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Ethanol acceptance as a function of genotype amounts of brain [Met]enkephalin

(endorphins/alcohol/radioimmunoassay)

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ABSTRACT **Our results indicate a negative correlation between the amount of ethanol (10%) consumed and endogenous levels of brain [Met]enkephalin in C57BL/6J (alcohol-preferring) and DBA/2J (alcohol-nonpreferring) inbred mice strains. Additionally, it was found that 8 wk after 1-day starved groups of both C57BL/6J and DBA/2J mice were challenged with ethanol (10%) for 1-day acceptance, they had significantly lower levels of brain [Met]enkephalin compared with their nonalcohol-treated controls. These results suggest that the brain endogenous peptidyl opiates may play a crucial role in alcohol-seeking behavior.**

Increasing evidence from both animals and man supports the interrelatedness between ethanol or condensation amine metabolites and opiates (1–5). Furthermore, there is support for the involvement of endogenous peptidyl opiates in the actions of ethanol (6–9). In this regard, experimental studies focused on the ability of the acute effects of opiate agonists to reduce the volitional consumption of ethanol in rats (10, 11) and in hamsters (12). In addition, ethanol consumption is increased after opiate withdrawal (13). With the characterization of multiple opioid receptors (14) and stereospecific interactions of ethanol (15) and condensation byproducts (2) at δ receptors, it is reasonable to suspect that some endogenous opioids may alter ethanol consumption in a way similar to opiates (16). Furthermore, it has been proposed (17) that ethanol-seeking behavior is genetically linked to a deficiency of the endogenous peptidyl opiates.

From previous studies (16) and the present one, we have determined that baseline amounts of [Met]enkephalin and alcohol preference vary significantly in both C57BL/6J and DBA/2J mice; so, in this study it was necessary to negate any inherent species variability by correlating individual ethanol acceptance with corresponding individual [Met]enkephalin levels. The experiments reported here demonstrate a negative correlation between the individual amount of ethanol consumed and the individual level of brain [Met]enkephalin in C57BL/6J (alcohol-preferring) and DBA/2J (alcohol-nonpreferring) inbred mice strains.

MATERIAL AND METHODS

Male C57BL/6J and DBA/2J mice (8 wk old) were obtained from The Jackson Laboratories, maintained at constant temperature (25 ± 2°C) and lighting (12 hr light/12 hr dark) conditions, and subsequently (14 wk old) tested for alcohol acceptance. Baseline tap water drinking was established in both strains from day-to-day monitoring for 1 wk. Then they were separated into a control water-drinking group and an ethanol-acceptance group. Control animals in both C57BL/6J and DBA/2J were matched with the experimental groups for the amount of water drinking. On the morning of day 8, fluid and food were removed from all groups for 24 hr. Then a 10% ethanol/tap water solution and tap water were administered to the treated group and control groups, respectively, for 24 hr. At 24 hr, their fluid volumes were recorded, and the animals were returned to food and water ad lib for 8 wk to minimize the effects of alcohol consumption. The animals then were decapitated, and their brains were removed (excluding the pituitary) and frozen on a dry block of dry ice. The samples were weighed and homogenized at 95°C in a solution of 2 M acetic acid and then were heated for 5 min at 95°C. The samples then were chilled to 4°C for 5 min and centrifuged at 14,000 × g for 15 min. The supernatant fractions were removed, shell-frozen in borosilicate tubes, and lyphilized overnight. The next day the residue was resuspended in 0.1 M phosphate buffer, pH 6.8/0.1% bovine serum albumin and centrifuged at 1,000 × g for 15 min. The supernatant fractions were then assayed for [Met]enkephalin (Immuno Nuclear kit, Stillwater MN). Duplicate samples were run, and a log/logit $y/1 - y$ graph was used to determine binding. In order to insure dependability of the Immuno Nuclear enkephalin kit and our procedure, a series of validation tests were accomplished. To corroborate the immunological specificity, Boehringer Mannheim's [Met]enkephalin was compared with the enkephalin supplied with the kit. The effect of freezing versus nonfreezing was determined because of the quantity of the samples to be processed; it had been necessary to freeze individual samples. Reliability of results was tested by comparing different assays of the same sample and comparing the loss of activity of a sample over a period of 30 days. Recovery was determined from both mouse brain and nonneural mouse liver. Finally, to test for nonspecific binding, dilutions of 1:250 to 1:2,000 of different samples were compared.

Fig. 1 compares the standard curve of [Met]enkephalin from Immuno Nuclear with the [Met]enkephalin from Boehringer Mannheim. The curves are closely parallel; from a linear regression analysis of the linear portion of the curve, it was found that the line of the Boehringer Mannheim enkephalin had a slope of −0.00894 and a y-axis intercept at 3.74, whereas [Met]-enkephalin from the kit had a slope of −0.00936 and a y-axis intercept of 3.87.

Five samples that had been frozen versus five that were left untreated were later assayed for [Met]enkephalin. The [Met]-enkephalin content ± SEM of the frozen brains was found to be 117.8 ± 10.2 ng/g versus 149.0 ± 9.3 ng/g for the unfrozen samples. Freezing had a decided although nonsignificant effect ($P < 0.1$).

Fig. 2 shows two dilutions of the same mouse brain sample assayed for [Met]enkephalin over a period of 30 days. Unexpectedly, the assayed amount at day 7 increased and returned

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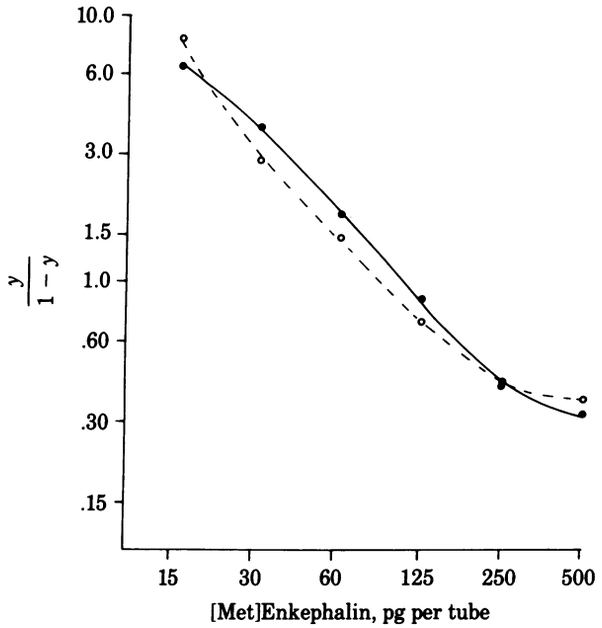

FIG. 1. Standard curve of [Met]enkephalin from Immuno Nuclear (○) and [Met]enkephalin from Boehringer Mannheim (●). [Met]Enkephalin from Immuno Nuclear had a slope of −0.00936 and a *y*-intercept of 3.87, and [Met]enkephalin from Boehringer Mannheim had a slope of −0.00894 and a *y*-intercept of 3.74.

to the value of day 1 on about day 20. A noticeable decrease in activity was seen for the 1:1,500 dilution after day 20. Subsequent material tested was assayed on the same day to avoid any differences due to aging.

Ten aliquots of another sample were tested for intraassay variability, and a coefficient of variation of 0.1008 was found for the 1:750 dilution, whereas a coefficient of variation of 0.1450 was found for a dilution of 1:1,500.

Aliquots (50 μl; 50 ng) of [Met]enkephalin were added to one-half of each of eight ICR Swiss mice brains and compared to their respective other halves on which 50 μl of saline had been added. A recovery of 64 ± 8% was found. However, subsequent analysis revealed in comparing both sides that large coefficients of variation were noted: side A had a coefficient of variation of 0.314 and side B had one of 0.275. Therefore, nonneuronal liver tissue was used. A recovery of 44.8 ± 2.5% was

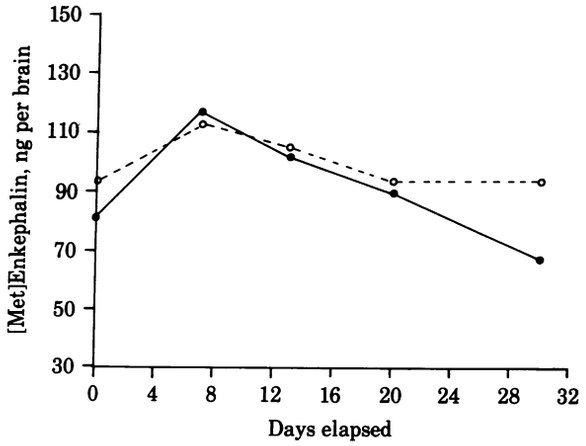

FIG. 2. Two dilutions (1:750 and 1:1,500) of the same mouse brain assayed for [Met]enkephalin over a period of 30 days: ○, 1:750 dilution; ●, 1:1,500 dilution.

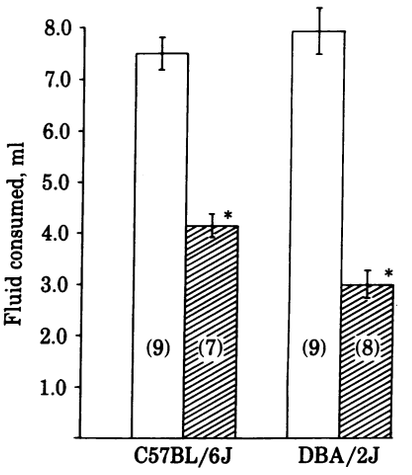

FIG. 3. Acceptance by C57BL/6J and DBA/2J of 10% (vol/vol) ethanol in tap water (▨) versus tap water-consuming controls (□). Vertical bars represent SEM. The number of animals is in parentheses. *, $P < 0.05$.

determined with a coefficient of variation of 0.112.

Four dilutions of three different samples were tested to determine the reliability of values at different areas of the standard curve. An analysis of variance revealed no significant difference between columns; $F(3, 8) = 1.23$; $P < 0.1$.

The neurotransmitter cross-reactivity of the Immuno Nuclear radioimmunoassay is as follows: [Met]enkephalin, 100%; [Leu]enkephalin, 2.8%; substance P, <0.002%; β-endorphin, <0.002%; α-endorphin (β-lipotropin$_{61-77}$), <0.10%; porcine dynorphin$_{1-13}$, <0.002%; and α-neoendorphin, <0.002%. An analysis of variance (1- or 2-way variance) and the Student *t* test were used to evaluate all statistical operations.

RESULTS

Fig. 3 shows C57BL/6J mice did not drink significantly more water than did DBA/2J mice (7.54 ± 0.29 ml/7.94 ± 0.46 ml), but C57BL/6J mice consumed significantly more 10% ethanol (4.12 ± 0.46 ml) than did the DBA/2J mice (2.98 ± 0.26 ml). A significant ($P < 0.05$) negative correlation was found ($R = -0.566$; $P < 0.05$) between ethanol acceptance by both mouse strains and their whole brain [Met]enkephalin levels (Fig. 4).

Surprisingly, when the ethanol-treated groups were com-

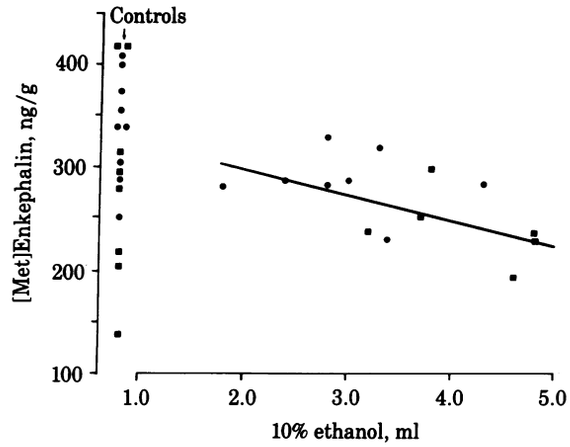

FIG. 4. Whole brain [Met]enkephalin levels versus corresponding 10% ethanol acceptance in DBA/2J (●) and C57BL/6J (■) mice. Each point represents either a DBA/2J or C57BL/6J mouse. For comparison, control values are shown. $R = -0.566$; $n = 14$; $P < 0.05$.

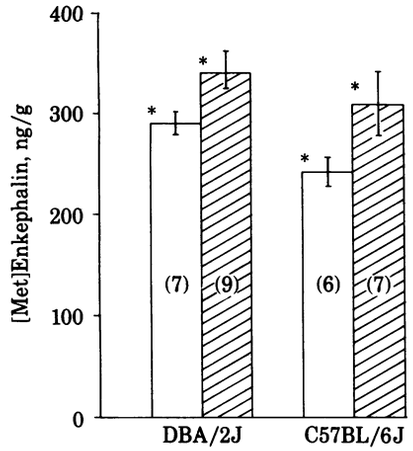

FIG. 5. Whole brain [Met]enkephalin levels 8 wk after one day of ethanol (10%) acceptance in C57BL/6J and DBA/2J mice (□) vs. levels in controls (▨). Bars represent SEM. The number of animals is in parentheses. *, $P < 0.05$.

pared with controls, the ethanol groups had significantly lower ($P < 0.0103$) amounts of [Met]enkephalin [2-way analysis of variance $F(1, 25) = 7.69$]. This effect also was found ($P < 0.05$) in each species (Fig. 5) when compared with their respective controls (DBA/2J, 290.01 ± 12.07/341.21 ± 17.38 ng/g; C57BL/6J, 242.82 ± 13.98/308.35 ± 32.89 ng/g). C57BL/6J when compared with DBA/2J mice showed lower levels of [Met]enkephalin, although the difference was not significant [$P < 0.071$; analysis of variance $F(1, 25) = 3.56$]. However, earlier work (17) in our laboratory did show a significant difference [DBA/2J, 339.17 ± 12 ($n = 19$); C57BL/6J, 306.61 ± 10.37 ($n = 20$]; $P < 0.05$).

DISCUSSION

Results of these experiments indicate that C57BL/6J (alcohol-preferring) mice had lower levels of whole brain [Met]enkephalin compared to DBA/2J (alcohol-nonpreferring) mice. This report illustrates a direct correlation between actual amounts of ethanol consumed and whole-brain [Met]enkephalin-like immunoreactive substances. Specifically, the finding of a negative correlation between ethanol consumption and brain peptidyl opiates supports in part the concept of a deficiency of endogenous opiate peptides genetically linked with alcohol preference (17, 18).

Of great surprise was the finding that after a 24-hr period of starvation, one day of acceptance of ethanol (10%), and an 8-wk lapse of time prior to analysis, significant reductions of brain [Met]enkephalin were obtained in both experimental C57BL/6J and DBA/2J mice compared to matched controls. The permanent nature of this finding may become significant in view of the fact that cerebrospinal fluid-injected condensation amine by-products of alcohol induce long-term alcohol consumption in rodents (19). There is animal evidence with regard to peptidyl opiate involvement in mediating alcohol-seeking behavior (16); and furthermore, it has been reported that human addicts have one-third the level of β-endorphin and significantly more adrenocorticotropic hormone in their cerebrospinal fluid than do normal volunteers (20). The results of this investigation indicate that the enkephalins may act as critical determinants for volitional consumption of ethanol (21). Furthermore, because we found significant subline variability in two divergent alcohol-preferring strains of mice, it is tempting to speculate that in man, alcohol preference may have to be determined from a comprehensive individualized approach rather than simple genetic phenotyping.

We pay tribute to Frederic Bartter (who originally sponsored this manuscript before his death), whose numerous accomplishments exemplify excellence. We thank Dr. Sheldon Miller of Advanced Health Systems for his enthusiastic support of this research and Dr. M. K. Ticku for critical comments regarding this work. S.F.A.E. is currently a doctoral thesis candidate, and this research is a partial requirement for his degree. This work was supported in part by a grant to K.B. from the Raleigh Hill Foundation.

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