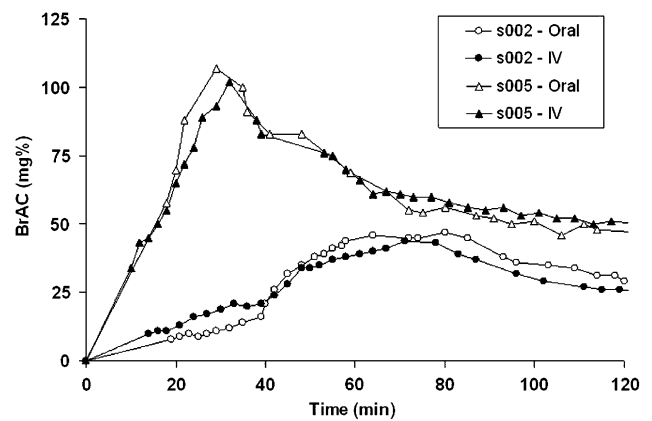

Fig. 2. Breath alcohol concentration (BrAC) versus time profiles for two subjects following oral alcohol and an intravenous (IV) infusion of alcohol designed to mimic the BrAC–time curve following oral dosing. The infusion profile was computed from a slightly modified PBPK model. All 40 subjects returned to the lab for an infusion session designed to test differences in brain function attributable to the route of administration. Preliminary results indicate strong correlations between nearly all dependent measures of brain function obtained at identical time points in both sessions.

Our applications of the methodology to date usually used a BrAC clamp at 60 mg% for several hours (O'Connor et al., 1998, 2000; Ramchandani et al., 1999). We used the exposure to explore the influence of a family history of alcoholism (FHA), personal RDH, and gender on the brain's initial and adaptive response to acute exposure to alcohol (Blekher et al., 2002; Morzorati et al., 2002; Ramchandani et al., 2002). Demographically similar individuals ($N = 118$; 21–38 years, 50% female) were classified as family history positive (FHP) based on the presence of at least 2 first- or second-degree relatives or as family history negative (FHN) if there was no alcoholism in any first- or second-degree relative. Subjects completed both an alcohol session (BrAC clamp at 60 ± 5 mg% for 150 minutes) and a placebo session (no alcohol in the infusate) in randomized order. Eight tasks of 4 kinds [3 subjective questionnaires, 2 neuropsychological tasks, visually guided and antisaccade eye-movement tasks, and 2 event-related potential (ERP) tasks] were used as dependent measures and measured on identical schedules at baseline, beginning at 29 and 125 minutes after the start of the infusion. A significant time×session interaction was required before an alcohol effect was inferred in any variable, and a significant FHA×time effect was required before an initial or adaptive response to alcohol was examined. Recent drinking history was measured using total drinks and number of drinking days in the 28-day interval prior to the study, gleaned from a daily diary. Multivariate regression was also used to test for the association between RDH and the initial and adaptive response to alcohol and the influence of gender on this relationship.

There were no statistically significant RDH differences between FHP and FHN groups, and there was no significant relationship between AER and RDH. Greater initial responses of subjective perceptions to alcohol were signif-

icantly correlated with lesser RDH, but vice versa for acute adaptation. Although unassociated with FHA in this study, RDH may be an important determinant of the initial and adaptive subjective response to alcohol during a 60 mg% clamp (Ramchandani et al., 2002). Both high and intoxicated feelings, and all subscales of the Biphasic Alcohol Effects Scale (BAES) and Sensation Scale, were sensitive to a BrAC of 60 mg% compared with control. Family history–positive subjects had significantly greater initial responses to alcohol and feeling more intoxicated, high, and stimulated and also reported more alcohol-related bodily sensations at the beginning of the clamp. Family history–positive subjects reported a significant decrease in perceptions of intoxication and high during the clamp ($p < 0.03$), i.e., demonstrated acute tolerance to alcohol, but FHN subjects did not. Both FHA groups reported feeling more sedated ($p < 0.0015$) as the clamp progressed. No gender differences in subjective perceptions obtained under any condition (Morzorati et al., 2002).

While the prototypical desired BrAC time course is the clamp, many others are possible. For example, in the study providing data for Figs. 1 and 2, the subject's own BrAC–time curve following oral dosing was used, and the ability to mimic distinctly individual unclamped time courses was demonstrated. This study was conducted in 44 subjects, and differences in alcohol responses attributable to the route of administration were evaluated. Preliminary results indicate strong correlations between nearly all dependent measures of brain function obtained at identical time points in both sessions (V. A. Ramchandani et al., in preparation).

We know from previous studies that brain function is sensitive to the level of alcohol exposure, per se, and the passage of time since the exposure began. Recently, we
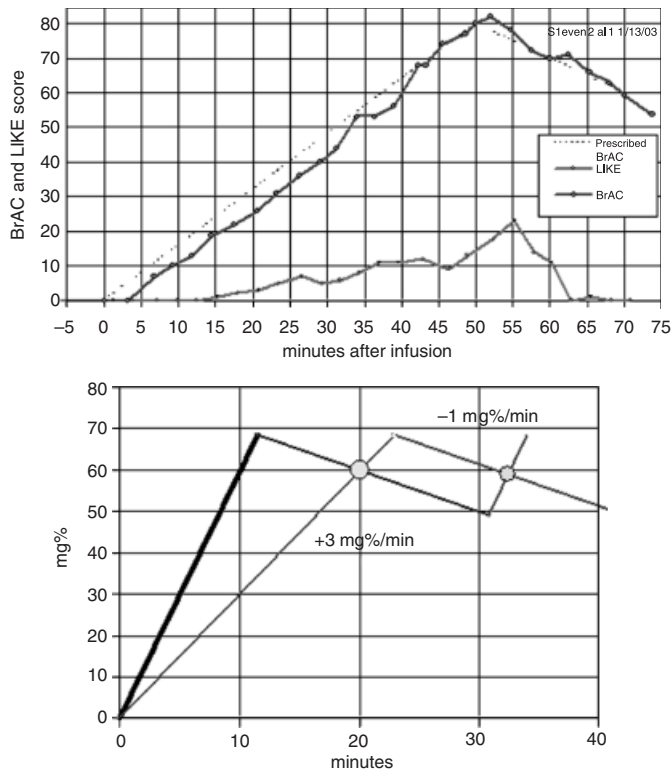
Fig. 3. (Top) An example of the time courses of breath alcohol concentration (BrAC) and the brain function measure "like the alcohol effect" in an infusion session where the BrACs were prescribed to ascend at 1.7 mg%/min for 50 minutes and then descend at −1.0 mg%/min for 20 minutes. Compared to the rates of change of BrAC (slopes) on the ascending and descending limbs, the subject manifested a greater slope sensitivity on the descending limb for this dependent variable. (Bottom) The time courses of BrAC in two separate sessions conducted in each individual twice cross over each other at the same BrAC and same experimental time, but with slopes of opposite sign. Values of the dependent measures obtained at (or interpolated to) these crossover points are of special interest in the current family history of study.

have begun using the ability of the PBPK model to pre-compute an infusion rate profile to explore more basic questions; e.g., does the human brain respond directly to the rate of change of alcohol? We are using the PBPK model–based technique to compute the infusion profile for each individual that achieves a prescribed linear ascending and descending limb of the resulting BrAC–time curve. During that infusion, we recorded subjective perceptions, electroencephalogram (EEG), heart rate, and gaze fixation every 3 minutes so that a time course of brain function can be reconstructed for each dependent measure and compared with the time course of brain exposure (Fig. 3A). The developmental study used ascending rates as high as 12 mg%/min and descending rates as steep as −3 mg%/min (the normal descending rate for humans is rarely steeper than −0.3 mg%/min). Having picked out the two most informative time courses for the purpose in a developmental study, we are now testing 40 subjects in a FHA design using the time courses shown in Fig. 3B.

Another distinct use of the combination of PBPK modeling and experimental infusion sessions is to optimally es-timate an individual's PBPK parameters from carefully recorded time series documenting the input (infusion profile) and output (time course of BrAC) in a particular experiment. This procedure requires many thousands of solutions of candidate PBPK model parameter sets, but the tedium is precisely where computers excel. Two near-term future developments are perceived: extraction of endophenotypical parameters that should be heritable (e.g., V_{max} and K_m) and identification of a very accurate PBPK model for an individual for use in experimental settings where BrAC readings cannot be obtained during the experiment (e.g., the fMRI scanner). Long-term developments may include letting the subject control the infusion rate (a form of computer-assisted self-administration of ethanol in humans) and development of a closed-loop approach when a suitable biosensor (that provides a real-time, accurate, and reliable measure of breath, blood, or interstitial fluid alcohol concentration) is available.

It remains our intent to promote the responsible use of PBPK-controlled infusion methods in the field. To this end, we offer to provide interested researchers with our current software at no cost and with training, in our laboratories or yours, at cost, in return for acknowledging the source in grant applications and presentations. Careful researchers who have experience with these methods are now available in America, Germany, Israel, and Japan.

TIME COURSE OF ETHANOL EFFECTS ON NEUROENDOCRINE FUNCTION IN HUMANS—*ULRICH S. ZIMMERMANN*

Acute tolerance to ethanol can be viewed as a result of the brain's neuroadaptive response to the presence of ethanol. Modest alcohol doses affect functioning of the hypothalamic-pituitary-adrenal and -gonadal (HPA and HPO) and system. For example, cortisol and adreno-corticotropin (ACTH) secretion after pharmacological stimulation by corticotropin-releasing hormone (CRH) (Waltman et al., 1993) and naloxone (Cami et al., 1988) is attenuated, while spontaneous cortisol secretion is unaltered (Inder et al., 1995; Sarkola et al., 1999; Waltman et al., 1993). On the other hand, the nausea that may be associated with high doses of alcohol is in itself a profound stimulus for vasopressin (AVP) secretion, which causes HPA activation in subjects being sick (Inder et al., 1995). Luteinizing hormone (LH) secretion also is affected by ethanol with more complex findings: Luteinizing hormone-releasing hormone (LHRH) in rat hypothalamic explants was found to be suppressed by alcohol (Fernandez-Solari et al., 2004; Rettori and McCann, 1997), while baseline LH secretion in normal women was unaffected Mendelson et al., 1981; Sarkola et al., 1999; Teoh et al., 1988). Stimulation of hypothalamic LHRH by naloxone was reduced by ethanol in male rats (Cicero et al., 1982), but LH response to oral naltrexone was increased by alcohol in women during the early follicular phase (Teoh et al., 1988).

Respective studies in male humans are not published to the best of our knowledge. These effects on neuroendocrine function are consistent with acute action of ethanol on 3 hypothalamic neurotransmitter systems involved in the control of pituitary hormone secretion, i.e., stimulation of endogenous opiates (EOP) (Gianoulakis, 1990; Madeira and Paula-Barbosa, 1999) and gamma-aminobutyric acid (GABA) and inhibition of N-methyl-D-aspartate (NMDA)-mediated glutamatergic neurotransmission (Boehm et al., 2005; Fernandez-Solari et al., 2004).

N-Methyl-D-aspartate-mediated excitatory transmission is involved in pharmacological (Farah et al., 1991; Iyengar et al., 1990) and stress-induced activation of the HPA system (Zelena et al., 1999), possibly by stimulation of AVP (Costa et al., 1992) and involving loci outside the hypothalamus (Gabr et al., 1995). In humans, oral administration of large doses of the excitatory amino acid glutamate produced mixed results. One study reported increased cortisol secretion (Carlson et al., 1989), which could not be replicated by other authors (Fernstrom et al., 1996). Excitatory amino acids and NMDA also stimulate LH secretion (Farah et al., 1991). These mechanisms might be involved in ethanol-induced attenuation of ACTH, cortisol, and LH secretion.

GABAergic activity in the hypothalamus exerts different influences on HPA and HPO system. Benzodiazepine administration, acting through GABAA receptors attenuates CRH release (Kalogeras et al., 1990) and naloxone-stimulated ACTH secretion (Torpy et al., 1993). GABAergic control over the HPO system appears to be more complex, since GABA can stimulate (McCann and Rettori, 1986) or inhibit LH depending on pubertal status (Grumbach, 2002) or estrous cycle (Jackson and Kuehl, 2002).

Endogenous opiates inhibit hypothalamic releasing hormones including CRH (Drolet et al., 2001) and LHRH (Wildt et al., 1993). This is the reason why acute opiate receptor blockade by naloxone administration stimulates secretion of ACTH, cortisol, and LH. Special relevance of this neuroendocrine function test comes from the observation that HPA response to naloxone stimulation varies according to family history of alcoholism in healthy nondependent subjects. Offspring of alcoholics show more HPA activation after opiate receptor blockade, suggesting that their endogenous opioidergic control over HPA activity is stronger than in FHN controls (Hernandez-Avila et al., 2002; Wand et al., 2001). This phenomenon appears not to be a general marker of psychiatric disorder, since HPA response to naloxone is unaltered in patients with affective disorders compared to healthy controls (Judd et al., 1981). Two studies investigated the combined effects of ethanol and naloxone administration in humans and found that ethanol largely abolished cortisol response (Cami et al., 1988) or slightly diminished ACTH and cortisol response (Jeffcoate et al., 1980).

For these reasons, we thought that investigating the neuroendocrine response to opiate receptor blockade might be a promising tool to observe central nervous adaptive processes occurring during the time course of an alcohol exposure experiment, i.e., acute tolerance. We therefore tested in a repeated-measures design whether the neuroendocrine response differed between experimental days involving short versus long ethanol exposure prior to naloxone administration.

We hypothesized that (i) the HPA response to naloxone injection would be reduced during ethanol versus placebo administration if naloxone was given 25 minutes after onset of alcohol exposure (i.e., initial response) and (ii) that this alcohol effect would be attenuated if naloxone was given 2 hours after onset of alcohol exposure (i.e., adaptation).

Ethanol in itself can dose-dependently affect neuroendocrine function. Therefore, we sought to keep the level of alcohol exposure constant throughout the entire experiment and between experimental days to be able to conclude on time rather than alcohol dose effects. Since control over blood alcohol levels is poor after oral administration, we used intravenous (i.v.) ethanol infusion according to the PBPK model suggested by S. O'Connor (O'Connor et al., 1998, 2000; Ramchandani et al., 1999) to achieve constant alcohol levels throughout the time of neuroendocrine testing.

Methods

Fifteen healthy young males without a family history of alcoholism were studied in a single-blind placebo-controlled crossover design with alcohol administration during the first and placebo during the second session in a fixed order. To minimize experimental variance, ethanol was administered intravenously according to a PBPK model (Ramchandani et al., 1999). Breath alcohol concentration was increased to 60 mg% within 20 minutes and clamped at this level for the entire experiment by adjusting ethanol infusion rate. Breath alcohol concentration was measured every 2 to 8 minutes by an Alcotest 7410 device (Draeger Sicherheitstechnik, Lübeck, Germany). Hypothalamic-pituitary-adrenal system was stimulated by an i.v. bolus of naloxone (0.125 mg/kg, Curamed, Karlsruhe, Germany) at 0:25 hour after beginning alcohol/placebo infusion in 8 subjects (i.e., "early" injection thought to reflect the initial response to alcohol) and at 2:25 hour of infusion in 7 subjects ("late" injection thought to reflect adaptation to alcohol). Clock time of naloxone injection was 17.25 hours in all experiments. Blood was taken for measurement of ACTH, cortisol, and LH before and at 15, 30, 45, 60, 90, and 120 minutes after naloxone. The area under the time curve (AUC) after naloxone administration was evaluated as the outcome parameter of endocrine response and was calculated according to the trapezoid rule. Statistical testing was performed by 2-factorial repeated-measures multivariate analysis of variance (MANOVA) with "alcohol" (placebo vs alcohol) as within-subjects factor and "adaptation" (early naloxone injection/initial response vs late

naloxone injection/adaptation) as between-subjects factor. Area under the curve of ACTH, cortisol, and LH were the dependent variables. Subjective alcohol effects (BAES) and body sway were measured repeatedly before and during infusion.

Results

Breath alcohol concentration clamping at 60 mg% was achieved within 20 minutes, and maintained within ± 5 mg% throughout the experiment. Multivariate analysis of variance with the AUC of hormone secretion during alcohol or placebo infusion, but before naloxone administration, showed no significant effects for the factors "alcohol" or "adaptation." The baseline hormone levels immediately prior to naloxone were also not significantly influenced by these 2 factors. Naloxone stimulated the secretion of ACTH, cortisol, and LH during all placebo and alcohol experiments. Multivariate analysis of variance with poststimulation hormone AUCs as dependent variables revealed a significant "alcohol" effect [$F(3, 11) = 3.88$, $p = 0.041$], which was because of a significant effect on LH AUC ($p < 0.05$ in univariate tests), but not on ACTH or cortisol. The effect of "adaptation" and the interaction between both factors was not statistically significant. Alcohol significantly increased ratings of the BAES sedation subscale (*t*-test for repeated measures, $df = 14$, $t = -4.97$, $p < 0.0001$), decreased stimulation subscale ratings ($df = 14$, $t = 2.35$, $p < 0.034$), and increased body sway ($df = 14$, $t = -3.05$, $p < 0.009$). The last ratings of these 3 measures did not significantly differ from prior measurements during alcohol infusion.

Discussion

Infusion of alcohol resulting in moderate breath concentrations did not per se influence hormone secretion. This is consistent with earlier reports (Inder et al., 1995; Waltman et al., 1993). No interaction between alcohol and time of naloxone injection was observed for stimulated secretion of ACTH, cortisol, or LH. This finding is contrary to our expectations and does not support the notion of acute adaptation of hypothalamic neuropeptide systems to the presence of constant alcohol levels. Alcohol exposure increased the LH response to opiate antagonism, regardless of the time of naloxone administration. This is in line with an earlier report of increased LH secretion upon alcohol administered 1 hour after an oral dose of the opiate antagonist naltrexone (Teoh et al., 1988) and provides indirect evidence of an interaction between alcohol and the hypothalamic EOP system in humans.

We were unable to demonstrate acute tolerance to the effects of alcohol on subjective intoxication and body sway. Acute tolerance is clearly established in humans after oral intake resulting in dynamic blood alcohol levels. However, some researchers questioned its occurrence during exposure to constant blood concentrations (Hiltunen et al., 2000; Kaplan et al., 1999). Our data add to these concerns. Possibly, dynamic changes of blood alcohol concentration over time are essential to initiate adaptive processes resulting in tolerance.

EFFECTS OF STEADY-STATE BLOOD ALCOHOL LEVELS ON AUDITORY ERPs IN RATS—*SANDRA MORZORATI*

We recently characterized the alcohol clamp for use in rats, making it possible to achieve and maintain predetermined steady-state arterial alcohol concentrations (AACs) throughout an experimental session (Morzorati and Stewart, 2005). As detailed elsewhere (Morzorati et al., 2002), an infusion rate profile was calculated by using a PBPK model of alcohol distribution and elimination in rats. A PBPK model is a series of mass balance equations that quantitatively describe the physiological behavior of alcohol in various bodily compartments over time. The infusion rate profile generated by the PBPK model was used to clamp AAC in rats close to steady state beginning 5 minutes after the alcohol infusion was started and maintained AAC at a relatively constant level for a prolonged period of time.

The purpose of this study was to determine whether neuronal activity develops acute tolerance during the alcohol clamp. Acute tolerance was defined as recovery of neuronal function while AACs were maintained at a constant level. Auditory ERPs were chosen as a measure of neuronal activity. Event-related potentials reflect the activity of an ensemble of neurons and are obtained by averaging a number of short EEG segments that are time-locked to onset of the auditory stimuli. The resulting ERP waveform consists of a series of deflections that are named by their polarity and succession and are thought to represent different aspects of information processing in the auditory system. The first long-latency ERP deflections are termed P1 and N1. These components correlate with the detection of stimuli and N1 was found to be consistently suppressed by alcohol in humans, monkeys, and rats (Campbell and Lowick, 1987; Cohen et al., 1998; Ehlers, 1988; Ehlers et al., 1992; Jääskeläinen et al., 1998; Krull et al., 1994; Pfefferbaum et al., 1980; Porjesz and Begleiter, 1983).

Adult male Wistar rats participated in 1 of 2 studies. The first study determined the test–retest reliability of the auditory ERPs over a 3- to 4-hour period. The second study examined the effects of alcohol on components of the auditory ERP during a blood alcohol clamp. All rats were implanted epidurally with stainless-steel screw electrodes overlying the frontal cortex and allowed to recover for 2 weeks. For the test–retest study, ERPs were acquired 2 or 3 times from a single rat, at time 0 (T0) and 60 (T60), 120 (T120), or 180 (T180) minutes thereafter. These experiments were critical to determine if ERPs of similar magnitude and morphology could be elicited multiple times in 1 day from a single rat. For the acute alcohol tolerance study, a rat underwent 2 surgeries: for implantation of

EEG electrodes followed by insertion of a jugular cannula. After a 2-day recovery from the cannulation surgery, ERPs were acquired twice, prior to the alcohol infusion (T0) and after steady-state AAC was achieved (T5, T15, T120, T135, or T195 minutes). A rat was clamped only once (at either 75 or 150 mg%) and recorded only twice. The infusate for the clamp was 20% (v/v) alcohol prepared in saline. Event-related potentials were collected when the rats were alert and immobile based on behavioral observation and the absence of slow-wave activity in the EEG. Dependent variables were calculated as change scores (Bonate, 2000) for each time point for P1-N1 amplitude and the latencies to the peaks of P1 and N1. Change scores were used to normalize the data as ERP component amplitudes vary from rat to rat. Thus, for the test–retest study, the dependent variables were calculated as T60-T0, T120-T0, and T180-T0, while, for the acute alcohol tolerance study, the dependent variables were calculated as T5-T0, T15-T0, T120-T0, T135-T0, and T195-T0. Separate analyses of variance were used to determine if changes in the dependent variables differed with time (test–retest study) and/or differed with AAC and time (acute alcohol tolerance study).

For the test–retest study, changes in P1-N1 amplitude and changes in the latencies to the peaks of P1 and N1 did not differ between recording sessions. These results indicated that, in our hands, ERP parameters were stable and repeatable when acquired multiple times over several hours from a single rat in the absence of alcohol. On the other hand, alcohol significantly ($p = 0.0001$) reduced changes in P1-N1 amplitude in a dose-related manner. Visual inspection of the ERPs prior to and following the alcohol infusion revealed that alcohol had little to no effect on P1 amplitude, consequently, decreases in P1-N1 amplitude reflect decreases in N1 amplitude (Morzorati and Stewart, 2005). This finding is consistent with published reports in humans and animals in which comparable or lower doses of alcohol reduced the amplitude of the N1 component elicited by auditory, visual, or somatosensory stimuli (Campbell and Lowick, 1987; Cohen et al., 1998; Ehlers, 1988; Ehlers et al., 1992; Jääskeläinen et al., 1998; Krull et al., 1994; Pfefferbaum et al., 1980; Porjesz and Begleiter, 1983).

The maximal suppressant effect of alcohol occurred 120 minutes after the start of the alcohol infusion in both clamps. Reductions in P1-N1 amplitude were significantly ($p < 0.03$) greater at this time point compared with all other time points. It has been demonstrated in humans that individual ERP components are generated by multiple neural processes rather than a single brain event (Näätänen and Pciton, 1987; Wood and Wolpaw, 1982). If the same is true in rats, then the delay to the peak effect may reflect the summation of alcohol's action on various mechanisms and/or structures over time. P1-N1 amplitude began to recover 15 minutes following the peak alcohol effect, indicating that acute tolerance had developed in this

dependent variable when AACs were maintained at 75 and 150 mg% (Morzorati and Stewart, 2005). Acute tolerance to the depressant effect of alcohol on N1 amplitude has been reported in human subjects (Cohen et al., 1998). To our knowledge, this is the first demonstration of alcohol tolerance in components of the rat ERP. In humans, the N1 component correlates with stimulus detection (Parasuraman and Beatty, 1980) and may be involved with the amount of stimulus information received by the detecting system (Hillyard and Kutas, 1983). In the present study, a reduction in N1 amplitude suggests that auditory signal detection was diminished by alcohol. Arterial alcohol concentrations of 75 or 150 mg% had no effect on the latencies to the peaks for the P1 or N1 components. Component latencies reflect the rate of stimulus detection. Thus, while alcohol decreased the amount of stimulus information being detected, the rate of detection was unaffected.

In summary, pharmacologically relevant AACs significantly decreased the amplitude of the N1 component of the auditory ERP in rats. As AACs were held constant, N1 amplitude recovered. Neuronal activity, as measured by ERPs, develops acute within-session tolerance during steady-state AACs in rats.

DISCUSSION—*HARRIET DE WIT*

The research summarized here illustrates the rich array of applications of the alcohol clamp procedure. Methods have now been fully developed for controlling breath alcohol levels with exquisite accuracy and control, including both the rate of rise of the ascending limb of the alcohol concentration curve and the maintenance of alcohol levels over extended periods of time. The studies described here illustrate at least 4 uses of the procedure. Dr. Neumark used the procedure to study genetic variations in alcohol kinetics by studying polymorphisms in the genes for enzymes involved in alcohol elimination, in a Jewish population. There is a growing interest in genetic sources of variation in both the pharmacokinetics and the pharmacodynamic responses to alcohol, and the alcohol clamp procedure provides an efficient and sensitive technique to investigate genetic determinants of alcohol elimination. Dr. O'Connor demonstrated how the clamp procedure can reveal the role of drinking history, genetics, and gender in both the initial responses to the drug and the rate of elimination of the drug. His group combines the rigorous control over plasma concentrations of alcohol with highly sensitive objective outcome measures of drug effect, including measures of electrical activity of the brain. Dr. Zimmerman applied the clamp procedure to study the role of the HPA and HPO axes in acute tolerance to alcohol. Studies of acute tolerance are especially difficult with oral administration of alcohol, because of the dynamic changes in plasma concentration. Therefore, the clamp technique is ideal for the purpose of studying changes in hormonal systems in responses to a single dose of alcohol. In an unusual

application of the procedure, Dr. Morzorati used the alcohol clamp technique in rats, using electrophysiological activity as the outcome measure. The development of parallel human and animal models will open the way to conducting more invasive interventions in the animal models that could investigate brain function in responses to alcohol, in ways that would not be possible with humans. Thus, the alcohol clamp procedure has already stimulated an interesting series of studies and promises to become a widely accepted method for studying the acute and sustained effects of alcohol.

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