Enkephalinase Inhibition: 
Regulation of Ethanol Intake in 
Genetically Predisposed Mice

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BLUM, K., A. H. BRIGGS, M. C. TRACHTENBERG, L. DELALLO AND J. E. WALLACE. Enkephalinase inhibition: Regulation of ethanol intake in genetically predisposed mice. ALCOHOL 4(6) 449-456, 1987.—This is the first report of alteration in alcohol intake in mice with a genetic predisposition to alcohol preference and known to have innate brain enkephalin deficiencies. We have been able to significantly attenuate both volitional and forced ethanol intake respectively by acute and chronic treatment with hydrocinnamic acid and D-phenylalanine, known carboxypeptidase (enkephalinase) inhibitors. Since these agents, through their enkephalinase inhibitory activity, raise brain enkephalin levels, we propose that excessive alcohol intake can be regulated by alteration of endogenous brain opioid peptides.

Enkephalinase Alcohol Alcohol consumption Genetics D-phenylalanine Amino acids

A growing body of evidence indicates two distinct relationships which correlate brain chemistry and alcohol consumption. The first, a neurochemical interaction, between the CNS actions of endorphins and enkephalins and the effects of alcohol and alcohol metabolites [19, 20, 28, 29, 32, 60, 65, 67]. The second, a chemical-behavioral interaction, between this neurochemistry and volitional consumption of alcohol, i.e. “drug hunger” [14]. The relationship between alcohol and opiate peptides and endogenous receptor systems are depicted in Fig. 1.

Collectively, the more than 100 publications generated in this area point to an interaction between opiates and ethanol in terms of their behavioral and pharmacological actions. These interactions have been characterized as the “link” hypothesis [2, 31, 66, 78, 81]; (1) opiates and ethanol act through the opiate receptors [81]; (2) TIQs directly or indirectly interact at delta and mu opiate receptor and/or allostERIC sites [47, 61, 72]; (3) narcotic antagonists significantly reduce TIQ-induced abnormal ethanol drinking behavior in animals [30]; (4) in animals and humans reduction in brain endorphins/enkephalins is correlated with pronounced alcohol consumption. Reduction in brain endorphins/enkephalins is evident in three model systems—genetic predisposition to alcohol, stress-induced alcohol intake and alcohol toxicity due to chronic alcohol intake [11].

Animal Evidence

Ethanol, as opiates, alters brain concentrations of B-endorphin, enkephalin and other opioid peptides [23, 69, 74]. Additionally, ethanol interferes with the synthesis of brain peptides [76]. Ethanol, acetaldehyde and TIQs preferentially bind to one or more multiple opiate receptors [46, 53, 80]. While much of the literature indicates preferential binding to the delta opioid site (endogenous enkephalin receptor) [27, 44, 57] this does not negate the possibility that other receptor sites may be occupied [51]. In fact there is evidence that under certain conditions salsolinol may act as an antagonist at delta sites and an agonist at mu sites [1].

Our laboratory has suggested the possible involvement of opioid peptides in the actions of alcohol. It has been shown that certain opioid peptides significantly reduce alcohol consumption in rodents [52]. Initially, ethanol consumption is increased after ethanol withdrawal with a concomitant decrease in brain methionine-enkephalin [56]. Morphine itself may even be involved as a mediator in alcohol seeking behavior. In fact, a nonpeptide opioid recently found in mammalian and other vertebrate tissues has been identified as morphine [34].

It is well established that isoquinolines can be formed in mammalian tissue from ingestion of ethanol [32, 64, 79].

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Administration of isoquinolines to mammals results in the in vivo formation of morphine [34]. It is possible then, that ethanol has part of its effect by virtue of either TIQs or morphine or both. In fact, TIQs can induce alcohol drinking behavior in alcohol avoiding rodents [35]. TIQ-induced alcohol drinking behavior may be the result of a compensatory mechanism whereby TIQs produce an enkephalin deficit [21]. Alcohol compensates for the deficiency of natural enkephalin. The initial acute consumption elicits a restoration of these functions, whereas in chronic consumption, a genetic deficiency is aggravated. This results in insufficient transmitter regulation, inducing formation of aberrant products with a potential for production of endogenous neurotoxins.

To demonstrate the existence of a neurochemical relationship between ethanol seeking behavior and the endorphin system, such that both ethanol and narcotic drugs share similar reward properties [9, 82], requires clear differences in brain endorphin chemistry and alcohol consumption in mice with a predilection towards or away from alcohol intake. Using a 14-day preference test, our laboratory found an estimated correlation of 0.909 between mouse whole brain methionine-enkephalin ([Met]-enk) levels in alcohol preferring (C57BL/6J, C58/J) and alcohol avoiding strains (DBA/2J) [8, 10, 12, 40].

Our laboratory further observed that one of the C57BL strains, C57BL/6N from the Simonsen Laboratories, had reverted to the more normal condition and avoids alcohol while the C57BL/6J, from the Jackson Laboratories, maintains its alcohol preference [13]. There is a statistically significant difference in brain levels of [Met]-enk in these two substrains [15].

Correlating the amounts of alcohol consumed by mouse strains revealed a significant relationship (p<0.5) such that the C57BL/6J mice, significantly deficient in [Met]-enk, drink more ethanol than do DBA/2J, [Met]-enk normal, mice [18]. Similarly, long-term ethanol consumption in hamsters significantly reduces the concentration of a leucine-enkephalin-like immunoactive substance in the basal ganglia [16, 26]. Long-term alcohol administration to rats and guineapigs decreases the synthesis of B-endorphin by lowering the activity of mRNA for proopiomelanocortin, the precursor for B-endorphin [54, 76].

Additional data suggest that stressful situations may exacerbate a tendency to alcohol craving [14, 62]. McGivern and associates (unpublished), using a Case-Stress paradigm, showed that following stress rodents had significantly lowered whole brain enkephalin levels. Animals subjected to this stress paradigm consumed increased amounts of alcohol in their home cages [62].

Recent findings point to the formation of adduct products following ethanol intake which are biologically inactive and/or sequestor viable neuropeptide transmitters [74]. For example, in the pituitary an N-acetylation of B-endorphin, observed following ethanol administration, rendered this endorphin devoid of opiate activity [75]. In vitro experiments by Summers [80] provide another mechanism by which aberrant adducts between acetaldehyde and [Met]-enk could interfere with opioid peptide mediation of alcohol intake in mammals.

**Human Evidence**

Genazzani et al. [43] recently reported a central deficiency of B-endorphins in alcohol addicts. B-endorphin levels, measured in CSF, were reduced by over 65% in the 29 chronic alcoholics. Other indications of brain endorphinergic abnormality in alcoholics come from Fachinetti et al. [41]. Additionally, P3 waves are lower in 80% of alcoholics as compared with normals [7]. Further, speaking to the genetic character of this illness, 35% of the sons of these alcoholics also exhibited abnormal P3 activity. Finally, extensive longitudinal studies with identical and fraternal twins raised apart show the offspring from an alcoholic parent to be far more prone to alcoholism even though reared in a nonalcoholic associated environment [45, 63, 73].

These animal and human data allow the hypothesis that craving for alcohol correlates with a decreased [Met]-enk level [11].

**Rationale for the Development of Enkephalinase Inhibitors as Anti-Alcohol Craving Agents**

In view of the inverse correlation between enkephalin levels and alcohol consumption it became of interest to raise brain enkephalin levels and to examine the effects on alcohol consumption. Direct endorphin replacement therapy does not appear to be a viable alternative for several reasons. First, the replacement drug will be addictive, e.g., Pert et al. [68]. Second, oral administration of endogenous opiates, for therapeutic purposes, is limited because of the extremely labile nature of the substances and poor penetration in the brain, resulting in short duration of action. An alternative strategy would be to use an antagonist such as naltrexone (Trexan) to occupy opiate receptors. However, narcotic antagonists are not clinically useful antidotes for acute alcohol intoxication. While naltrexone and some delta and mu receptor blockers can reduce certain actions of alcohol, they probably do not affect alcohol craving per se and only weakly attenuate intoxication [50, 58]. This fact is borne out in both animal [2] and human investigations [58].

An alternate strategy to increase enkephalin levels directly would be to reduce destruction. Endogenous opiates are rapidly destroyed by endogenous enzymes that cleave amino acid peptides such as enkephalins and endorphins. At least three enzymes act by this mechanism—carboxypeptidase A and B and leucine aminopeptidase [33].

Ehrenpreis and coworkers [39] developed the concept of using inhibitors of enzymes which degrade opiate peptides as possible therapeutic agents to avoid the disadvantages of exogenous administration of an endorphin or endorphin surrogate. Further, there appears to be a correlation between the level of opioid receptors and responsiveness to D-phenylalanine (DPA), a carboxypeptidase A inhibitor, in inbred mice [25].
The effects of these inhibitors (termed "enkephalinase" inhibitors) on morphine tolerance withdrawal [4, 24, 36] coupled with the interaction between morphine, endorphins, and alcohol consumption, stimulated our laboratory to investigate the possibility that enkephalinase inhibitors would alter alcohol seeking behavior and thereby act as potential anti-alcohol craving agents.

Two different enkephalinase inhibitors were examined: hydrocinnamic acid (HC), which crosses the blood-brain-barrier (BBB) poorly, and D-phenylalanine (DPA), which crosses the BBB more readily. These were chosen because preliminary data by Ehrenpreis and others indicated that they were relatively nontoxic and alter tissue enkephalin levels [33, 38, 49, 71].

DPA has been administered to mice at a variety of doses and for different durations without untoward effect. The LD 50 for DPA is 5,452 mg/kg, a value slightly greater than that for the comparable L-form. For a standard human male this toxicity level translates (if a one to one relationship is assumed) to an LD 50 dose of 436,160 mg.

No toxic effects were seen following acute administration to monkeys of 3000 mg/kg or chronic administration of 1000 mg/kg/day for 30 days [38]. Ehrenpreis (personal communication) has carried out 2-month and 6-month oral toxicity studies of DPA in mice. No deaths occurred with acute doses of 10,000 mg/kg. No toxic effects were seen, in a 2-month study, at a dose range of 1 g/kg/day. Using the same dose, and examining 35 tissues for pathology, mice showed no observable toxic effects after 6-months of chronic oral administration. In addition, no behavioral changes were seen in mice over this time period. Heller has comparable results after 2 continuous years administration at 10 times the equivalent human dose. His study, using several dose levels and examination of 8 tissues, focuses on mortality, teratology, carcinogenicity and pathology. No negative findings were reported [49] and personal communications.

Although alternate possibilities exist which might be inferred from the available literature, several conclusions can be stated. First, genetically alcohol preferring mice have alterations in brain enkephalin levels. Second, enkephalinase inhibitors produce an elevation in brain enkephalins. Third, as a direct or indirect consequence of enkephalinase inhibition alcohol consumption may be reduced.

METHOD

Animals, Housing and Acclimation

In this experiment a total of 102 inbred mice were used. Male C57BL/6J and DBA/2J mice were obtained from Jackson Laboratories. All animals were maintained on a 12L/12D lighting schedule, under conditions of constant temperature (25±2°C). Acclimation required 7 days. Animals were weighed daily. The animals were tested at 14 weeks of age. Fluids were provided in sealed containers to prevent evaporation (inverted 12 ml syringes with a standard laboratory angled drinking spout). Animals were housed in individual cages to measure baseline water consumption and thereby allow division into matched experimental and control groups for each of the C57BL/6J and DBA/2J mouse groups.

1-Day Ethanol Acceptance Test

C57BL/6J mice were maintained as naive animals for 6 weeks as described and then divided into matched experimental and control groups. The experimental group received IP injections of DPA (500 mg/kg/day in saline as two divided doses) for 18 days. Control groups were treated similarly save that the injectate was saline alone. Following the last injection each group was deprived of food and water for 24 hr before being challenged by 10% ethanol in tap water. Total fluid volume consumed was measured to provide a baseline
The inflection points evident in Fig. 3 were extracted for analysis intracerebral ventricular injection (ICV) at a dose of 25 micrograms mean alcohol consumption for 7 days prior to injection. All data are using the Duncan Multiple Range test. The square represents the to C57BL/6J mice in a 14-day three choice 2-bottle preference test.

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of ethanol acceptance so that individual variation could be compensated.

The 1-day acceptance test data were evaluated (n=30) using ANOVA procedures. Paired t-tests were applied to pre- and post-drug ethanol challenge data to evaluate proband specific changes. These are necessary because even among C57BL/6J animals there is a distribution of ethanol preference which we have previously shown to covary with brain [Met]-enk levels [18].

14-Day Preference Test

Each group of C57BL/6J mice received a single injection into the cerebral ventricle (ICV) before ethanol challenge. The experimental group injectate was 25 µg of HC in artificial CSF, the control group, CSF alone.

The standard 14-day, 3 bottle, 2-choice preference method of Holman and Myers was used to determine the effects of HC on alcohol preference [55]. This test required that 3 drinking spouts be provided—one leading to tap water, a second to the ethanol supply and the third to an empty container. The bottles were rotated daily to avoid positional cues. Consumption of ethanol (10% in tap water) and tap water was measured daily over a 14-day baseline period. Readings were taken at 9:00 a.m. daily. Calculations of total fluid, total water and water/ethanol ratio were performed. All calculations were corrected for body weight. A mean of the daily preference ratios, corrected for weight, for the pretest 14-day period was calculated for each animal. Substantial changes in weight or total fluid volume consumption warranted exclusion from the study. Food was available ad lib.

Three statistical analyses were performed—ANOVA, Duncan Multiple Range test, and paired t-tests. ANOVA—drug and group effects were analyzed in a 2 factor repeated measures analysis of covariance. Repeated measures of pretreatment (as a mean) for the two independent groups and the group by time interaction was tested with animal weight, ethanol and water intake as covariates. Duncan test—within group means adjusted for the covariates, water and ethanol intake, were compared. The error mean square and degrees of freedom from the main effect for time was used as the error term.

Whole Brain Methionine-Enkephalin Analysis

Eight week old C57BL/6J mice, weighing 18–22 g, were utilized to analyze the effect of HC on whole brain [Met]-enk levels. The mice were divided into four groups of 5–7 mice per group.

The animals were decapitated, the brains removed and placed on dry ice. The samples were weighed and homogenized at 95°C in a solution of 2 M acetic acid for 5 min. Samples were then chilled to 4°C and centrifuged at 14,000×g for 15 min. The supernatant was removed, shell frozen in borosilicate tubes and lyophilized overnight. The next day the residue was resuspended in 0.1% BSA in 0.1 M phosphate buffer, pH 6.8 and centrifuged at 1,000×g for 15 min. The supernatant fractions were assayed for [Met]-enk (Immunonuclear Kits, Stillwater, MN). Duplicate samples were run and a log/logit Y/1-Y graph was used to determine binding. A complete description of our procedure as well as the validation tests performed has been described [18].

Four dilutions of three different samples were tested to determine the reliability of values at different areas on the standard curve. An analysis of variance revealed no significant difference between columns (p < 0.1).

RESULTS

D-Phenylalanine Effect on 1-Day Acceptance Test

Figure 2 illustrates that alcohol-preferring mice exhibited a statistically significant (Student’s t-test, p < 0.01) 21% decline in alcohol consumption consequent to DPA injection. The alcohol consumption of the DPA-treated mice is now comparable to and not significantly different from that of the alcohol-aversive saline treated DBA/2J controls.

C57BL/6J mice treated with DPA drank significantly less 10% ethanol (2.87±0.14 ml; n=11) than did the saline-treated group (3.72±0.14 ml; n=11). This decline is statistically significant at the p < 0.01 level. The ethanol-aversive DBA/2J mouse strain drank comparable amounts of alcohol (3.0±0.21 ml; n=8). In other studies we have determined that reducing the course of injections to 10 days resulted in a decreased ethanol consumption which was not significant (p < 0.1) relative to saline treated controls.

Hydrocinnamic Acid (HC) Effect on 14-Day Preference Test

Figure 3 shows that on the first (day 14) and eighth (day 21) days, following ICV injection of HC, the experimental group exhibited a significant decrease in ethanol consumption. Data of this sort are normally highly variable [57]. To compensate, in part, for this variability scores were adjusted by use of the regression coefficient. HC itself had no effect on total fluid volume consumed on the day of injection or throughout the experiment.

Drug and group effects were analyzed in a 2 factor repeated measures analysis of covariance. Repeated measures from pretreatment, at inflection points, days 1, 2, 3, 8, 9 post-injection were used for the two independent groups. The group by time interactions were tested with animal weight, ethanol and water intake as covariates. Water and ethanol intake covariates were significant with a negative regression for water; weight was not a significant con-
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hydrocinnamic Acid

Enhancement of Whole Brain Methionine-Enkephalin by Hydrocinnamic Acid

HC was provided to 25, 8 week old, C57BL/6J, alcohol-craving mice, by IP injection 2 hours before sacrifice. IP injection was used to avoid direct and unintended effects on brain chemistry possibly associated with the ICV route; consequently the dose had to be significantly greater than that used with ICV injections. The dose range chosen was based on prior work by Ehrenpreis who showed an optimal analgesic response, measured by tail-flick, of 125 mg/kg [38]. HC was delivered in 2 equally divided injections over an 8 hr period at doses of 100, 150 and 250 mg/kg; control animals received saline injections similarly to control.

Figure 5 shows a significant (p<0.02) enhancement of whole brain [Met]-enk levels in C57BL/6J mice treated with HC relative to controls. Although there was no dose-dependent relationship found between 100, 150 and 250 mg/kg all doses resulted in significant increase in [Met]-enk over controls and were 178±8.0 (n=7), 180±8.0 (n=5), and 188±20.0 (n=6) picomoles per gram, respectively, compared to controls at 140±12.0 (n=7).

FIG. 5. Whole brain methionine-enkephalin levels in picomoles per gram following HC (hydrocinnamic acid) at 250 mg/kg intraperitoneally, compared to saline controls. n = number of mice tested. *Indicates significance at p<0.02 as determined by Student’s t-test.

In terms of ethanol and opiate seeking behavior the "genotype" theory proposed [11] is that individuals prone to such behavior possess a genetic deficiency of the endorphinergic system and both environmental conditions and long-term exposure to ethanol and opiates result in marked reduction of endogenous peptidyl opiates. Animal and human evidence which support opioid peptides a gene defect in the posttranslational processing of endorphins has been forthcoming and includes: (1) ethanol preferring C57BL/6J mice exhibit less brain enkephalin than non-ethanol preferring DBA2J mice, suggestive of an inverse relationship whereby lowered opioids equates to higher ethanol desire [18]; (2) stress reduces brain endorphins in rodents [62]; (3) long-term exposure consumption of ethanol by hamsters and other rodents results in marked reduction of brain enkephalin and endorphin [16]; at the mRNA level there is a decrease in posttranslation of the pre-enkephalin during chronic ethanol consumption in rodents [76]; and (5) a central deficiency of B-endorphin in human alcoholics [43].

This is the first report of reduced ethanol consumption in genetically bred mice utilizing DPA, a very safe and effective enkephalinase inhibitor [33] and HC, another known enkephalinase inhibitor [36].

The first experiment was designed to evaluate the efficacy of DPA to reduce ethanol intake in inbred mice. Results of this experiment indicate that ethanol consumption in alcohol-prefering mice (C57BL/6J) can be markedly reduced by inhibiting the endogenous metabolism of brain endorphins by utilization of "neuropeptidase" inhibitors. The report illustrates that DPA, by virtue of enkephalinase inhibition [4], significantly reduces ethanol acceptance in C57BL/6J mice so as to be comparable to that of DBA2J alcohol-aversive animals.

HC was utilized to evaluate the possibility that enkephalinase inhibition would result in a suppression of volitional ethanol consumption. Analysis of these data allows the interpretation that the magnitude of the effect was a function of the severity of the presumed enkephalin deficit as measured by ethanol preference. That is, specific drug dose produced a greater effect in low preference mice as compared to high preference animals. All animals received a single dose of HC; weight was determined not to be a covariate. This observation explains the significant negative regression coefficient for weight, and suggests a dose-dependent effect of HC to act as a pharmacologic modulator of ethanol intake.

Furthermore, the data reveal the HC produced a significant (p<0.015) reduction of volitional ethanol consumption 24 hr after ICV injection. This finding was all the more significant as it was evident despite the trauma induced by the injection.

Of great interest is the rebound effect observed on post-injection day 2 in both groups (drug and control) toward pre-injection levels (Fig. 4). The rebound effect is currently unexplainable, but suggests a complex behavioral compensation. The finding that a significant difference was observed in the control group only at day 1 and 3 suggests that HC tended to dampen the rebound or overshoot effect possibly due to the stress of the injection. This stress may have caused reduced brain opioid peptide levels which induced the possible increased ethanol preference. Furthermore, there was a day to day trend in the drug group to have lower preference ratio scores from day 3 to day 8 when it reached...
significance ($p < 0.03$) compared to pre-drug level. On day 9, once again, a significant ($p < 0.02$) rebound or overshoot effect occurred for only the drug groups. The 8 day time period is not surprising in that other enzyme inhibitors (e.g., p-chlorophenylalanine) show similar behavioral time effects probably due to regeneration of new enzyme molecules [42].

The data show a reduction in alcohol preference in HC injected mice as compared to controls receiving CSF only. At present we can say with certainty that alcohol preference was reduced while water intake was unaltered. Further studies must enlarge the issue and address questions of tem as a potential critical determinant for both volitional and forced [70] consumption of ethanol. We can then examine the specificity of the enzyme inhibitor actions.

With regard to the third experiment, at all doses there was significant increase over control ($p < 0.02$) though there were no dose related differences (Fig. 5). These results are not surprising in view of the finding of Ehrenpreis et al. [38] who showed that with HC greater analgesia was seen at 125 mg/kg than at 200 mg/kg. This suggests that these concentrations were on the plateau portion of the curve and that dose response data should be obtained. Other reports reveal the enkephalinase inhibitory properties of HC as well as its ability to raise levels of opioid peptides [37].

Although additional intensive systematic research is required to fully characterize this novel finding, it further suggests the possible involvement of the endorphinergic system as a potential critical determinant for both volitional and forced [70] consumption of ethanol.

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REFERENCES


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