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Withdrawal from alcohol in withdrawal seizure-prone and -resistant mice: evidence for enkephalin resistance

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Abstract

Methionine enkephalin (Met-enkephalin) functions as an endogenous anticonvulsant. Peptide transport system-1 (PTS-1) is an important regulator of Met-enkephalin levels in brain and transports the peptide from brain to blood. In outbred mice, alcohol dependence is associated with decreased PTS-1 activity and increased levels of Met-enkephalin. In contrast, alcohol withdrawal is associated with recovery of PTS-1 activity, decreased levels of Met-enkephalin, and seizures. In this study, we examined the PTS-1/Met-enkephalin system in two replicates of withdrawal seizure-resistant (WSR) and withdrawal seizure-prone (WSP) mouse lines. We measured levels of preproenkephalin (PPE) mRNA and Met-enkephalin peptide in brain and the activity of PTS-1 during alcohol-naive, -dependent, and -withdrawal states. In alcohol-naive animals, Met-enkephalin levels were higher in WSP than in WSR mice. In alcohol-withdrawal animals, Met-enkephalin levels remained elevated in WSP mice, whereas they increased in WSR mice. Peptide levels were unrelated to levels of PPE mRNA or activity of PTS-1. Factorial analysis showed that proneness to seizures was genetically linked to Met-enkephalin levels in alcohol-naive, -dependent, and -withdrawing mice but not to mRNA levels or PTS-1 activity. Overall, these results may be explained by resistance to enkephalin in WSP mice and suggest that the dysregulation of the PTS-1/Met-enkephalin system contributes to susceptibility to seizures in WSP mice.

Keywords: Blood–brain barrier; Ethanol; Preproenkephalin; Alcoholism; Genetics

1. Introduction

Animal models demonstrate the role of genetics in the various effects of ethanol (Crabbe et al., 1994). Genes regulate, in part, physiological responses to ethanol such as alterations in locomotion, changes in temperature, and the development or inheritance of tolerance, sensitivity, or dependence. In some cases, a specific gene or gene product has been associated with an alcohol-related behavior. The levels of methionine enkephalin (Met-enkephalin) in brain, for example, are related to the consumption of ethanol and to withdrawal seizures.

The role of Met-enkephalin in seizure activity has been studied extensively. Early reports showed that exogenously administered opiates produce epileptiform discharges on EEG associated with "wet-dog" shakes (Gall et al., 1988; Hong et al., 1984). These studies hypothesized that Met-enkephalin was a proconvulsive peptide. However, the studies were flawed because the dose of opiates was non-physiologic. Most researchers feel that the EEG changes and wet-dog shakes reflect toxic or nonspecific effects of Met-enkephalin (Bajorek et al., 1984, 1986; Lee et al., 1983).

Subsequent studies have supported an anticonvulsant role for endogenous opiates. Endogenous opiates are released during seizures provoked by a variety of experimental conditions (Asai et al., 1995; Bing et al., 1997; Carrillo et al., 1992; Frenk, 1983; Urea et al., 1977; Frenk et al., 1978; Kanamatsu and Hirano, 1988; Patel et al., 1991; Lee et al., 1987). These opiates cause postictal suppression of cortical excitability and inhibit future seizure activity.

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This suppression is reversed by naloxone (Bajorek et al., 1984; Carrillo et al., 1992; Frenk, 1983; Koide et al., 1992; Koide et al., 1993; Lee et al., 1983). Preliminary studies suggest that delta opiate (enkephalinergic) agonists can reduce alcohol-withdrawal seizures in animals and humans (Aliyev, 1992; Kotslinka and Langwinski, 1986).

Previous work in outbred ICR mice has shown that Met-enkephalin levels in brain are regulated by posttranslational mechanisms (Plotkin, 1997). One such mechanism is by transport across the blood–brain barrier (BBB) (Banks and Kastin, 1997). Peptide transport system-1 (PTS-1) shuttles Met-enkephalin from brain to blood and is regulated by many compounds (Banks and Kastin, 1989a, 1989b, 1991; Banks et al., 1986, 1987). Consumption of ethanol by naive animals leads to a reduction in PTS-1 activity and a corresponding increase in Met-enkephalin in brain. In contrast, withdrawal of ethanol from dependent animals leads to the recovery of PTS-1 activity and a decrease in Met-enkephalin levels to baseline (Banks and Kastin, 1989b; Plotkin et al., 1997). These studies suggest that PTS-1 is a major regulator of Met-enkephalin in ICR mice and that recovery of PTS-1 activity during withdrawal lowers Met-enkephalin levels and increases susceptibility to seizures.

Withdrawal seizure-prone (WSP) and withdrawal seizure-resistant (WSR) mice are lines of mice that have been selectively bred for 26 generations for susceptibility and resistance to alcohol-withdrawal seizures, respectively. These mice exhibit a 10-fold difference in the severity of withdrawal from alcohol as measured by the handling-induced convulsion (HIC) test (Philips et al., 1989; Crabbe and Philips, 1993). After selection, alleles relevant to withdrawal seizures are fixed in the homozygous state. The WSP/WSR lines do not differ significantly in the rates of disposal of alcohol, sensitivity to GABA, or in the degree of membrane fluidity after exposure to alcohol (Philips et al., 1989). These lines exist as genetic replicates; that is, two independent breeding efforts have been conducted so that two sets exist (WSP_1/WSR_1 and WSP_2/WSR_2). As measured by HIC scores, the relative severity in withdrawal symptoms is $WSP_1 > WSP_2 > WSR_2 > WSR_1$, where subscripts indicate genetic replicates.

We have shown that alcohol-naive WSP mice have higher levels of Met-enkephalin in brain than alcohol-naive WSR mice (Plotkin et al., 1998), but the effect of ethanol consumption on peptide levels has not been studied. Here, we examined the PTS-1/Met-enkephalin system in three groups: (1) animals not exposed to ethanol (“naive” group), (2) animals physically dependent on and currently consuming ethanol (“dependent” group), or (3) animals physically dependent on but withdrawing from ethanol (“withdrawal” group). We examined the role of brain-to-blood transport of Met-enkephalin, as estimated by the activity of PTS-1, and the role of transcription, as measured by levels of preproenkephalin (PPE) mRNA, in the regulation of Met-enkephalin within the brain.

2. Materials and methods

2.1. Animal lines

WSP_1, WSR_1, WSP_2, and WSR_2 mice representing the 26th generation of selective inbreeding were obtained from the laboratory of Dr. John Crabbe (Portland, OR). WSP_1 and WSR_1 mice (genetic replicate 1) and WSP_2 and WSR_2 mice (genetic replicate 2) are the products of two independent breeding efforts to establish lines of mice with different susceptibility to withdrawal-induced seizures. WSP and WSR mice diverged in their sensitivity to withdrawal-induced seizures by the 11th generation and were sufficiently divergent by the 26th generation to permit relaxation of selection (Crabbe and Philips, 1983).

The selection process and characteristics of WSP and WSR mice have been reviewed previously (Kosobud and Crabbe, 1995). In brief, the use of selectively bred mouse lines simplifies the study of behavior by controlling for genetic variation among individual mice. By selecting for sensitivity and resistance to seizures over multiple generations, genes that influence withdrawal-induced seizures become fixed in the homozygous state. In contrast, genes that influence unrelated behaviors continue to vary normally (Kosobud and Crabbe, 1995). The use of two independent replicates in this study permits the differentiation of chance fixation of a trait, which may occur in one replicate, from true association between genes and behavior, which occurs in both replicates (Kosobud and Crabbe, 1995).

Female mice (15–25 g) were housed in a 12-h light/12-h dark cycle with free access to food and water until at least 8 weeks of age. All mice were housed and treated according to protocols approved by AAALAC.

2.2. Chronic oral ethanol

Kahn’s formula liquid diet (Bioserv, Frenchtown, NJ) was used for the administration of ethanol since this diet has been shown to produce the least variation in blood alcohol concentration and to produce the most severe effects of ethanol toxicity (Vaurousek-Jakuba et al., 1991). Two days prior to the experiment, all mice were switched from standard chow to Kahn’s formula, which was made isocaloric to a 5% (v/v) ethanol solution by the addition of sucrose. Free access to water was maintained. During the 7-day experiment, animals in the naive group consumed the sucrose diet, while animals in the dependent and withdrawing groups consumed the alcoholic diet. Consumption of the liquid diet by ICR mice does not differ for alcoholic and nonalcoholic feeds. These mice ingest 20–21 kcal per mouse and about 25 g/kg of ethanol per day (Banks and Kastin, 1989b). Substitution of sucrose feed for alcoholic feed precipitated withdrawal symptoms in dependent animals. All assays in withdrawing mice were performed between 8 and 12 h after the removal of ethanol since this

time course produced withdrawal symptoms in similarly treated mice (Banks and Kastin, 1989b).

2.3. Harvesting of tissues for peptide and mRNA levels

Thirty mice from each line (WSP_1, WSR_1, WSP_2, and WSR_2) were divided into groups based on exposure to ethanol — naive, dependent, or withdrawing. Mice from all groups (naive, dependent, and withdrawing) were sacrificed between 1 and 3 p.m. The brains were removed, placed on ice, and hemisected with a scalpel. Individual hemispheres were frozen in liquid nitrogen and stored at $-70°C$ until further processing. Determination of peptide and mRNA levels were alternated in right and left hemispheres to control for possible hemispheric asymmetry.

2.4. Radioimmunoassay (RIA) of Met-enkephalin

Peptides were extracted from brain as described previously (Plotkin et al., 1998). Concentrations of Met-enkephalin-like immunoreactivity were determined with a RIA kit (INCSTAR, Stillwater, MN) previously validated by high-performance liquid chromatography (HPLC) (Duka et al., 1978). All samples were processed in duplicate. Cross-reactivity of the antiserum was 2.8% for leucine enkephalin, 0.1% for α-endorphin, and $<0.002\%$ for substance P, β-endorphin, porcine dynorphin 1–13, and α-*neo*-endorphin. The minimum detectable amount was 2 pg, and the inter-assay and intraassay variability were 13% and 9%, respectively (Martinez et al., 1991). Values were expressed as picograms per microgram of total protein and as a percent of the mean concentration for naive animals.

2.5. Preparation of digoxigenin-labeled probes

Digoxigenin-labeled probes were generated by polymerase chain reaction (PCR) amplification of plasmid templates, as described previously (Plotkin et al., 1998). The plasmid pYSEA1 (kindly donated by Dr. Steven Sabol) was used to generate the probe for PPE mRNA, and the plasmid pBluescript SK M13(-) (Stratagene, La Jolla, CA) containing a cDNA clone for mouse actin (Leader et al., 1986) was used for β-actin.

Pure plasmid DNA (100 ng for PPE and 1 μg for actin) was used as template for PCR. The reaction mixture was heated to 94°C for 10 min, followed by 25 rounds of thermocycling according to the following specifications: 1 min of denaturing at 94°C, 1 min of annealing at 57°C, and 1 min of elongation at 72°C. A final extension step was performed at 72°C for 10 min. PCR products were separated by gel electrophoresis to confirm the amplification of PPE and actin cDNA. Probe concentrations were determined by dot–blot analysis according to manufacturer's protocol and diluted to a final concentration of 50 ng/ml in hybridization solution.

2.6. Measurement of PPE mRNA

RNA was isolated from about 0.25 g of brain tissue with TRI reagent (Molecular Research Labs, Cincinnati, OH), as described previously (Plotkin et al., 1998). Samples of RNA were reconstituted in 20 μl of DEPC-treated water and the resulting concentrations determined by spectrophotometry.

RNA samples were separated by gel electrophoresis and stained with ethidium bromide (0.5 mg/ml) to permit visualization of ribosomal bands. If distinct ribosomal bands were present, the sample was considered intact and 20 μg of each sample was separated on a 1% agarose–2.2 M formaldehyde gel in $1\times$ MOPS buffer. RNA was transferred overnight to a Hybond-N membrane (Amersham, Arlington Heights, IL) by capillary blotting in $20\times$ saline sodium citrate (SSC). RNA was cross-linked to membranes by UV light in a Stratalinker (Stratagene).

Membranes were hybridized overnight at 43.5°C with the digoxigenin-labeled probe for PPE. After post-hybridization, washes with $2\times$ SSC–0.1% SDS and $0.5\times$ SSC–0.1% SDS at room temperature, membranes were incubated with anti-digoxegenin-alkaline phosphatase antibody (1:10,000). Application of CPSD (Boehringer Mannheim, Indianapolis, IN) induced luminescence. Membranes were exposed to Kodak X-OMAT film (Rochester, NY) at room temperature and signals quantified by densitometry using the MCID image analysis system (Imaging Research, Saint Catharines, Ontario, Canada).

Membranes were stripped and rehybridized overnight with the probe for β-actin. After processing as described for PPE mRNA, membranes were exposed to film at room temperature and the signals quantified by densitometry. Signal density for PPE was normalized for loading variation by division with the corresponding signal density for β-actin.

2.7. Iodination of Tyr-MIF-1

L-Tyr-MIF-1 was labeled with ^{125}I by incubation with chloramine T for 1 min. The reaction was stopped with sodium metabisulfite, and the resulting mixture separated by HPLC.

2.8. Determination of $t_{1/2}$ and %T

A method for determination of the rate of transport for substances out of the brain has been described previously (Banks and Kastin, 1989c). In this study, radiolabeled Tyr-MIF-1 was used to measure PTS-1 activity since Tyr-MIF-1 and Met-enkephalin produce comparable results in transport studies (Banks and Kastin, 1990; Banks et al., 1987) and is less subject to oxidation during experimentation. Briefly, mice were anesthetized with ethyl carbamate (40% w/v) and the scalp removed to reveal the

bregma. A hole was made in the skull 1 mm lateral, 1 mm posterior, and 3.5 mm deep to the bregma with the exposed tip of a 26-gauge needle covered with polypropylene tubing. At time 0, a 1-μl Hamilton syringe (Reno, NV) was used to inject 25,000 cpm of ^{125}I-L-Tyr-MIF-1 (I-Tyr-MIF-1) into the lateral ventricle through the hole in the skull.

The presence of PTS-1 in WSP and WSR mice was confirmed by inhibition experiments with elemental aluminum. This test is based on the ability of aluminum to function as a noncompetitive inhibitor of PTS-1 (Banks et al., 1988) and has been used to identify functional systems in other species (Banks and Kastin, 1994). In these studies, mice from each line were given 0.2 ml of either aluminum chloride (100 mg/kg) or 0.9% saline by intraperitoneal injection. Thirty minutes later, mice were injected with I-Tyr-MIF-1 as described above. After 10 min, mice were decapitated and the brains removed without the pineal and pituitary glands. The level of ^{125}I remaining in the brain (residual counts per minute) was counted in a gamma counter. Previous studies with HPLC indicate that the majority of the radioactivity transported represents intact L-Tyr-MIF-1 (Banks et al., 1990).

In kinetic studies, mice were injected with I-Tyr-MIF-1 at 0 min. At 2, 5, 10, and 20 min after injection, mice were decapitated and the brains removed without the pineal and pituitary glands. Values at 0 min were estimated by injection of 25,000 cpm of I-L-Tyr-MIF-1 into the lateral ventricles of mice that had been overdosed with urethane. Residual counts per minute were counted in a gamma counter.

The activity of PTS-1 was expressed in two ways. The half-time disappearance ($t_{1/2}$) represents the amount of time required for half of the injected material to exit the brain. The $t_{1/2}$, expressed in minutes, was calculated using the following equation:

$$t_{1/2} = \frac{-0.301}{A}$$

where A is the slope of the regression between the log (residual counts per minute) and transport time.

The second measure of transport velocity, percent transported per minute per gram of brain tissue (%T), differs from $t_{1/2}$ by reflecting differences in brain weight among the lines and differences in the amount of material available for transport at time 0. %T was calculated according to the equation:

$$\%T = \frac{(I - R_{10})(100)}{I(\mathrm{Br})(t)}$$

where I is the antilog of the y-intercept of the regression between log (residual counts per minute) and transport time, R_{10} is the residual counts per minute in a given brain at 10 min, Br is the average brain weight, and t is transport time (10 min).

2.9. Statistics

Means are reported with their standard errors. Separate analyses were performed to examine the influence of alcohol treatment (naive, dependent, and withdrawing) and line (WSP and WSR) on concentrations of Met-enkephalin. To study the effect of treatment, values for Met-enkephalin (expressed as picograms per miligram of total protein and as percent of mean values for naive controls) and %T were compared by two-way analysis of variance (ANOVA) using line and treatment as independent variables. PPE mRNA was compared by one-way ANOVA for each treatment group. Residual counts per minute for WSP and WSR mice in inhibition studies were compared by two-way ANOVA for line and treatment (saline vs. aluminum).

To study the effect of line, the two-way factorial ANOVA method recommended by Crabbe et al., (1990) was used. This method uses replicate and line as independent factors in two-way ANOVA followed by a range test when there are significant line or interaction (Line × Replicate) effects. The evidence for a genetic correlation of the measured trait with the WSP/WSR trait is then graded as strong (significant ANOVA effect for line and the lines for both replicates significantly different in the same direction by range test), moderate, weak, or none (no effect for line and no consistent differences in the range test). In all analyses, Duncan's Multiple Range Test (DMRT) was performed when P values reached statistical significance by ANOVA ($P < .05$).

Slopes and y-intercepts used in the calculation of $t_{1/2}$ were determined by linear regression of log (residual counts per minute) and transport time and were evaluated for significance by the Prism 2.0 program (GraphPad Software, San Diego, CA). A measure of dispersion for $t_{1/2}$ was calculated by multiplication of the coefficient of variation for each slope by the value for $t_{1/2}$. Disappearance curves were compared across treatment groups within a given line to evaluate the effect of ethanol on PTS-1 in each line. Disappearance curves also were compared across lines within a given treatment group to compare the response of different lines to a given exposure to ethanol.

The Prism 2.0 program was used to correlate levels of Met-enkephalin and PPE mRNA within individual mice and to correlate mean values for concentrations of Met-enkephalin, $t_{1/2}$, and %T among the groups.

3. Results

3.1. Concentrations of Met-enkephalin in whole brain

The concentrations of Met-enkephalin in whole brain of WSP and WSR mice were significantly different among naive, dependent, and withdrawing animals, as determined by two-way ANOVA [treatment: $F(2,94) = 1.9$, $P = .15$; line:

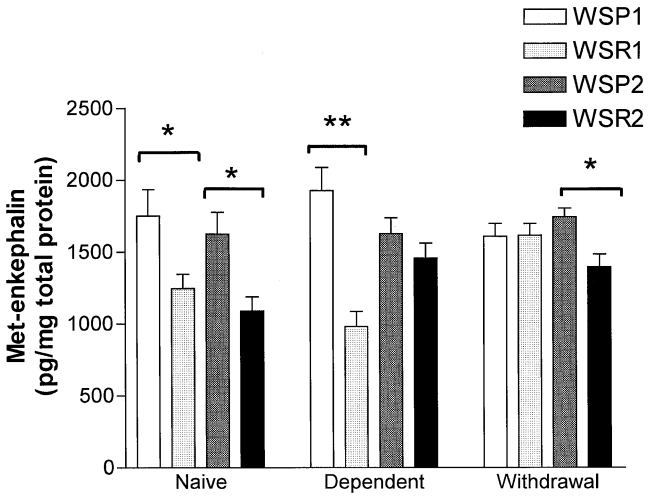

Fig. 1. Comparison of absolute concentrations of Met-enkephalin (picograms per microgram of protein) in naive, dependent, and withdrawing animals from WSP_1, WSR_1, WSP_2, and WSR_2 lines ($n=109$). Values for WSP mice were significantly greater than for WSR mice in both genetic replicates for naive animals ($*P<.05$) and in one genetic replicate for dependent animals ($**P<.001$). In the range test, following two-way factorial analysis for lines and replicates, WSP_2 and WSR_2 were significantly different in withdrawing mice.

$F(3,94)=12.5$, $P<.0001$; interaction: $F(6,94)=3.3$, $P<.01$; $n=109$]. In naive animals, WSP mice expressed greater concentrations of Met-enkephalin than WSR mice for both genetic replicates ($WSP_1>WSR_1$, $WSP_2>WSR_2$) ($P<.05$). In dependent animals, the concentration of Met-enkephalin was significantly greater in WSP mice for genetic replicate 1 only ($WSP_1>WSR_1$) ($P<.001$). In withdrawing animals, there were no significant differences between WSP and WSR mice in either genetic replicate (Fig. 1).

Crabbe's factorial analysis for Met-enkephalin levels in naive mice showed a significant effect for line [$F(1,35)=15.67$, $P<.01$, $n=39$], but not for replicate or interaction. The range test showed that WSP mice had significantly ($P<.05$) higher Met-enkephalin levels than WSR mice for both replicates. This constitutes strong evidence, the highest rating on Crabbe's scale, for a genetic correlation. Dependent mice had a significant

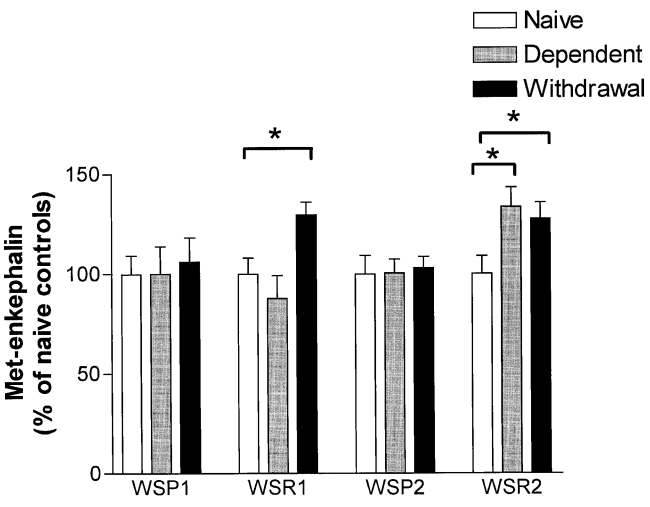

Fig. 2. Comparison of relative concentrations of Met-enkephalin (percent of mean values for naive controls) in naive, dependent, and withdrawing animals from WSP_1, WSR_1, WSP_2, and WSR_2 lines ($n=109$). Values for WSR mice were significantly increased in withdrawing animals for both genetic replicates and in dependent animals for genetic replicate 2 ($*P<.05$, $n=109$).

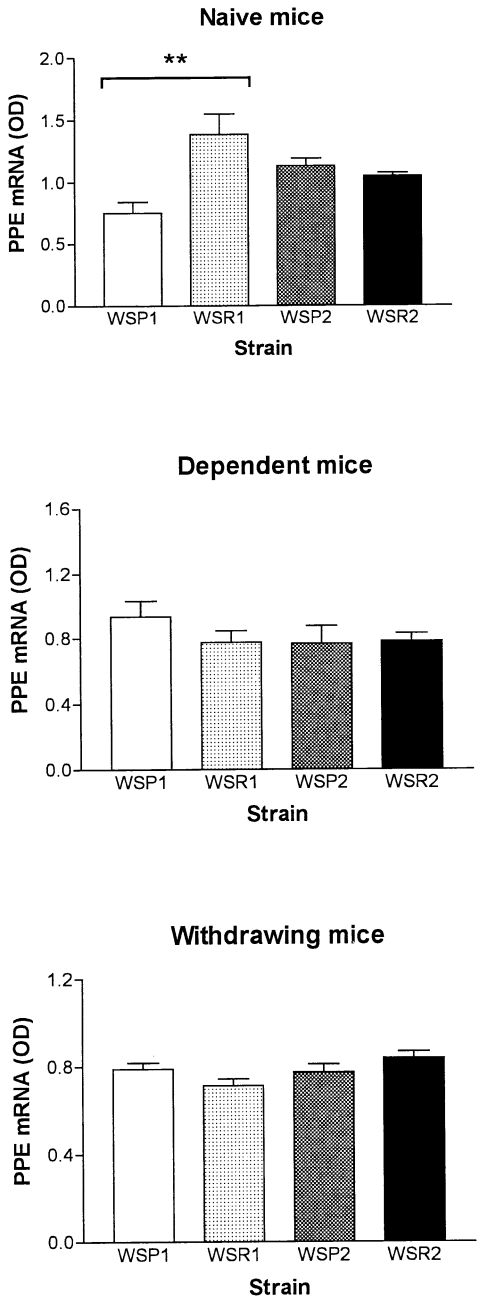

Fig. 3. Comparison of levels of PPE mRNA in naive ($n=19$), dependent ($n=38$), and withdrawing animals ($n=37$) from WSP_1, WSR_1, WSP_2, and WSR_2 lines. PPE mRNA levels were significantly less in WSP mice than in WSR mice in naive animals for genetic replicate 1 only ($**P<.01$).

effect of line [$F(1,29)=17.4$, $P<.01$] and interaction [$F(1,29)=8.32$, $P<.01$, $n=33$] but not replicate with the range test showing a statistically significant difference between WSP_1 and WSR_1. This constitutes a moderate level of evidence on Crabbe's scale. Withdrawing mice showed an effect for line [$F(1,31)=4.20$, $P<.05$, $n=35$] and interaction [$F(1,31)=4.71$, $P<.05$, $n=35$] but not replicate. The range test showed difference between WSP_2 and WSR_2, constituting moderate evidence for a genetic correlation in the withdrawing state.

The percent concentration of Met-enkephalin in whole brain was significantly different among the treatment groups when expressed relative to mean values in naive animals [treatment: $F(2,99)=3.2$, $P<.05$; line: $F(3,99)=2.5$, $.05<P<.1$; interaction: $F(6,99)=1.8$, $P>.1$; $n=109$]. The concentration of Met-enkephalin was elevated in dependent WSR_2 mice and in withdrawing WSR_1 and WSR_2 mice when compared with naive animals ($P<.05$, Fig. 2). The concentration of Met-enkephalin did not change in dependent or withdrawing WSP mice exposed to ethanol.

3.2. Levels of PPE mRNA in whole brain

Fig. 3 summarizes the levels of PPE mRNA in the different groups. In naive mice, the levels of PPE mRNA in whole brain differed significantly among the lines [$F(3,15)=6.59$, $P<.005$, $n=19$]. WSP_1 mice expressed lower levels of PPE mRNA than do WSR_1 ($P<.001$), WSP_2 ($P<.05$), and WSR_2 ($P<.05$) mice as determined by DMRT. The levels of PPE mRNA in whole brain did not differ significantly among the lines in dependent mice [$F(3,34)=0.90$, $P>.05$, $n=38$] or in withdrawing mice [$F(3,33)=2.46$, $P>.05$, $n=37$].

Crabbe's factorial analysis yielded similar results. For the naive group, ANOVA showed a significant effect for line [$F(1,15)=6.93$, $P<.05$] and interaction [$F(1,15)=11.6$, $P<.01$] but not replicate. Duncan's Range Test showed a significant difference between the WSP_1 and WSR_1 mice.

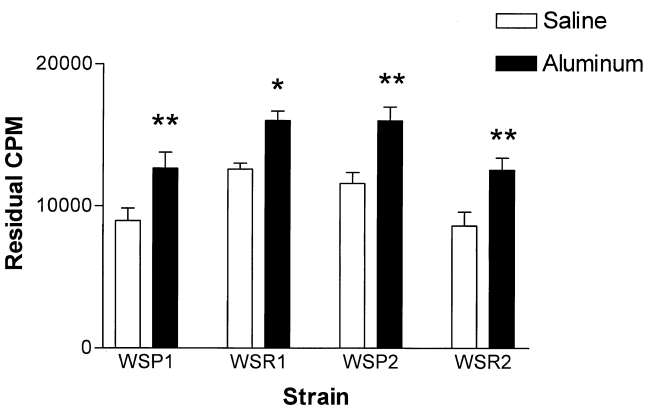

Fig. 4. Presence of PTS-1 in all four lines, as shown by the significant increase in residual counts per minute in animals pretreated with aluminum (* $P<.05$, ** $P<.01$, $n=68$).

Table 1
Half-time disappearance rates ($t_{1/2}$, in minute) ± coefficient of variation for WSP_1, WSR_1, WSP_2, and WSR_2 mice ($n=10$ per entry)

	Alcohol status		
Line	Naive	Dependent	Withdrawing
WSP_1	16.2 ± 3.9	17.3 ± 2.6	16.2 ± 4.2
WSR_1	14.5 ± 4.6	17.7 ± 4.6	11.9 ± 1.3
WSP_2	11.2 ± 1.0	13.2 ± 1.9	16.1 ± 3.2
WSR_2	15.2 ± 1.5 *	13.7 ± 2.3	11.5 ± 2.1

Within a given line, no differences occurred among naive, dependent, or withdrawing groups.
Within a given alcohol status, only naive WSP_2 and WSR_2 mice differed significantly.
* $P<.05$.

This constitutes a moderate level of evidence that a genetic correlation exists between Met-enkephaln mRNA and the WSP/WSR phenotype. Dependent mice showed no effect by ANOVA. Withdrawing mice showed no effect of line or replicate but did have a significant interaction [$F(1,33)=4.67$, $P<.05$]. Range test showed that the only differences were between WSR_1 and WSR_2. This shows that there is no evidence for a genetic linkage with Met-enkephalin mRNA levels in the dependent and withdrawing states.

3.3. Transport studies: inhibition of PTS-1 by aluminum

There was a significant difference in the residual counts per minute found in brain of mice pretreated with aluminum, as determined by two-way ANOVA [treatment: $F(1,59)=37.9$, $P<.0001$; line: $F(3,59)=9.7$, $P<.0001$; interaction: $F(3,59)=0.1$, $P=.96$; $n=68$]. DMRT revealed significant inhibition in WSP_1 ($P<.01$), WSR_1 ($P<.05$), WSP_2 ($P<.01$), and WSR_2 ($P<.01$) mice pretreated with aluminum (Fig. 4).

3.4. Transport studies: disappearance curves, $t_{1/2}$, and %T

In naive, dependent, and withdrawing mice, the disappearance curves for I-L-Tyr-MIF-1 were significant for all lines ($P<.05$ for all groups, $n=10$ per line, each point represents three animals). When analyzed by treatment group, the half-time disappearance ($t_{1/2}$) of I-L-Tyr-MIF-1 differed significantly in naive mice, with $t_{1/2}$ greater in

Table 2
Percent transported per minute per gram of brain weight (%T) expressed as % per minute-gram ± S.E. for WSP_1, WSR_1, WSP_2, and WSR_2 mice ($n=6$ per entry)

	Treatment group		
Line	Naive	Dependent	Withdrawing
WSP_1	10.6 ± 3.5	5.0 ± 1.1	5.1 ± 1.1
WSR_1	8.2 ± 2.2	5.5 ± 1.7	9.9 ± 2.3
WSP_2	13.9 ± 2.7	7.7 ± 2.5	6.7 ± 1.3
WSR_2	6.9 ± 1.4	8.0 ± 0.8	7.6 ± 1.0

No statistical differences existed for a given line across treatment groups or across lines for a given treatment group.

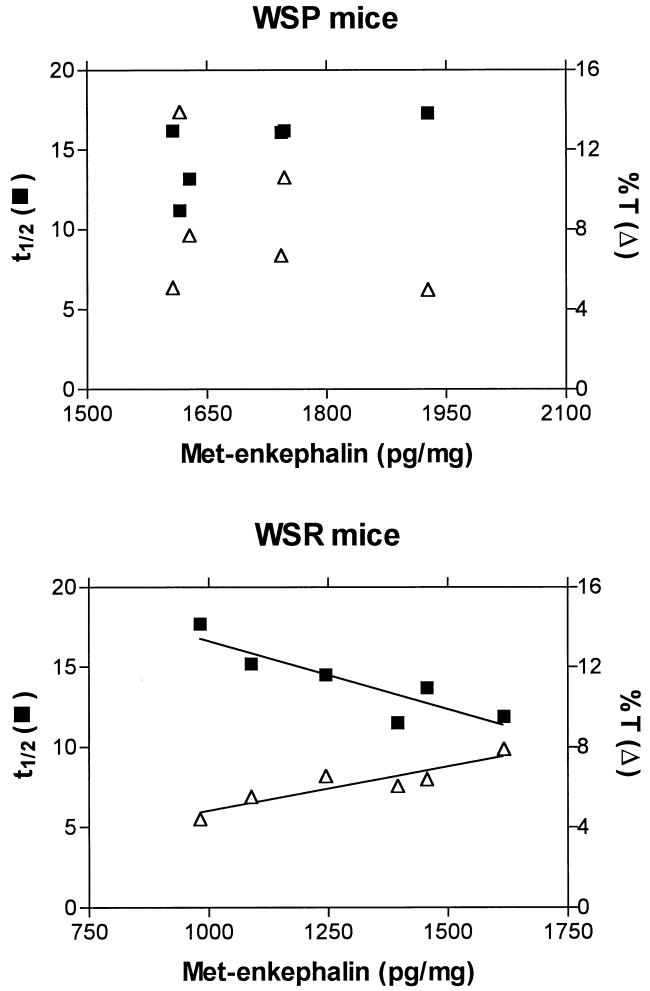

Fig. 5. (a) Correlation of Met-enkephalin, $t_{1/2}$ (left y-axis), and $\%T$ (right y-axis) in WSP mice. Concentrations of Met-enkephalin did not correlate with $t_{1/2}$ ($n=6$ with each point representing about three animals) and $\%T$ ($n=6$ with each point representing about three animals). (b) Correlation of Met-enkephalin, $t_{1/2}$ (left y-axis), and $\%T$ (right y-axis) in WSR mice. Concentrations of Met-enkephalin correlated with $t_{1/2}$ ($r=-.89$, $P<.05$ $n=6$ with each point representing about three animals) and $\%T$ ($r=.91$, $P<.05$, $n=6$ with each point representing about three animals) in WSR mice.

WSR_2 mice than in WSP_2. When analyzed by line, $t_{1/2}$ did not differ among naive, dependent, and withdrawing mice (Table 1). The percent transported per minute per gram ($\%T$) did not differ significantly among any of the lines or across groups (Table 2). Factorial analysis with Crabbe's method showed no effect for line, replicate, or interaction in naive, dependent, or withdrawing mice for either $t_{1/2}$ or $\%T$.

3.5. Correlations between Met-enkephalin, PPE mRNA, and transport rate

In naive, dependent, and withdrawing mice, the concentrations of Met-enkephalin did not correlate with levels of PPE mRNA when analyzed by individual line or analyzed as a single group. The mean concentrations of Met-enkephalin for all 12 groups did not correlate with transport rates when expressed as $t_{1/2}$ ($r=-.04$, $P=.91$, $n=12$) or as $\%T$ ($r=.17$ $P=.60$, $n=12$). However, a significant relationship was present when groups were separated by proneness to ethanol. In WSR mice, the concentration of Met-enkephalin correlated with $t_{1/2}$ ($r=-.89$, $P<.05$, $n=6$) and with $\%T$ ($r=.91$, $P<.05$, $n=6$), as shown in Fig. 5b. In contrast, no correlation existed for WSP mice for $t_{1/2}$ ($r=.67$, $P=.14$, $n=6$) or for $\%T$ ($r=-.38$, $P=.45$, $n=6$), as shown in Fig. 5a.

4. Discussion

The purpose of these studies was to characterize the response of the Met-enkephalin/PTS-1 system to alcohol dependence and withdrawal in WSP and WSR mice. Overall, we found that regulation of Met-enkephalin differed between WSP and WSR mice. WSR mice also differed in some respects from outbred ICR mice investigated previously.

WSP and WSR mice differ in their response to ethanol as shown by analysis of treatment effects. In naive mice, the concentration of Met-enkephalin was significantly greater in WSP mice than in WSR mice, consistent with previous results (Plotkin, 1998). After exposure to ethanol, the concentration of Met-enkephalin increased in WSR mice (Figs. 1 and 2). This increase occurred in WSR_1 mice during withdrawal and WSR_2 mice with dependence and withdrawal, so that in the withdrawal state, no differences existed among the four WSP/WSR lines. Levels of PPE mRNA did not correlate with levels of Met-enkephalin in brain for WSP or WSR mice (Fig. 3), indicating that transcriptional mechanisms do not determine Met-enkephalin levels.

PTS-1 activity is a major posttranscriptional regulator of brain Met-enkephalin levels in ICR and other lines of mice (Banks and Kastin, 1977). PTS-1 existed in all four lines of the WSR/WSP mice as shown by inhibition of peptide efflux with aluminum (Fig. 4). However, correlations did not exist among measures of PTS-1 activity and brain Met-enkephalin levels in any group. In addition, changes in PTS-1 activity were not inversely related to brain Met-enkephalin changes and no relation existed between PTS-1 activity and brain Met-enkephalin levels in WSP mice. This shows that other posttranscriptional mechanisms, such as propeptide cleavage, peptide release, or enzymatic degradation control Met-enkephalin levels in the brain (Asai et al., 1995; Borsook and Hyman, 1995; de la Baume et al., 1983; Lynchand Snyder, 1986; Zubieta, 1985).

The factorial analysis recommended by Crabbe et al., 1990 gives insight into the genetic underpinnings of these differences. Strong evidence exists for a genetic linkage between Met-enkephalin levels in brain and the WSP/WSR phenotype in naive mice. This strongly suggests

that levels of Met-enkephalin are intimately related to proneness/susceptibility to withdrawal-induced seizures. Moderately strong evidence supports a link between Met-enkephalin levels and proneness to seizures in dependent and withdrawing animals. No such linkage existed for the measures of PTS-1 activity or for Met-enkephalin mRNA levels. Therefore, PTS-1 and Met-enkephalin mRNA are unlikely to be influenced by that set of genes common to Met-enkephalin levels and prone-ness/resistance to seizures.

One explanation for the persistently elevated levels of Met-enkephalin in WSP mice is resistance to enkephalin. The paradoxic finding of an elevated hormone with a deficient biologic action is the hallmark of an endocrine resistance syndrome, as exemplified by elevated insulin levels in the face of hyperglycemia in type 2 diabetes mellitus. The lack of hormonal action negates the negative feedback loop between response and hormonal release, resulting in increased hormone levels. In the case of Met-enkephalin and seizures, it is not clear whether this resistance is caused by receptor/postreceptor mechanisms, as fits the classic definition of resistance syndrome, or by the lack of some intermediary in the negative feedback loop. It may also be that the defect lies at some point downstream of Met-enkephalin stimulation, so that subclinical seizure activity in the WSP mouse is maximally stimulating Met-enkephalin release. The maximally stimulated resistance model would explain the finding of tonically elevated levels of Met-enkephalin that do not further increase during withdrawal and seizure activity.

PTS-1 activity correlated with Met-enkephalin in WSR mice when replicates and states (alcohol-naive, -dependent, and -withdrawal) were combined (Fig. 5). Such a positive correlation is seen across strains (Banks and Kastin, 1997) and suggests the feed forward portion of a negative feedback loop between PTS-1 and Met-enkephalin levels exists in the WSR line. That is, as brain levels of Met-enkephalin increase, PTS-1 activity shows a corresponding increase to return Met-enkephalin levels to baseline. No such correlation existed for WSP mice, demonstrating different regulatory mechanisms than WSR mice.

In summary, we propose that levels of Met-enkephalin rise during withdrawal in WSR mice and protect them from seizures. In these mice, the PTS-1/Met-enkephalin system is intact although it is not the main regulator of Met-enkephalin levels after exposure to ethanol. In contrast, dysregulation of the Met-enkephalin/PTS-1 system contributes to the maladaptive response of WSP mice to withdrawal from ethanol dependence. This line has higher levels of Met-enkephalin at baseline, which do not correlate with levels of PPE mRNA or PTS-1 activity. This lack of correlation indicates that Met-enkephalin in brain is regulated differently in WSP mice than in WSR or ICR mice. During withdrawal, levels of Met-enkephalin remain elevated but do not rise further in WSP mice, suggesting an enkephalin resistance.

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References

Aliyev NA. Application of a synthetic analogue of Leu-enkephalin (Dalargin) in alcohol withdrawal. Drug Alcohol Depend 1992;30:209–13.

Asai M, Martinez A, Fernandez-Mas R, Fernandez-Guardiola A. Penicillin-G induced interictal activity increases both opioid peptide tissue content and in vitro release in the rat brain. Neuropeptides 1995;29:163–70.

Bajorek JG, Lee RJ, Lomax P. Neuropeptides: a role as endogenous mediators or modulators of epileptic phenomena. Ann Neurol 1984;16: S31–8 (Supplement).

Bajorek JG, Lee RJ, Lomax P. Neuropeptides: anticonvulsant and convulsant mechanisms in epileptic model systems and in humans. Adv Neurol 1986;44:489–500.

Banks WA, Kastin AJ. Effect of neurotransmitters on the system that transports Tyr-MIF-1 and the enkephalins across the blood–brain barrier: a dominant role for serotonin. Psychopharmacology 1989a;98:380–5.

Banks WA, Kastin AJ. Inhibition of the brain to blood transport system for enkephalins and Tyr-MIF-1 in mice addicted or genetically predisposed to drinking ethanol. Alcohol 1989b;6:53–7.

Banks WA, Kastin AJ. Quantifying carrier-mediated transport of peptides from the brain to the blood. In: Conn PM, editor. Methods in enzymology. San Diego: Academic Press, 1989c. pp. 652–60.

Banks WA, Kastin AJ. Leucine modulates peptide transport system-1 across the blood–brain barrier at a stereospecific site within the central nervous system. J Pharm Pharmacol 1991;43:252–4.

Banks WA, Kastin AJ. Peptide transport systems for opiates across the blood-brain barrier. Am. J. Physiol 1990;259:E1–E10.

Banks WA, Kastin AJ. Leucine modulates peptide transport system-1 across the blood-brain barrier at a stereospecific site within the central nervous system. J.Pharm.Pharmacol 1991;43:252–4.

Banks WA, Kastin AJ. The role of blood-brain barrier transporter PTS-1 in regulating concentrations of methionine enkephalin in blood and brain. Alcohol 1997;14:1–9.

Banks WA, Kastin AJ, Fischman AJ, Coy DH, Strauss SL. Carrier-mediated transport of enkephalins and *N*-Tyr-MIF-1 across blood–brain barrier. Am J Physiol 1986;251:E477–82.

Banks WA, Kastin AJ, Michals EA. Tyr-MIF-1 and Met-enkephalin share a saturable blood–brain barrier transport system. Peptides 1987;8: 899–903.

Banks WA, Kastin AJ, Michals EA, Barrera CM. Stereospecific transport of Tyr-MIF-1 across the blood–brain barrier by peptide transport system-1. Brain Res Bull 1990;25:589–92.

Banks WA, Kastin AJ, Fasold MB. Differential effect of aluminum on the blood-brain barrier transport of peptides, technetium, and albumin. J. Pharmacol. Exp. Ther. 1988;244:579–85.

Banks WA, Kastin AJ. Effects of aluminum on blood-brain barrier structure and function. Life Chemistry Reports 1994;11:141–9.

Bing G, Wilson B, Hudson P, Jin L, Feng Z, Zhang W, Bing R, Hong JS. A single dose of kainic acid elevates the levels of enkephalins and activator protein-1 transcription factors in the hippocampus for up to 1 year. Proc Natl Acad Sci USA 1997;94:9422–7.

Blasberg RG, Fenstermacher JD, Patlak CS. Transport of α-aminoisobutyric acid across brain capillary and cellular membranes. J Cereb Blood Flow Metab 1983;3:8–32.

Borsook D, Hyman SE. Proenkephalin gene regulation in the neuroendocrine hyphothalamus: a model of gene regulation in the CNS. Am. J. Physiol 1995;269:E393–E408.

Carrillo E, Fuente T, Laorden ML. Hyperthermia-induced seizures alter the levels of methionine-enkephalin in immature rat brain. Neuropeptides 1992;21:139–42.

Crabbe JC, Belknap JK, Book KJ. Genetic animal models of alcohol and drug abuse. Science 1994;264:1715–23.

Crabbe JC, Philips TJ. Selective breeding for alcohol withdrawal severity. Behav Genet 1993;23:171–7.

Crabbe JC, Phillips TJ, Kosobud A, Belknap JK. Estimation of genetic correlation: interpretation of experiments using selectively bred and inbred animals. Alcohol: Clin Exp Res 1990;14:141–51.

de la Baume S, Yi CC, Schwartz JC, Chaillet P, Marcais-Collado H, Costentin J. Participation of both 'enkephalinase' and aminopeptidase activities in the metabolism of endogenous enkephalins. Neuroscience 1983;8:143–51.

Duka T, Hollt N, Przewlocki R, Wesche D. Distribution of methionine- and leucine-enkephalin within the rat pituitary gland measured by highly specific radioimmunoassays. Biochem. Biophys. Res. Comm. 1978;85:1119–27.

Frenk H. Pro- and anticonvulsant actions of morphine and the endogenous opioids: involvement and interactions of multiple opiate and non-opiate systems. Brain Res Rev 1983;6:197–210.

Frenk H, Urca G, Liebeskind JC. Epileptic properties of leucine- and methionine-enkephaline: comparison with morphine and reversibility by naloxone. Brain Res. 1978;147:327–37.

Gall CM. Localization and seizure-induced alterations of opioid peptides and CCK in the hippocampus. NIDA Res Monogr 1988;82:12–31.

Hong JS, McGinty JF, Grimes L, Kanamatsu T, Obie J, Mitchell CL. Seizure-induced alterations in the metabolism of hippocampal opioid peptides suggest opioid modulation of seizure-related behaviors. NIDA Res Monogr 1984;82:48–65.

Kanamatsu T, Hirano S. Differences in ME-LI and VIP-LI in discrete brain regions of seizure-naive and seizure-experienced El mice. Neurochem Res 1988;13:983–8.

Koide S, Onishi H, Katayama M, Yamagami S. Endogenous methionine enkephalin may play an anticonvulsant role in the seizure-susceptible El mouse. Neurochem. Res. 1993;18:1259–62.

Koide S, Onishi H, Yamagami S, Kawakita Y. Effects of morphine and D-Ala^2-D-Leu^5-enekphalin in the seizure-susceptible El mouse. Neurochem. Res. 1992;17:779–83.

Kosobud AE, Crabbe JC. Genetic influences on the development of physical dependence and withdrawal in animals. In: Begleiter H, Kissin B, editors. The genetics of alcoholism. New York: Oxford University Press. 1995;221–56.

Kotslinska J, Langwinski R. Audiogenic seizures during ethanol withdrawal can be blocked by a delta opioid agonist. Drug Alcohol Depend 1986;18:361–7.

Leader DP, Gall I, Campbell P, Frischauf AM. Isolation and characterization of cDNA clones from mouse skeletal muscle actin mRNA. DNA 1986;5:235–8.

Lee RJ, Bajorek JG, Lomax P. Opioid peptides and seizures in the spontaneously epileptic Mongolian gerbil. Life Sci 1983;33:567–70.

Lee RJ, Hong JS, McGinty JF, Lomax P. Increased enkephalin and dynorphin immunoreactivity in the hippocampus of seizure sensitive Mongolian gerbils. Brain Res 1987;401:353–8.

Lynch DR, Snyder SH. Neuropeptides: multiple molecular forms, metabolic pathways, and receptors. Ann. Rev. Biochem. 1986;55:773–99.

Martinez AM, Padbury JF, Burnell EE, Thio SL. Plasma methionine enkephalin levels in the human newborn at birth. Biol Neonate 1991;60:102–7.

Patel VK, Abbott LC, Rattan AK, Tejwani GA. Increased methionine-enkephalin levels in genetically epileptic (tg/tg) mice. Brain Res. Bull 1991;27:849–52.

Philips TJ, Feller DJ, Crabbe JC. Selected mouse lines, alcohol and behavior. Experientia 1989;45:805–27.

Plotkin SR, Banks WA, Kastin AJ. Enkephalin, PPE mRNA, and PTS-1 in Alcohol Withdrawal Seizure-Prone and -Resistant Mice. Alcohol 1998;15:25–31.

Plotkin SR, Banks WA, Waguespack PJ, Kastin AJ. Ethanol alters the concentration of Met-enkephalin in brain by affecting peptide transport system-1 independent of preproenkhephalin mRNA. J. Neurosci. Res. 1997;48:273–80.

Urca G, Frenk H, Liebeskind JC, Taylor AN. Morphine and enkephalin: analgesic and epileptic properties. Science 1977;197:83–6.

Vavrousek-Jakuba EM, Baker RA, Shoemaker WJ. Effect of ethanol on maternal and offspring characteristics: comparison of three liquid diet formulations fed during gestation. Alcohol. Clin. Exp. Res 1991;15:129–35.

Zubieta M, Vindrola O, Talavera E, Asai M, Massarini A, Linares G. Pentylenetetrazol-induced seizures produce an increased release of IR-Met-enkephalin from rat striatum in vitro. Brain Res 1985;360:101–7.