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# Genotypic differences in mesolimbic enkephalin gene expression in DBA/2J and C57BL/6J inbred mice

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**Abstract**

The DBA/2J and C57BL/6J (herein referred to as DBA and C57) inbred mouse strains exhibit low and high predispositions for voluntary ethanol consumption, respectively, but the neurobiological basis underlying this differential drug vulnerability remains poorly understood. Comparison of endogenous brain proenkephalin gene expression showed the C57 mouse, compared to the DBA mouse, had lower preproenkephalin mRNA abundance, proenkephalin concentration and processed [Met$^5$]enkephalin-immunoreactive peptide levels in the mid brain. No strain differences in enkephalin gene expression was observed in the striatum, hypothalamus, or medulla pons. Neurochemical analysis of C57 mice, following high voluntary ethanol consumption (~ 17 g/kg/day), revealed markedly higher enkephalin gene expression in the striatum and mid brain compared to ethanol-naive animals. These findings suggested that mesolimbic enkephalin is augmented following ethanol consumption, and that endogenous low enkephalin biosynthesis may be associated with an increased vulnerability for ethanol abuse. However, the neurobiological basis of this behaviour may not be quite this simple. C57 mice pretreated with the dopamine receptor agonist, bromocriptine, had reduced striatum and mid brain preproenkephalin mRNA levels, and showed a 41% lower voluntary ethanol consumption compared to controls. We conclude that functional connectivity exists between enkephalin and dopamine systems, and although low mesolimbic enkephalin may predispose to high ethanol preference, dopamine is a more important determinant than enkephalin in the hierarchy of neurotransmitter pathways that mediate the increased vulnerability for ethanol consumption in the C57 mouse.

*Keywords:* Enkephalin; Dopamine; (Inbred mouse); Ethanol drinking

## 1. Introduction

Enkephalin opioid peptides and enkephalin analogues have been reported to have analgesic activity (Heymann et al., 1988; Hansen et al., 1992), and reinforcing properties (Belluzzi and Stein, 1973; Singh et al., 1994; Devine and Wise, 1994). In fact, injections of such compounds into the intracerebroventricles of animals have shown them capable of establishing conditioned place preference (Shippenberg et al., 1987; Bals-Kubik et al., 1990). It has therefore been proposed that enkephalins contribute to the neurobiology of drug reward and maintenance of drug addiction (reviewed by Di Chiara and North, 1992). Further definition of the role of enkephalin on a molecular level seems important.

It is well known that enkephalin functions physiologically as a neuromodulator that presynaptically regulates mesolimbic and mesocortical dopamine neurotransmission originating from the ventral tegmental area of the mid brain (Kalivas et al., 1983; Latimer et al., 1987; Kalivas and Richardson-Carlson, 1986). This regulation is reciprocal, and enkephalin gene expression, ranging from preproenkephalin mRNA transcription through processing of the precursor to [Met$^5$]enkephalin peptide generation have been shown to be under tonic inhibitory control mediated by dopamine (Hong et al., 1978; Mocchetti et al., 1985; George and Kertesz, 1987). Thus, the challenge now is to clarify the importance of enkephalin as a principal

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mediator of drug abuse vulnerability. For this purpose, we chose the ethanol-avoiding DBA/2J (DBA) and ethanol-preferring C57BL/6J (C57) inbred mouse strains which have been used by some investigators for identifying candidate neurobiological mechanisms responsible for drug abuse (reviewed by George and Goldberg, 1989; Crabbe et al., 1994). A clue comes from neurochemical studies reporting low brain [Met$^5$]enkephalin peptide levels in the C57 mouse compared to the DBA mouse (Blum and Briggs, 1988; George et al., 1991). The simplest interpretation is that high ethanol consumption is a function of a genetically determined low central [Met$^5$]enkephalin peptide level. However, we recently reported an association between high ethanol consumption and preference and low mesolimbic dopaminergic function in these animals (Ng et al., 1994; Ng and George, 1994; George et al., 1995) raising the need to clarify the importance of central enkephalin as a mediator of drug abuse vulnerability. In particular, it was of interest to determine whether the enkephalin system was functionally integrated with the activity of dopamine in the murine model. The neurochemical and pharmacological evidence now show that the vulnerability to and acquisition of high ethanol consumption by C57 animals could be a function of genetically determined low mesolimbic enkephalin content, but that low mesolimbic dopamine level is the predominant hierarchical neurotransmitter pathway regulating this behaviour.

## 2. Materials and methods

### 2.1. Materials

[$^{125}$I][Met$^5$]enkephalin was from DuPont-New England Nuclear (Boston, MA, USA), [Met$^5$]enkephalin rabbit antibody from Immunonuclear (Stilwater, MN, USA), and [Met$^5$]enkephalin standards from Peninsula Laboratories (Belmont, CA, USA). The plasmid (PYSEA1) containing the rat preproenkephalin cDNA was a generous gift from Dr. S. Sabol (NIH, Bethesda, MD, USA). Restriction enzymes were from Pharmacia (Toronto, Ontario, Canada), and all pharmaceuticals were purchased from Research Biochemical (Natick, MA, USA). All other chemicals were from commercial sources. The DBA/2J and C57BL/6J inbred mouse strains were obtained from Jackson Laboratories (Bar Harbor, ME, USA).

### 2.2. Animal care

7-Week-old male DBA/2J and C57BL/6J mice were acclimatized prior to studies. Animals were housed individually for 1 week in an environmental room under 12-h light and 12-h dark cycle, with food and water ad libitum, and weighed daily to minimize handling stress during the test period.

### 2.3. Tissue dissection

Sacrifice alternated among mice groups to minimize diurnal effects. Striatum (caudate putamen and nucleus accumbens), mid brain (no thalamus), and other brain regions were dissected as defined by Glowinski and Iversen (1966) and quickly frozen on dry ice and stored at −70°C.

### 2.4. Radioimmunoassay determination of [Met$^5$]enkephalin content

Basal [Met$^5$]enkephalin was extracted from tissues by boiling in 1 N acetic acid containing 1 N HCl solution for 10 min. Tissues were subjected to polytron homogenization, centrifuged at 30 000 × $g$ for 20 min, and supernatant collected, lyophilized, and reconstituted for [Met$^5$]enkephalin assay. [Met$^5$]enkephalin content in samples was determined in triplicate by radioimmunoassay using rabbit [Met$^5$]enkephalin antiserum, [$^{125}$I][Met$^5$]enkephalin and [Met$^5$]enkephalin standards as previously described (George and Kertesz, 1985). Precursor cryptic [Met$^5$]enkephalin in the longer enkephalin-containing peptides was released by sequential enzymatic digestion with trypsin for 20 min at 37°C, followed by incubation with carboxypeptidase B for 10 min at 37°C. Cryptic [Met$^5$]enkephalin content was the difference in [Met$^5$]enkephalin content measured by radioimmunoassay in aliquots obtained before and after enzyme treatment. An internal assay standard, hydrolysis of [Met$^5$]enkephalin-Arg-Gly-Leu, was performed in each assay. Reactions were terminated by the addition of glacial acetic acid and boiling. Protein content was determined by the method of Bradford (BioRad) using bovine serum albumin as the standard.

### 2.5. Northern blot analysis

Dissected tissues were homogenized in 4 M guanidium thiocyanate buffer, phenol/chloroform extracted, and total RNA obtained by ethanol precipitation according to the method of Chomczynski and Sacchi (1986). Extracted total RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and diluted in loading buffer consisting of 6% formaldehyde, 50% formamide, 1 × 3-[$N$-morpholino]propanesulfonic buffer (MOPS), 10% glycerol, 0.01% bromophenol blue, and 5 $\mu$g ethidium bromide. Samples were heat denatured at 65°C for 10 min and size-fractionated on a 1% agarose-formaldehyde gel. Gels were denatured, neutralized and RNA transferred by positive pressure blotting onto Nytran membranes (Schleicher and Schuell) and UV cross-linked. Nytran membranes were prehybridized in hybridization buffer consisting of 50% formamide, 5 × Denhardt's solution, 200 $\mu$g/ml salmon sperm DNA, 5 × NaCl/$NaHPO_4$/EDTA (SSPE) buffer, 0.1% sodium dodecyl sulphate (SDS) and 10% dextran for 4 h at 42°C. The 1.5-kb preproenkephalin mRNA was

detected by hybridization with a $P^{32}$-labeled 970-kb *Sac*I-*Sma*I restriction endonuclease fragment of the rat enkephalin gene ($1-2 \times 10^6$ cpm/ml) for 12 h at 42°C. The 2-kb β-actin mRNA was detected with a $P^{32}$-labeled 1.8-kb β-actin *Bam*H1 restriction digest cDNA probe ($1-2 \times 10^6$ cpm/ml) to correct for slight differences in loading of samples. Blots were routinely washed at 45°C in 0.5 × SSC, 0.1% SDS for 2–3 h and exposed to Kodak XAR autoradiographic film with an intensifying screen at −70°C for 2–7 days. $P^{32}$-labeling of cDNAs was performed using random hexamers with the Klenow fragment of *E. coli* DNA polymerase I (Pharmacia), which were purified from unincorporated nucleotide using Nensorb 20 columns (NEN-Dupont). Blots were routinely stripped in 50% formamide/6 × SSPE at 65°C for 30 min, and rinsed in 2 × SSPE buffer before re-use. Autoradiograms were scanned using an Imaging Research MCID image analysis system (St. Catherines, Ontario, Canada) and the optical density units recorded.

### 2.6. Free-choice ethanol vs. water consumption test

Voluntary ethanol consumption was evaluated over a 7-day, 24-h free-choice ethanol solution vs. water in a two-tube paradigm. Mice were handled and weighed daily and conditioned to receive water from two graduated drinking tubes for 7 days to minimize place preference during the test. During the voluntary ethanol test, one tube was filled with distilled water and the other with a 10% v/v ethanol/water solution. The tubes were rotated daily and the volumes of water and ethanol drink consumed measured. The mice used for any given experiment had no prior experimental use and originated from the same shipment.

### 2.7. Drug treatments

The effects of dopamine $D_2$ receptor activation on enkephalin gene expression was assessed using bromocriptine mesylate. An amount of 5 mg/kg/day was chosen since a similar dose was shown to decrease enkephalin in rat pituitary (George and Kertesz, 1985). Bromocriptine was dissolved in 0.05 M tartaric acid containing ethanol (75:25 v/v).

The consequence of dopamine $D_2$ receptor-induced regulation of enkephalin gene expression on voluntary ethanol consumption in C57 mice was examined. Bromocriptine mesylate was administered as a daily dose of 5 mg/kg for 14 consecutive days (7 days before the start and 7 days during the ethanol preference test), since this dosing regiment has been previously shown in these mice to regulate dopamine receptor gene function (Ng and George, 1994). A 7-day pretreatment period was chosen based on the assumption of steady-state blood levels following 5 drug half-lives, and a maintenance dose was given for sustained drug effect during the test. All drug injections were prepared fresh, and administered 2 h before the onset of the dark cycle. As a control group, mice received injections of the vehicle. Each study was independently replicated twice.

## 3. Results

### 3.1. Endogenous enkephalin gene expression in brain regions of the DBA and C57 mice

The levels of basal [Met$^5$]enkephalin peptide and precursor cryptic [Met$^5$]enkephalin in various brain regions of the DBA and C57 mice were determined by radioimmunoassay and are shown in Table 1. When compared to the DBA mouse, basal and cryptic levels of [Met$^5$]enkephalin peptides in the C57 mouse were 16% ($P < 0.05$) and 32% ($P < 0.05$) lower, respectively, in the mid brain, reduced in hypothalamus, but not different in the striatum, or medulla pons. The rabbit antiserum for [Met$^5$]enkephalin had essentially no cross-reactivity to the extended enkephalin peptides, [Met$^5$]enkephalin-Arg$^6$, [Met$^5$]enkephalin-Arg$^6$-Phe$^7$, [Met$^5$]enkephalin-Arg$^6$-Gly$^7$-Leu$^8$, or the metabolites Tyr, Tyr-Gly, Tyr-Gly-Gly, and Gly-Gly-Phe-Met; with 2.8% cross-reactivity to Leu-enkephalin, but negligible (< 0.002%) to β-endorphin, α-neoendorphin, and dynorphin. Intra-assay variation of samples assayed in triplicate was 5–10% whereas interassay variation was under 5%. The limits of assay detection were 3–6 pg [Met$^5$]enkephalin/tube.

As shown by Northern blot analysis, a 1.5-kb message corresponding to the rat preproenkephalin mRNA was detected with the $P^{32}$-radiolabeled preproenkephalin cDNA

Table 1
Enkephalin gene expression in brain regions of ethanol-naive DBA and ethanol-naive C57 mice

| Brain regions | | Levels of enkephalin gene expression | | |
|---|---|---|---|---|
| | | ME (pmol/μg protein) | | PPE mRNA (O.D.) |
| | | Basal | Cryptic | |
| Striatum | DBA | 0.246 ± 0.017 | 0.099 ± 0.029 | 100.0 |
| | C57 | 0.234 ± 0.032 | 0.112 ± 0.019 | 99.2 ± 1.8 |
| Hypothalamus | DBA | 0.044 ± 0.004 | 0.049 ± 0.010 | 100.0 |
| | C57 | 0.059 ± 0.012 | 0.018 ± 0.008 | 91.6 ± 2.4 |
| Mid brain | DBA | 0.090 ± 0.005 * | 0.062 ± 0.009 * | 100.0 |
| | C57 | 0.076 ± 0.002 | 0.044 ± 0.005 | 76.8 ± 4.8 * |
| Medulla pons | DBA | 0.140 ± 0.012 | 0.095 ± 0.010 | 100.0 |
| | C57 | 0.120 ± 0.004 | 0.107 ± 0.005 | 129.9 ± 11.0 |

The table lists the mean ± S.E.M. values of 3 experiments for basal [Met$^5$]enkephalin peptide content, precursor cryptic [Met$^5$]enkephalin peptide content (pmol/μg protein) and preproenkephalin mRNA abundance (relative O.D. units) in brain regions for the DBA and C57 mice. [Met$^5$]enkephalin peptides were determined by radioimmunoassay and mRNA contents were assessed by Northern blot and densitometric scanning analysis as described in Materials and methods. mRNA values are expressed as % O.D. units of the C57 mouse normalized to β-actin expression in the same region. Statistical significance at $P < 0.05$ for DBA vs. C57 is indicated (*).

probe in mouse neuronal tissues (Fig. 1). Scanning densitometric analysis of autoradiograms determined 23% lower ($P < 0.05$) preproenkephalin mRNA abundance in the mid brain of the C57 compared to the DBA mouse (Table 1). Preproenkephalin mRNA abundance was 30% higher in the medulla pons of the C57 mouse but this was not statistically significant in this study. Consistent with this finding, neither basal or cryptic enkephalin peptide levels differed in the medulla pons. Differences were not observed in other brain regions examined, indicating a region-specific, mid brain deficit in enkephalin gene expression in the C57 mouse compared to the DBA mouse (Table 1).

### 3.2. Effects of ethanol on enkephalin gene expression in the C57 mouse

Ethanol consumption led to a 41% increase ($P < 0.05$) in basal [Met$^5$]enkephalin peptide content in the striatum, and an accompanying 32% increase ($P < 0.05$) in the mid brain but not in any other brain regions of the C57 mouse compared to ethanol-naive animals (Fig. 2). The basal [Met$^5$]enkephalin peptide levels in the ethanol-drinking mouse compared to the ethanol-naive mouse in pmol/$\mu$g protein were as follows: in the hypothalamus $0.059 \pm 0.012$ vs. $0.051 \pm 0.004$ (not significant), and in the medulla pons $0.120 \pm 0.004$ vs. $0.130 \pm 0.014$ (not significant). Cryptic enkephalin levels also did not vary, and were as follows in the ethanol-drinking mouse compared to the ethanol-naive mouse in pmol/$\mu$g protein: in the striatum $0.112 \pm 0.019$ vs. $0.129 \pm 0.028$ (not significant), in mid brain $0.044 \pm 0.015$ vs. $0.039 \pm 0.002$ (not significant), in hypothalamus $0.018 \pm 0.008$ vs. $0.029 \pm 0.008$ (not significant), and in medulla pons $0.107 \pm 0.005$ vs. $0.094 \pm 0.012$ (not significant). At the level of transcription, ethanol consumption led to a 12% higher preproenkephalin mRNA abundance in the mid brain (Fig. 2), but not in other regions (data not shown), suggesting that ethanol actions were selective in enhancing enkephalin gene expression and processing to [Met$^5$]enkephalin in the mesolimbic regions of the mouse brain. Additionally, these data might be interpreted to show that high ethanol consumption may be a function of low brain enkephalin in light of previous findings in which augmenting endogenous enkephalin levels attenuated ethanol consumption and preference (Blum and Briggs, 1988; George et al., 1991). We have however recently reported an association between low mesolimbic dopamine and high ethanol consumption (Ng et al., 1994), which prompted us to clarify the nature of the interaction among mesolimbic enkephalin, dopamine and high ethanol consumption.

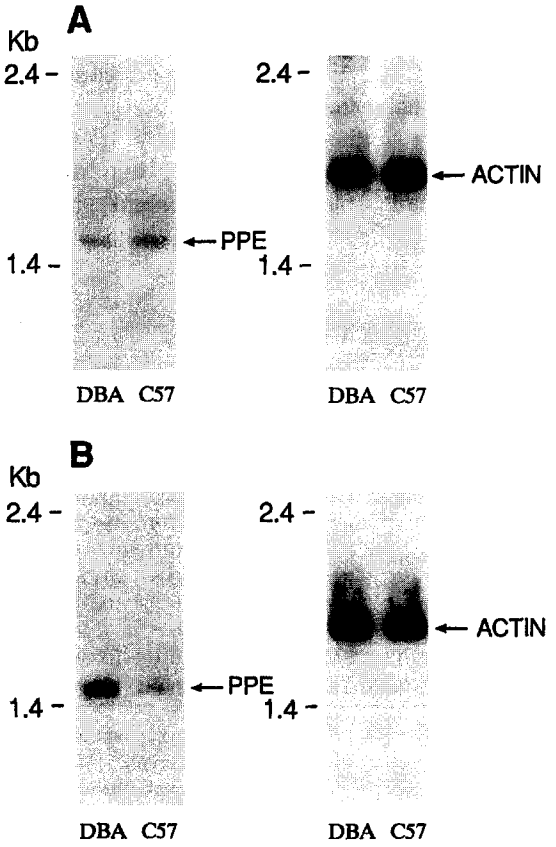


Fig. 1. Preproenkephalin mRNA in brain regions of ethanol-naive DBA and ethanol-naive C57 mice. Representative autoradiograms of Northern blots performed on (A) hypothalamus and (B) mid brain regions from DBA and C57 mice. Hybridized preproenkephalin (PPE) mRNA and β-actin mRNA are shown. Autoradiograms are from 3-day exposures.

### 3.3. Effects of bromocriptine treatment on murine brain preproenkephalin mRNA abundance

An enkephalin-dopamine interaction has not been critically demonstrated in the murine brain. No strain differences were observed in the sensitivity of the enkephalin system to the dopamine $D_2$ receptor agonist bromocriptine at the dosing schedule tested (5 mg/kg/day for 7 days). Bromocriptine treatment decreased preproenkephalin mRNA abundance by 30–35% in the striatum and by 10–11% in the mid brain as determined by densitometric scanning analysis of Northern blots (Table 2). It has been proposed that striatal [Met$^5$]enkephalin neurons are under tonic inhibitory control by nigrostriatal dopaminergic neurons (Sabol et al., 1983), and that blockade of dopamine $D_2$ receptors by haloperidol produces a disinhibition leading to increased [Met$^5$]enkephalin synthesis (Mocchetti et al., 1985, 1987; George and Kertesz, 1987; Abood et al., 1990; Jaber et al., 1994). It would appear that mid brain [Met$^5$]enkephalin neurons are also subjected to dopaminergic regulation, albeit to a lesser degree. We have shown previously that bromocriptine (5 mg/kg) affects both mesolimbic/nigrostriatal dopamine $D_1$ and $D_2$ receptor expression in these mice (Ng et al., 1994), indicating that striatum and mid brain enkephalin biosynthesis in bromocriptine-treated animals may owe to the net effect of dopamine $D_1$ receptor-mediated enkephalin gene activation

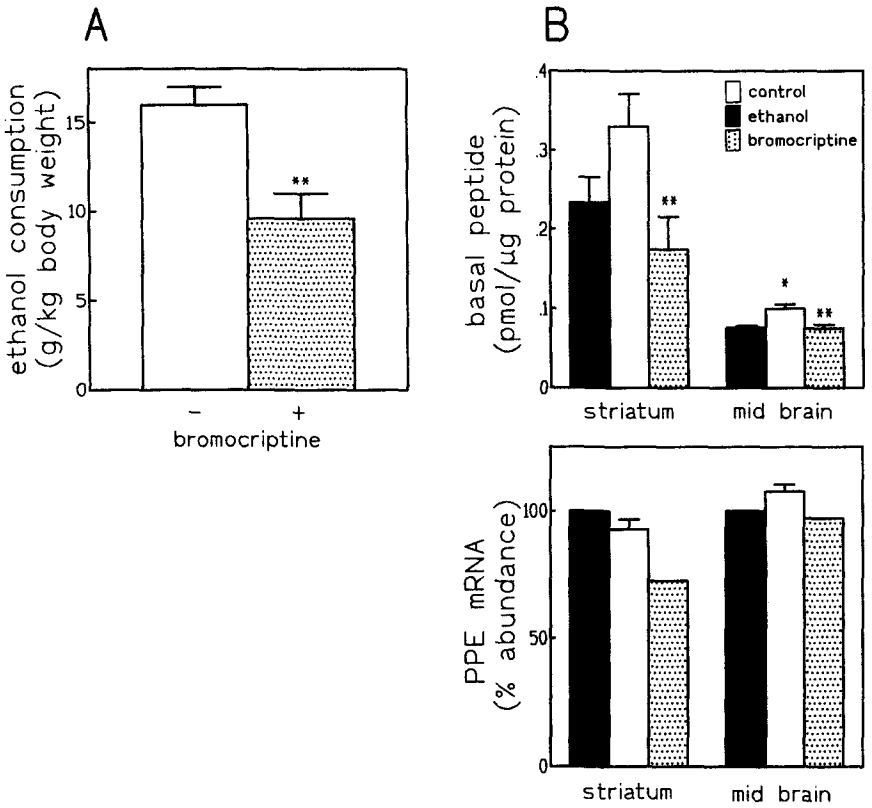


Fig. 2. Behavioral and neurochemical consequences of bromocriptine pretreatment in C57 mice. (A) The mean voluntary ethanol consumption of vehicle-pretreated and bromocriptine-pretreated C57 mice are shown. The results are from 6 mice representative of 2 independent studies. Statistical significance (*) at $P < 0.05$ (vehicle vs. bromocriptine) was determined using Student's $t$-test. (B) The top bar graph shows the basal [Met$^5$]enkephalin peptide content in brain regions of the control, untreated mouse (open bar), vehicle-pretreated, ethanol-drinking mouse (black bar), and bromocriptine-pretreated mouse exhibiting reduced ethanol intake (stippled bar). Basal [Met$^5$]enkephalin peptide content (pmol/μg protein) is the mean ± S.E.M. of 3 separate experiments using tissues from 6 animals. Significant differences ($P < 0.05$) between the control, untreated animals compared to vehicle-pretreated mice (*), and vehicle-pretreated mice compared to bromocriptine-pretreated mice (**) are shown and were determined using Student's $t$-test. Bottom bar graph shows preproenkephalin (PPE) mRNA abundance as % of untreated mouse. The % preproenkephalin (PPE) mRNA abundance for the vehicle-pretreated group was from 2 blots and data for the bromocriptine-pretreated mice was from a single blot of tissues from 3–6 animals.

and $D_2$ receptor-mediated enkephalin gene inhibition. These data illustrated for the first time that enhancement of synaptic dopamine activity inhibited enkephalin biosynthesis in the mesostriatal regions but not in other regions of these mouse strains.

Table 2
Effects of dopaminergic regulation on preproenkephalin mRNA abundance in DBA and C57 mice

| | Bromocriptine-induced regulation of PPE mRNA abundance (% decrease) | |
|---|---|---|
| | Brain region | |
| | Striatum | Mid brain |
| DBA | 30.7 ± 10.5 | 10.8 ± 2.5 |
| C57 | 35.0 ± 7.0 | 9.7 ± 3.2 |

The table shows preproenkephalin mRNA abundance in the striatum and mid brain of bromocriptine-treated DBA and C57 mice compared to vehicle-treated control animals. Receptor mRNA was revealed by Northern blot analysis and levels determined by densitometric scanning and corrected for β-actin expression. Values were normalized to 100% of the vehicle-treated animals and are the mean ± S.D. from 2 blots ($n = 4$ for each condition). Statistical analysis was not determined for $n = 2$ blots.

### 3.4. Effects of bromocriptine pretreatment on voluntary ethanol consumption in the C57 mouse

The contribution of the dopaminergic component of the enkephalin-dopamine system to ethanol abuse was examined. C57 mice were pretreated with bromocriptine prior to ethanol preference testing as described in methods. Bromocriptine-pretreated mice consumed a mean value of 10.0 ± 1.5 g ethanol/kg/day ($n = 12$) in comparison to vehicle-pretreated C57 mice who consumed 17.0 ± 1.0 g ethanol/kg/day ($n = 12$) (Fig. 2). This represented a significant 41% reduction ($P < 0.05$) in the absolute amount of ethanol consumed. In control animals given the drug or vehicle alone without ethanol preference testing, total mean daily water intake was 8.5 ± 1.0 ml ($n = 6$) in bromocriptine-treated mice and 7.6 ± 1.3 ml ($n = 6$) in vehicle-treated mice. Water intake was not different between these groups indicating that lower ethanol drinking

exhibited by bromocriptine-pretreated mice was attributed to the actions of bromocriptine.

### 3.5. Enkephalin gene expression in bromocriptine-pretreated C57 mouse exhibiting reduced voluntary ethanol consumption

We examined enkephalin biosynthesis in bromocriptine-pretreated C57 mice that exhibited marked reductions in voluntary ethanol drinking. Bromocriptine-pretreated mice had 28% lower preproenkephalin mRNA abundance in keeping with a 47% lower ($P < 0.05$) basal peptide content in the striatum compared to vehicle-pretreated, ethanol-drinking animals (Fig. 2). In the mid brain, bromocriptine-pretreated mice had similar preproenkephalin mRNA abundance but 25% ($P < 0.05$) lower basal [Met$^5$]enkephalin peptide content (Fig. 2). No other changes were evident in other brain regions examined, and are as follows for the bromocriptine-pretreated mouse compared to vehicle-pretreated mouse (pmol/$\mu$g protein): $0.056 \pm 0.010$ vs. $0.051 \pm 0.004$ in the hypothalamus (not significant), and $0.115 \pm 0.005$ vs. $0.130 \pm 0.014$ in the medulla pons (not significant). Cryptic [Met$^5$]enkephalin content also did not vary in the bromocriptine-pretreated mouse compared to vehicle-pretreated mouse and are as follows in pmol/$\mu$g protein: $0.096 \pm 0.009$ vs. $0.129 \pm 0.028$ in the striatum (not significant), $0.044 \pm 0.015$ vs. $0.039 \pm 0.002$ in the mid brain (not significant), $0.027 \pm 0.004$ vs. $0.029 \pm 0.008$ in the hypothalamus (not significant), $0.097 \pm 0.0012$ vs. $0.094 \pm 0.012$ in the medulla pons (not significant). Similarly, we did not find any differences in preproenkephalin mRNA abundance in striatum, mid brain, hypothalamus and medulla pons between these two groups (data not shown). In summary, bromocriptine-pretreated mice (exhibiting reduced ethanol consumption) had lower enkephalin gene expression compared to vehicle-pretreated mice (consuming 41% more ethanol) as indicated by lower preproenkephalin mRNA abundance and basal [Met$^5$]enkephalin contents in afferent and presynaptic regions of the mesolimbic/nigrostriatal dopamine pathway.

## 4. Discussion

It is well known but poorly understood why the DBA mouse is drug avoiding whereas the C57 mouse exhibits a high vulnerability for abused substances such as morphine, cocaine and ethanol (Belknap et al., 1993; Horowitz et al., 1977; Alexander et al., 1993; George and Goldberg, 1989; McClearn and Rogers, 1959). The present neurochemical and pharmacological study shows a genetically determined mesolimbic enkephalin deficit in the C57 mouse that together with the low central dopamine we have documented previously (Ng et al., 1994; Ng and George, 1994; George et al., 1995) forms important neurobiological determinants influencing the predisposition for high ethanol consumption in this model.

Murine brain proenkephalin gene expression appears to be under genetic and tissue-specific control. Basal [Met$^5$]enkephalin (representing processed peptide), cryptic [Met$^5$]enkephalin (representing unprocessed [Met$^5$]enkephalin in the form of proenkephalin and its smaller peptides), and preproenkephalin mRNA abundance were lower in the mid brain, but not in other regions of the C57 compared to the DBA mouse. Lower [Met$^5$]enkephalin peptide content was reported in the hypothalamus of the C57 mouse compared to the DBA mouse (Blum and Briggs, 1988), which we found reduced but not significant. Steady-state proenkephalin gene expression measurements however do not provide information about [Met$^5$]enkephalin release and degradation, and care must be taken in the interpretation of these results for the development of an integrated mechanistic hypothesis concerning ethanol drinking in these animals. In the rat, enkephalin in the ventral tegmental area of the mid brain has been reported to induce potentiation of mesolimbic dopamine activity (Broekkamp et al., 1979; Kelley et al., 1980; Kalivas et al., 1983). By analogy, innate low enkephalin activity in the mid brain of the C57 mouse may contribute to the reported endogenous mesolimbic/nigrostriatal dopaminergic deficit in these animals (Ng et al., 1994; Ng and George, 1994; George et al., 1995). The fact that enkephalin activity is not increased to compensate for the reduced mesolimbic dopaminergic function, as one might expect from decreased negative feedback by dopamine suggests possibly that enkephalin gene expression and/or enkephalin release is repressed or not fully activated under steady-state conditions in the C57 mouse. In fact, increased endogenous enkephalin degrading activity in striatal tissue of C57 mice and the attenuation of ethanol consumption following enhancement of enkephalin activity have been reported (George et al., 1991) in support of our hypothesis. These data suggest that steady-state proenkephalin gene expression is complex and subject to multiple levels of regulation. Dampened responsiveness of the enkephalin system resulting from low enkephalin activity may manifest in the reduced ability of naltrexone, a non-selective opioid receptor antagonist, to diminish locomotor activity in C57 compared to DBA mice (Castellano and Puglisi-Allegra, 1982). Taken together, these findings support the notion that high ethanol consumption may be related to low mesolimbic enkephalin activity in the C57 mouse. Consistent with this concept, we show that striatal and mid brain basal [Met$^5$]enkephalin peptide levels but not cryptic [Met$^5$]enkephalin peptide or preproenkephalin mRNA abundance are selectively increased following ethanol intake indicating that the enkephalin peptide is a substrate of ethanol action. Since basal [Met$^5$]enkephalin levels represent the balance of peptide synthesis, precursor processing, release and degradation, the ethanol-induced increase in peptide levels may be secondary to alterations in enkephalin re-

lease and/or post-translational processing and/or inhibition of enkephalin degrading enzymes. In keeping with the hypothesis that high ethanol consumption may be related to low mesolimbic enkephalin activity, these results also suggest that high ethanol consumption could be attenuated by increasing synaptic enkephalin activity in the C57 mice. In fact, it has been shown that augmenting central enkephalin concentrations (by preventing enkephalin breakdown) significantly decreases voluntary ethanol drinking in the C57 mouse (Blum and Briggs, 1988; George et al., 1991). This has also been demonstrated in other animal models. Preconditioning administration of Leu-enkephalin to ethanol-trained rats impairs the acquisition of ethanol preference and is antagonized by naloxone (Sandi et al., 1990). Many other studies have shown that opiate agonists reduce volitional consumption of ethanol in rats (Sinclair et al., 1973; Ho and Rossi, 1982). In addition, ethanol and morphine have also been shown to increase dopamine metabolism in nigrostriatal and limbic forebrain of the C57 mouse (Reggiani et al., 1980; George et al., 1995). Further, dopamine receptor agonists have been reported to reduce the propensity for ethanol drinking in these inbred mouse strains (Ng et al., 1994; George et al., 1995), in ethanol-trained rats and outbred rats selected for ethanol preference (Weiss et al., 1990; Pfeffer and Samson, 1988; Dyr et al., 1993; Rassnick et al., 1993). The most likely interpretation of these collective data is that deficits in mesolimbic enkephalin or dopamine activity are associated with increased vulnerability for ethanol abuse. Our findings show that the dopamine-enkephalin link is indeed intact in the C57 mice just as in DBA mice, since preproenkephalin mRNA levels were decreased by the dopamine receptor agonist bromocriptine. The data convinces us to believe that the mesolimbic dopamine component is likely a more important determinant than enkephalin in mediating ethanol abuse vulnerability. We show that bromocriptine-treated mice exhibit significantly reduced voluntary ethanol consumption even thought enkephalin gene expression is low in mesolimbic regions. Further, microinjection of sulpiride into the nucleus accumbens has been shown to increase ethanol drinking in alcohol-preferring P rats (Levy et al., 1991), suggesting the importance of reduced dopamine $D_2$ receptor activity in the accumbens, and strengthening the proposed importance of the hierarchy of the dopamine pathway, in mediating ethanol vulnerability. We propose that an endogenous deficit in mesolimbic enkephalin contributes to the hypodopamine state, but that dopamine appears to be the final determinant in the hierarchy of neurotransmitter pathways associated with mediating the increased vulnerability to ethanol abuse in the C57 mouse.

It should be noted that a strain difference in hypothalamic $\beta$-endorphin content has also been reported in these inbred mouse strains (De Waele et al., 1993), but the causal link to ethanol drinking is unclear at this time. It would be premature to exclude the possibility that other opioid mechanisms play an important role in the maintenance of continued ethanol drinking and ethanol reward (reviewed by Di Chiara and North, 1992). The findings of this report constitute the first neuropharmacological evidence to support an unifying hypothesis relating differences in mesolimbic enkephalin and dopamine function to increased vulnerability to high voluntary ethanol consumption in the C57 mouse. These findings may provide greater understanding of the factors, more complex and integrated than previously thought, underlying alcohol abuse.

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